MOLECULAR MECHANISMS OF TRANSPORT-P

A thesis submitted for the degree of
Doctor of Philosophy,
Faculty of Science,
University of London

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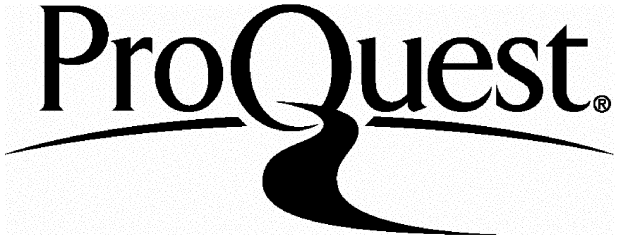
June 2001



1. ABSTRACT

The alpha-1 adrenergic ligand [^{3}H]prazosin binds to peptidergic neurones and is displaced by unlabelled prazosin in concentrations up to 10^{-7} M. However, at greater concentrations of unlabelled prazosin, there was a paradoxical increase in accumulation of [^{3}H]prazosin. This work investigated the causes of this new observation. The conclusions were as follows:

1. Alpha-1 adrenoceptors are not involved in the prazosin paradox. A cDNA library was constructed from the peptidergic neurones and a clone encoding an alpha-1 adrenoceptor was isolated. The affinity of prazosin for the binding sites in the peptidergic neurones was similar to its affinity for alpha-1 adrenoceptors expressed in cultured cells transfected with the alpha-1 adrenoceptor cDNA. However, there was no prazosin paradox in these transfected cells.

2. The prazosin paradox is due to an unusual uptake process which is activated by its ligand. The increase in accumulation of [^{3}H]prazosin is inhibited competitively by antidepressants which inhibit neuronal transporters. Specific binding sites for antidepressants are present in the peptidergic neurones. The prazosin paradox was not seen in membrane preparations, indicating that it requires intact cells or storage organelles. Antidepressant-sensitive prazosin uptake was not seen in aminergic neurones or in non-neuronal cells, indicating that it is a specialised feature of peptidergic neurones. The prazosin paradox is dependent on the energy of the electrochemical proton gradient which is generated by V-ATPase. A fluorescent analogue of prazosin accumulated in a granular distribution, indicating a vesicular location. Accumulation was predominantly in neurones rather than in glial cells.

3. There are specific structural requirements for accumulation via the uptake process. Ligands which have the greatest affinity for the uptake process consist of a basic amine and a skeleton of 18-20 carbons which are in a condensed cyclic structure. Halogens increase affinity. A furan group is essential for activation of the uptake process.

4. Release of amines: following uptake by the peptidergic neurones, the amines can be released from acidified cellular stores by an energy-dependent process.

This novel uptake process is designated Transport-P. It is likely to be encoded by an allosteric transport protein. The peptidergic neurone cDNA library was screened for known and unknown members of the families of amine transporters. Two cDNAs were isolated but neither accounts for Transport-P. This is consistent with data which indicate that Transport-P differs radically from known transport processes. An expression-cloning strategy is required to isolate a cDNA which encodes Transport-P, and techniques for this are proposed.

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4. ACKNOWLEDGEMENTS

I am indebted to my supervisor, Professor EA Barnard FRS, for his interest in my work and unfailing support. I benefited from his accumulated knowledge and experience in biochemistry, molecular biology, neuroscience and pharmacology, and I am grateful for the constructive criticism and technical advice which he provided. When the technical expertise was not available here, Professor Barnard advised me on how to obtain it elsewhere; this has opened new avenues which I hope to pursue in my future research on Transport-P.

I thank Professor CR Ganellin FRS for his interest in this project and for discussing the chemistry. Professor Ganellin and Dr Patricia Zunszain synthesised novel molecules for this project, for which I am grateful. I am indebted to Dr IJ Kopin, Scientific Director of NINDS. From our first meeting, he was generous in his support. Dr Kopin is a leading authority on biogenic amines; when I showed him the surprising data which I had obtained on [^{3}H]prazosin, he acknowledged the paradox and he provided generous support which enabled me to pursue the finding. I thank Dr KJ Catt for giving me the opportunity to visit the NIH where I enjoyed the intellectual and technical environment; Dr LZ Krsmanovic for teaching me techniques of cell culture and ligand binding; Dr M Brownstein, Dr T Usdin, Dr K Sandberg and Dr M Hediger for teaching me recombinant DNA and related techniques; Dr J Knutson and Miss D Porter for teaching me fluorescence techniques.

I thank the Department of Medicine and the Medical School for providing time and resources which enabled me to pursue this work at the Royal Free. I thank Professor AF Winder for his support and Professors KR Bruckdorfer and MW Waterfield for accommodating my laboratory within the Department of Biochemistry & Molecular Biology. Support was gratefully received from the University of London Central Research Fund and from the Wellcome Trust (Sir Henry Wellcome Commemorative Award for Innovative Research).

I thank my parents for bringing me up among books and for sending me to England to study. I hope that I have passed some of their values to my own children.

As ever, my wife's support has been invaluable. She is the ballast; the balance without which the direction is unsustainable. I thank her for her affection and patience, for bringing up our sons during my long absences and for organising our home. I dedicate this second thesis to her.

5. AUTHOR'S STATEMENT

I trained in neuroendocrinology at Bart's. I discovered that activation of α_1 adrenoceptors which are located in the brain stimulated the secretion of the adrenocorticotrophic hormone. I then moved to Dr Catt's laboratory in NICHD at the NIH in Bethesda, where I wanted to examine the molecular mechanisms of my earlier work. I examined primary cultures of rat hypothalamus for the presence of α_1 adrenoceptors using the ligand [^{3}H]prazosin. I found that [^{3}H]prazosin was displaced by unlabelled prazosin in concentrations up to 10^{-7} M. However, at greater concentrations of unlabelled prazosin, there was a paradoxical increase in the binding of [^{3}H]prazosin. I was intrigued by this finding. I formulated a hypothesis and began to test it while still at NICHD (Al-Damluji et al, 1993). I then moved to NINDS, where the Scientific Director, Dr IJ Kopin, had taken an interest in my work. I continued to test the hypothesis both at NINDS and later at the Royal Free. This thesis describes work which I carried out at NINDS and at the Royal Free.

The experiments which are described in this thesis were conceived by myself. Having learned the techniques of cell culture and ligand binding from Dr Krsmanovic, I continued to work on my own. I wrote the experimental protocols, carried out the procedures and analysed the data for all the pharmacological experiments which are reported in this thesis. I learned the techniques of spectrophotofluorimetry from Dr Knutson and I then developed the fluorescence microscopy method on my own. I worked on my own to isolate the orphan clone, which was the first cDNA to be isolated in this project. Having learned the techniques of recombinant DNA from my colleagues who are listed on the previous page, I worked on my own to extract RNA, prepare poly(A)RNA and to test the RNA by injection of Xenopus oocytes. I also worked on my own in constructing and analysing the cDNA library, performing PCR, ligating and manually sequencing the PCR products, preparing radiolabelled probes and in screening the library. I carried out the 5'RACE reactions on my own, and I ligated the 5' end to the rest of the molecule. At a later stage, I taught two post-doctoral fellows, Dr WB Shen and Dr C Fernandes who helped me with manual sequencing and with isolation of some of the clones. Some automated sequences were performed for me by Mr C Odell at the Ludwig Institute. I also taught two trainee Clinical Biochemists, Susan White and David Housley who performed some of the PCRs, according to protocols which I had written. This thesis was written by myself and approved by my supervisor. I also wrote the publications and the patent applications which arose from this thesis.

6. ABBREVIATIONS

ABC transporters: ATP-binding casette transporters

ACTH: adrenocorticotrophic hormone

Adrenoceptor: adrenergic receptor

ADP: adenosine diphosphate

ANOVA: analysis of variance

ATP: adenosine triphosphate

βARK: β adrenergic receptor kinase

BNPI: brain-specific Na^+-dependent inorganic phosphate transporter

BODIPY FL: 1,3-dimethyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene

C elegans: Caenorhabditis elegans

CFTR: cystic fibrosis transmembrane conductance regulator

CGAT: chromaffin granule amine transporter

CHO cells: Chinese hamster ovary cell line

COMT: catechol-O-methyl transferase

COS cells: CV-1 origin defective SV40 transfected cell lines

CRH or CRF: corticotrophin-releasing hormone (or factor)

CV: coefficient of variation

DEAE: diethylaminoethyl

DBH: dopamine β-hydroxylase

ddNTPs: 2',3'-dideoxynucleotide triphosphates

dNTPs: 2'-deoxynucleotide triphosphates

DEPC: diethyl pyrocarbonate

[^{3}H]DHE: [^{3}H]dihydroergocryptine

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethylsulphoxide

DNAseI: deoxyribonuclease I

DOPA: dihydroxyphenylalanine

DOPE: dioleoylphosphatidylethanolamine

DOSPA: 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate

DOTMA: N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium

EDTA: ethylenediaminetetraacetic acid

EST: expressed sequence tag

F-ATPase: F_1/F_0-ATPase (ATP synthase in mitochondria)

FBS: fetal bovine serum

GABA: γ-aminobutyric acid

GAT-1: GABA Transporter-1

GHRH: growth hormone releasing hormone

GnRH: gonadotrophin releasing hormone

G-protein: guanine nucleotide binding protein

GTC: guanidinium thiocyanate

HEAT: 2-[β-(4-hydroxyphenyl)ethylaminomethyl]tetralone

HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)

IC_{50}: 50% of maximal inhibitory concentration

icv: intracerebroventricular

IPTG: isopropylthio-β-D-galactoside

KRH buffer: Krebs-Ringer-HEPES buffer

LAAD: L-amino acid decarboxylase

LB medium: Luria/Bertani medium

MAO: monoamine oxidase

MDR: multiple drug resistance pump

MIBG: *meta* -iodobenzylguanidine

MOPS: 3-[N-morpholino]propanesulphonic acid

MPP^+: 1-methyl-4-phenylpyridinium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

OCT: organic cation transporter

P-ATPase: phosphorylated-type ATPase

PBS: phosphate-buffered saline

PC12 cells: rat adrenomedullary phaeochromocytoma cell line

PCR: polymerase chain reaction

PNMT: phenylethanolamine-N-methyltransferase

RNAse: ribonuclease

rTth: recombinant DNA polymerase/reverse transcriptase from *Thermus thermophilus*

SDS: sodium dodecyl sulphate

SSRIs: serotonin-selective re-uptake inhibitors

SV2: synaptic vesicle protein 2

SV40: simian virus number 40

SVMAT: synaptic vesicle monoamine transporter

SVOP: SV2 related protein

T antigens: tumour antigens (large and small) of the simian virus 40

Taq : DNA polymerase from *Thermus aquaticus*

TE Buffer: 10 mM Tris hydrochloride, 1 mM EDTA

TH: tyrosine hydroxylase

Tm: melting temperature of a duplex

TRH: thyrotrophin-releasing hormone

V-ATPase: vacuolar-type ATPase

7. INTRODUCTION:

The discovery of Transport-P was unexpected and was the result of experimental work which had been intended to extend my studies on α_1 adrenoceptors in the hypothalamus. In my work in the Medical College of St Bartholomew's Hospital, I had studied the role of the catecholamines in regulating the secretion of the anterior pituitary hormones in humans and in rats. That work formed my MD thesis which was supervised by Professor Michael Besser, Professor Lesley Rees and Sir John Vane. Then at the National Institutes of Health in Bethesda, I looked for α_1 adrenoceptor binding sites in hypothalamic peptidergic neurones, using the α_1 adrenoceptor ligand [^{3}H]prazosin. That work unmasked an unsuspected uptake process for amines in peptidergic neurones of the hypothalamus. This new uptake process was designated Transport-P. This thesis describes the discovery of Transport-P and the work which has been done since on the molecular mechanisms of this uptake process and its possible relevance to the mechanism of action of antidepressant compounds.

The peptidergic neurones of the hypothalamus are densely innervated by catecholamine nerve terminals and noradrenaline plays an important role in regulating their functional activity. Further, α_1 adrenoceptors and the central noradrenergic system are believed to be involved in the aetiology and the clinical manifestations of depressive illness. The thesis will therefore start by describing the central catecholamine system and α_1 adrenoceptors, and how these systems influence the activity of specific functions of the peptidergic neurones of the hypothalamus. Subsequent sections will describe the discovery of Transport-P and its mechanisms.

I wrote parts of Sections 7.1 and 7.2.5 for my MD Thesis (Al-Damluji, 1990). They are included in the present Thesis in order to provide a comprehensive description of the anatomical distribution of catecholamines and α_1 adrenergic receptors in the hypothalamus.

7.1 CATECHOLAMINE INNERVATION OF PEPTIDERGIC NEURONES IN THE HYPOTHALAMUS

7.1.1 Catecholamine systems in the brain

The concept of cellular communication via chemical substances was formulated in the years 1894-1905. Prior to that, it was thought that cells communicated with each other by electrical induction, also known as Galvanism. In 1791, Luigi Galvani of Bologna reported that preparations of frogs' muscles contracted vigorously during a thunder storm and when a spark was emitted from an electric machine. Muscle contractions were also observed when the muscle preparations were connected by a metallic circuit to frogs' nerves. Galvani proposed that 'animal electricity' was present in muscles. He likened muscles to a Leyden jar (ie, a capacitor) which induced a reciprocal electric charge in the nerves. Alessandro Volta of Pavia challenged Galvani's interpretation of these experiments: he asserted that the muscle preparations were responding to external electric shocks, rather than internal 'animal electricity'. Despite the controversy, Galvani's ideas were generally accepted during the 19th century and still had many prominent adherents until the 1930s. In the light of current knowledge, Galvani's idea of the presence of intrinsic electricity in animals is correct, in that it is now thought that conduction of impulses along a nerve takes place by depolarisation of nerve membranes consequent upon abolition of the electrochemical gradient of ions, predominantly sodium. In this regard, Galvani's discovery of electrically-induced muscle twitches was a landmark which led to the development of electrophysiology. However, Galvani was wrong in asserting that cells communicate with each other by electrical induction, in the manner of a capacitor; modern evidence suggests that cells communicate with each other via chemical messenger molecules such as neurotransmitters, hormones and growth factors. An appraisal of Galvani's work was published recently (Piccolino, 1997).

The newer concept of cellular communication via chemical substances originated in 1894 in the Physiological Laboratory at University College London. George Oliver and Edward Schafer (1894) discovered that injection of extracts of the adrenal medulla caused a dramatic increase in arterial blood pressure in anaesthetised animals. They concluded: "It appears to be established as the result of these investigations that, like the thyroid gland, the suprarenal capsules are to be regarded although ductless, as strictly secreting glands. The material which they form and which is found, at least in its fully active condition, only in the medulla of the gland, produces striking physiological effects upon the muscular tissue generally and especially upon that of the heart and arteries. .. On the other hand the removal of the

suprarenal capsules produces extreme weakness of the heart and muscular system generally, and great want of tone in the vascular system.... It may fairly be concluded therefore that one of the main functions, if not the main function, of the suprarenal capsules is to produce a material which is added in some way or another to the blood, and the effect of which is to assist by its direct action upon the various kinds of muscular tissue in maintaining that amount of tonic contraction which appears to be essential to the physiological activity of the tissue" (Oliver & Schafer, 1895a). The chemical structure of the active material was elucidated as a result of the work of Moore (1895 & 1897) at University College London, Abel (1897) at Johns Hopkins University and finally Takamine (1901) at Parke, Davis & Co. Thus, adrenaline became the first signalling molecule to be identified.

The first demonstration of catecholamines in the brain was by von Euler (1946) who screened various tissues for the presence of catecholamines. von Euler detected catecholamines in the brain but he assumed that they were largely localised in the vasomotor nerves which accompany the blood vessels. Vogt (1954) identified adrenaline and noradrenaline in brain tissue using bioassays and found that their concentrations were unaltered by cevical sympathectomy. She correctly concluded that the catecholamines were likely to be located in the neurones of the brain, and she postulated that they may serve a neurotransmitter function, in analogy to their function as chemical transmitters in the peripheral sympathetic nervous system. Vogt (1954) presented the first map of the distribution of catecholamines in the brain and she examined the effects of drugs on the concentrations of catecholamines in various brain regions. Shortly thereafter, Amin, Crawford & Gaddum (1954) described the detailed distribution of serotonin in various regions of the brain.

Thus, by 1954, it had become clear that the biogenic amines were present in some brain regions where they may serve a neurotransmitter function. The presence of catecholamines in brain neurones was demonstrated visually using fluorescence techniques. Gaddum & Schild (1934) had demonstrated the fluorescent properties of catecholamine derivatives when adrenaline was treated with an alkaline solution, and they used this property to develop a fluorimetric assay for adrenaline. They noted that this assay was more sensitive than other known chemical tests. It seems possible that Gaddum & Schild had generated the fluorescent compound 6,7-dihydroxyisoquinoline by cyclic condensation of the side chain of adrenaline (Figure 7.1.1). This is particularly likely as Gaddum & Schild noted that fluorescent

products could not be generated when noradrenaline was reacted under those conditions; absence of an N-alkyl group in noradrenaline makes it difficult to generate a cyclic structure from the side chain (Figure 7.1.1). Eranko (1955) found that when the adrenal medulla was fixed in formalin, it became strongly fluorescent in ultraviolet light. He also reported that *in vitro* mixing of pure noradrenaline with formaldehyde produced a poorly soluble, fluorescent precipitate (Eranko, 1955). Falck and Hillarp discovered that exposure of freeze-dried tissue sections to formaldehyde gas resulted in much more intense fluorescence, presumably because dehydrating the tissue prevented the diffussion of the water soluble catecholamine molecules (Falck, 1962; Falck et al, 1962). The chemical basis of these observations is a condensation reaction between noradrenaline and formaldehyde, in which fluorescent isoquinoline derivatives were produced (Figure 7.1.1). Viewed in a fluorescent microscope, catecholamine derivatives produced a green fluorescence while serotonin derivatives were yellow. The differentiation of noradrenaline from dopamine required a spectrofluorimetric technique: the noradrenaline derivative 6,7-dihydroxyisoquinoline (Figure 7.1.1) is a fully aromatic compound which has an excitation peak at 330 nm; this compound cannot be generated using this method from dopamine, whose equivalent fluorescent derivative is the reduced compound 6,7-dihydroxy-3,4-dihydroisoquinoline (excitation peak 370 nm; Bjorklund et al, 1968). Secondary amines such as adrenaline were less reactive than noradrenaline and it was not possible to distinguish adrenaline from noradrenaline as they have the same excitation and emission spectra. Phenolic substituents such as hydroxyl or methoxyl groups increased the intensity of the fluorescent compounds. In addition to formaldehyde, other carbonyl compounds were investigated and in a modification of the original procedure, formaldehyde was substituted with glyoxilic acid, resulting in greater fluorescence intensity (Figure 7.1.1; Bjorklund et al, 1972; Lindvall et al, 1974).

The distribution in the brain of formaldehyde-induced histofluorescence was similar to the distribution of catecholamines and serotonin, as defined by chemical assays. Further, formaldehyde-induced histofluorescence was abolished by prior treatment with reserpine which depletes brain monoamines and by α-methyl tyrosine which inhibits the synthesis of catecholamines. Conversely, fluorescence was enhanced by monoamine oxidase inhibitors which prevent the degradation of monoamines These findings confirmed that the formaldehyde-induced histofluorescence represented the presence of catecholamines or serotonin in brain neurones (Carlsson et al, 1962a & b).

The histofluorescence techniques detected monoamines in cell bodies and nerve terminals but concentrations along axons were too low to permit detection by this method. In order to follow the course of axon bundles in serial sections, stereotaxic lesions were made, causing "pile up" of the neurotransmitter proximal to the lesion and disappearance of the neurotransmitter from the areas which are supplied by the lesioned neurones (Anden et al, 1966; Ungerstedt, 1971). The lesions were mechanical hemisections, electrocoagulations or localised injections of the neurotoxin 6-hydroxydopamine (Ungerstedt, 1971). Fluorescence in axons could also be enhanced by administration of monoamine oxidase inhibitors (Dahlstrom & Fuxe, 1965), and by local or systemic injections of the synthetic catecholamine analogue α-methyl noradrenaline, which is accumulated in noradrenergic neurones via the pre-synaptic $Uptake_1$ noradrenaline transporter (Bjorklund et al, 1973). Much information was obtained with these methods on the localisation and distribution of the catecholamines in the rat and human brain. Dahlstrom & Fuxe (1965) produced an atlas in which they identified the locations of the cell bodies of the monoaminergic neurones in rat brain. Those early findings were subsequently confirmed and extended with other methods including chemical assays, autoradiography, electron microscopy, immunocytochemistry with antibodies to the enzymes of the catecholamine synthesis pathway and *in situ* hybridisation histochemistry using probes which are complementary to the RNA which encodes the synthetic enzymes. Figure 7.1.2 includes schematic representations of the general pattern of the origin and distribution of catecholamines in rat brain. Most studies were done in rats but the findings in humans are essentially the same.

Noradrenergic neurones are distributed throughout the brain but almost all their cell bodies are situated in the brain stem, in the network of neurones which is known as the reticular formation. Much attention was focused on the locus coeruleus (Area A6 in Figure 7.1.2), so called because of the dark blue colour of the melanin pigment which it accumulates at the time of puberty in humans (Foley & Baxter, 1958). This nucleus lies in the rostral part of the reticular formation, in the floor of the fourth ventricle in the dorsal pons and extends into the midbrain, ventromedial to the mesencephalic nucleus of the trigeminal nerve (Russell, 1955). Together with the supraoptic and paraventricular nuclei, the locus coeruleus has the densest capillary bed in the brain (Finley & Cobb, 1940); this presumably serves some chemoreceptor function whose significance remains speculative. Although the locus coeruleus had been discovered in 1809, little was known of its connections or function; the histofluorescence techniques demonstrated that the locus coeruleus is the largest group of

noradrenergic cell bodies in the brain, and is a major source of the noradrenergic innervation of the cerebral cortex, cerebellum and the hippocampus (Dahlstrom & Fuxe, 1965). Other important collections of noradrenergic cell bodies include the locus subcoeruleus (Area A7) which lies ventrolateral and caudal to the locus coeruleus (Olson & Fuxe, 1972); the nucleus tractus solitarius (A2 area) in the dorsomedial medulla and the A1 area in the ventrolateral medulla (Figure 7.1.2; Ungerstedt, 1971). Efferent noradrenergic fibres from these brainstem cell groups supply the spinal cord and the brain stem, the cerebellum via the superior cerebellar peduncle, the forebrain via the dorsal noradrenergic bundle and the diencephalon, including the hypothalamus, via the ventral noradrenergic bundle (Anden et al, 1966; Nobin & Bjorklund, 1973; Pickel et al, 1974; Pearson et al, 1983). In addition, there are noradrenergic axonal projections which form interconnections between these neuronal groups, and these are presumably important in coordinating the functions of these brain stem regions (Ungerstedt, 1971; Sawchenko & Swanson, 1982).

The neurones within noradrenergic cell groups such as the locus coeruleus are organised into units with respect to their target innervation, suggesting a functional segregation of catecholamine subunits (Foote et al, 1983; Holets, 1990). Further, there are subsets of noradrenergic neurones which contain peptides such as galanin and neuropeptide Y, suggesting functional specialisations of these neuronal subgroups (Sawchenko et al, 1985; Levin et al, 1987).

In contrast to the widespread distribution of noradrenaline, dopamine is confined to three main areas in the brain (Figure 7.1.2). Within the hypothalamus, the tuberoinfundibular tract (Area A12) has its cell bodies in the arcuate nucleus and will be discussed below; the other two dopaminergic systems are the nigrostriatal and the mesolimbic systems (Areas A8-10; Anden et al, 1966) which are concerned with coordination of motor function and are involved in movement disorders such as Parkinson's disease.

Unlike noradrenaline, adrenaline is not detectable in all parts of the brain (Figure 7.1.2; Koslow & Schlumpf, 1974; Van Der Gugten et al, 1976). In the areas where it is detectable, adrenaline is found in much lower concentrations than noradrenaline (Vogt, 1954; Gunne, 1962; Koslow & Schlumpf, 1974; Van Der Gugten et al, 1976), suggesting that the adrenergic system is much smaller than the noradrenergic system. The distribution of 'adrenergic neurones' was mapped with the indirect method of immunocytochemistry for

phenylethanolamine-N-methyltransferase (PNMT), the enzyme which converts noradrenaline to adrenaline (Hokfelt et al, 1973 & 1974). The cell bodies of the 'adrenergic neurones' are in two cell groups which were designated C1 and C2 (Hokfelt et al, 1974), corresponding to the A1 and A2 noradrenergic cell groups in the atlas of Dahlstrom & Fuxe (1965). These two cell groups are located within the reticular formation in the upper medulla; the C1 group is in the ventrolateral medulla, lateral to the olivary complex and caudal to the nucleus of the facial nerve. The C2 group is located at the same coronal level as the C1 group in the dorsomedial medulla near the floor of the 4th ventricle, adjacent to the nucleus tractus solitarius of the dorsal vagal complex (Figure 7.1.2; Hokfelt et al, 1974). Axons ascend within the ventral noradrenergic bundle to the hypothalamus where nerve terminals were observed in the paraventricular and arcuate nuclei and the median eminence (Hokfelt et al, 1973 & 1974). Other axons project to the thalamus, spinal cord, some cranial nerve nuclei, pons and midbrain. The greatest density of PNMT-immunoreactive nerve terminals is in the paraventricular nucleus of the hypothalamus and in the dorsal vagal complex (Hokfelt et al, 1974). The conclusions of these immunocytochemical findings were subsequently confirmed by chemical assays for the activity of PNMT (Saavedra et al, 1974), and with measurement of adrenaline concentrations using gas chromatography-mass spectrometry (Koslow & Schlumpf, 1974) in individual brain nuclei using the Palkovits punch biopsy technique. Subsequently, a third group of 'adrenergic neurones' with cell bodies around the medial longitudinal fasciculus was described; they were designated the C3 group, corresponding to the A3 group of noradrenergic neurones (Howe et al, 1980). Although this C3 group is adjacent to the C2 group, they appear to represent distinct groups of neurones, particularly as the C3 neurones appear later than the C2 neurones during the embryonic development of the rat (Foster et al, 1985).

Many of the PNMT neurones in the C1, C2 and C3 areas contain neuropeptide Y which may act as a co-transmitter or neuromodulator of the actions of adrenaline (Sawchenko et al, 1985). In contrast, neuropeptide Y is colocalised in only a few noradrenergic neurones which are predominantly located in the A1 area (Sawchenko et al, 1985). The PNMT neurones whose cell bodies are in the brain stem do not appear to synthesise tyrosine hydroxylase until a late stage of embryonic development in the rat (Foster et al, 1985); this has led to speculation regarding their function during embryonic development (see below). Neuronal cell bodies which were immunoreactive to PNMT were also identified in the hypothalamus of colchicine-treated rats (Ross et al, 1984; Ruggiero et al, 1985). These will

be discussed below.

In the human brain, PNMT-immunoreactive neurones have been described in areas which correspond to the C1 and C2 areas of the rat brain which are described above (Kitahama et al, 1985).

7.1.2 Peptidergic neurones

The concept of neurotransmission can be traced to the discovery of adrenaline by Oliver and Schafer (1894 & 1895a). The concept of peptidergic neurones can also be traced to these investigators who discovered the vasopressor activity of extracts of the pituitary gland (Oliver & Schafer, 1895b). This pressor activity is due to the presence in the pituitary of the peptides oxytocin and vasopressin, which are synthesised in the magnocellular neurones of the supraoptic and paraventricular nuclei of the hypothalamus and are transported along the axons to the posterior pituitary for storage and subsequent release. The second set of peptides which are located in neurones are the hypothalamic releasing factors which control the synthesis and release of the hormones of the anterior pituitary. These releasing factors are synthesised in the parvicellular neurones of the paraventricular nucleus and are transported to the zona externa of the median eminence, where they are released into the hypothalamo-hypophysial portal system. The anatomical distribution of these peptides will be described below, together with their catecholaminergic innervation.

Apart from the presence of peptides, peptidergic neurones do not have unique anatomical or morphological characteristics, nor are there any ultrastructural features which are unique to peptidergic neurones. Many peptidergic neurones possess large, dense core vesicles in which the peptides are stored; these are also found in neuroendocrine cells which secrete circulating hormones (Hokfelt et al, 1980). A distinguishing characteristic of peptidergic neurones is the mechanism which is used to replenish the neurotransmitter; in aminergic, cholinergic and amino acid neurones, the replenishment of neurotransmitter occurs mainly by re-uptake from the synapse of the neurotransmitter or its precursor (choline, in the case of acetylcholine). In contrast, in the case of peptidergic neurones, the peptides are replenished by synthesis in the cell body followed by transport along the axon (Hokfelt et al, 1980). This mechanism. which is slow and inefficient, probably explains why the concentrations of the peptides are far smaller than the concentrations of other neurotransmitters. However, peptides activate their receptors at far greater potency than other neurotransmitters, and this

compensates for their presence in smaller amounts (Hokfelt et al, 1980). Detection of the small amounts of peptides which are present in the brain necessitates the use of sensitive anatomical procedures such as immunocytochemistry, *in situ* hybridisation and anterograde and retrograde transport studies.

Oxytocin, vasopressin and the hypothalamic releasing factors mediate their effects as classical hormones. In addition, many aminergic and cholinergic neurones possess peptides whose functions are less clear. Everson Pearse at Hammersmith described the coexistence of peptides and amines in the neuroendocrine cells of the periphery, including the C cells of the thyroid, the adrenal medullary chromaffin cells, the intestinal chromaffin cells and the pancreatic islet cells. He postulated that these cells are of neural origin, as evidence indicated that they are derived from the neural crest (Reviewed in Pearse, 1974). It was subsequently demonstrated that many aminergic neurones in the brain also possess peptide hormones; this was consistent with Pearse's view that the peripheral neuroendocrine cells are of neural origin. All the circulating peptide hormones have been described in the nerve cells in the brain. There appears to be no fidelity in the association of amines with peptides, as there is no evidence that a particular amine transmitter is consistently colocalised with a particular peptide; rather, peptides appear to be present in subsets of aminergic neurones, presumably suggesting a sub-specialisation of aminergic cell groups. The basis of the specialisation is likely to be that the peptide modulates the actions of the amine, usually in a synergistic manner (Hokfelt, 1980). These neuropeptides exert their effects on their own specialised receptors in the brain. Some of these neuropeptides have trophic effects or they may be involved in regulating the expression of receptors on their target cells (Hokfelt, 1991).

A characteristic of neuropeptides is the plasticity of their expression in response to changes in the physiological environment (Hokfelt et al, 2000). In the hypothalamus, this is most evident in the parvicellular neurones of the paraventricular nucleus; some of these neurones have no detectable vasopressin under basal conditions but they express the peptide under conditions of stress or when there is a deficiency of glucocorticoids (Whitnall et al, 1987; Section 7.3.1).

7.1.3 General organisation of catecholamines in the hypothalamus

Most anatomical descriptions of the catecholaminergic innervation of the hypothalamus are based on observations in the rat. The few detailed studies on the human hypothalamus

indicate a similar pattern to what has been described in rodents (Nobin & Bjorklund, 1973; Pearson et al, 1983). In the original histofluorescence studies, it was noted that there were four areas in the hypothalamus which became intensely fluorescent following exposure to formaldehyde: the supraoptic nucleus, the paraventricular nucleus, the preoptic area and the region adjoining the wall of the third ventricle (Carlsson et al, 1962b). It subsequently became clear that the fluorescene in the supraoptic and paraventricular nuclei was due mostly to the presence of noradrenergic nerve terminals whose cell bodies are in the brain stem; and the fluorescence in the preoptic area and the periventricular zone was due predominantly to the presence of cell bodies of dopaminergic neurones. These regions will be discussed separately below.

The hypothalamus contains the greatest concentration of noradrenaline in the brain; this applies in humans, rats, dogs, sheep, pigs and cats (Vogt, 1954; Bertler & Rosengren, 1959; Bertler, 1961; Laverty & Sharman, 1965; Palkovits & Brownstein, 1989). All the hypothalamic nuclei which regulate neuroendocrine functions are densely innervated by noradrenergic nerve terminals (Palkovits et al, 1974; Palkovits & Brownstein, 1989). The origin of this noradrenaline is almost all extrinsic, as surgical isolation of the hypothalamus with knife cuts *in vivo* results in a drastic reduction of the noradrenaline content; in contrast, the dopamine content of the hypothalamus is unaffected by such knife cuts (Weiner et al, 1972; Brownstein et al, 1976). The cell bodies of the noradrenergic neurones which innervate the hypothalamus are in the brain stem, predominantly in the A1, A2 and A6 areas; the axons ascend in the ventral noradrenergic bundle both crossed and uncrossed, so that each set of brain stem nuclei appears to innervate both ipsilateral and contralateral halves of the hypothalamus. The axons then enter the hypothalamus through the medial forebrain bundle (Anden et al, 1966; Ungerstedt, 1971; Lindvall & Bjorklund, 1974; Palkovits et al, 1980). Noradrenergic nerve terminals have been identified in every hypothalamic nucleus but some of the most densely innervated regions are the paraventricular and supraoptic nuclei and the median eminence (Carlsson et al, 1962a & b; Bjorklund et al, 1970; Ungerstedt, 1971; Nobin & Bjorklund, 1973; Lindvall & Bjorklund, 1974; Palkovits & Brownstein, 1989). This will be described in more detail below.

The hypothalamus contains the greatest concentration of adrenaline in the brain, although the adrenaline content of the hypothalamus respresents only 10% of the noradrenaline content (Vogt, 1954; Palkovits & Brownstein, 1989). As described above, axons of the PNMT-

immunoreactive neurones in the C1 and C2 cell groups of the brain stem have been traced to the hypothalamus. In addition, neuronal cell bodies which were immunoreactive to PNMT were identified in the caudal part of the lateral hypothalamus, perifornical nucleus and the zona incerta (Ross et al, 1984; Ruggiero et al, 1985). These cell bodies are not evident in control rats but when animals are pre-treated with colchicine which blocks axonal transport of proteins, many PNMT-immunoreactive cell bodies became evident in cell bodies in the hypothalamus (Ross et al, 1984). This was accompanied by an increase in enzymatic methylating activity in the hypothalamus, confirming that the PNMT-immunoreactive material was bioactive. Surprisingly, the other enzymes of the catecholamine synthetic pathway (TH, LAAD and DBH) were not detectable in these PNMT-immunoreactive cell bodies (Ross et al, 1984; Ruggiero et al, 1985). The role of adrenaline as a neurotransmitter in the hypothalamus is therefore unclear and there are data which suggest that the hypothalamic neurones which contain PNMT are not adrenergic neurones in the conventional sense:

1. The neurotoxin 6-hydroxydopamine depletes hypothalamic adrenaline content but does not destroy PNMT neurones (Jonsson et al, 1976; Reid et al, 1976; Fuller, 1982; Mefford 1987). This suggested that adrenaline may be stored in other neurones.
2. As can be expected, the PNMT neurones of the brain stem contain the other enzymes which are involved in the synthesis of catecholamines (Foster et al, 1985). In contrast, in the immunocytochemical studies on the PNMT neurones in the hypothalamus, the other enzymes of the catecholamine synthesis pathway (TH, LAAD and DBH) could not be detected in the hypothalamic PNMT neurones; as these investigators were able to detect TH, LAAD and DBH in the brain stem of the same animals, it seems reasonable to conclude that these enzymes are either present in very small amounts or are absent from the PNMT neurones in the hypothalamus (Ross et al, 1984; Ruggiero et al, 1985).

A hypothesis was suggested to explain these data (Ross et al, 1984; Mefford, 1987). This is that noradrenaline released from the noradrenergic neurones is accumulated by 'PNMT neurones' and converted to adrenaline. This is then released back into the extracellular space, from where it is accumulated and stored with noradrenaline as a co-transmitter in noradrenergic neurones.This may explain why 6-hydroxydopamine depletes adrenaline and noradrenaline but not PNMT. An alternative possibility which was considered by these investigators is that the PNMT neurones of the hypothalamus may methylate some substrate other than noradrenaline (Ross et al, 1984). Similar speculations were considered regarding

the possible functions of the PNMT neurones in the brain stem of the rat embryo, in view of the relative delay in appearance of tyrosine hydroxylase in these neurones (Foster et al, 1985).

Dopamine is present in all regions of the hypothalamus (Palkovits et al, 1974). In contrast to the noradrenergic innervation of the hypothalamus which is almost all extrinsic, the dopaminergic innervation of the hypothalamus is predominantly intrinsic; the cell bodies of dopaminergic neurones are located predominantly in the periventricular region and in the arcuate nucleus which will be discussed below.

7.1.4 Catecholamine innervation of the supraoptic and paraventricular nuclei

The supraoptic nucleus contains large (magnocellular) vasopressinergic and oxytocinergic neurones which project to the posterior pituitary via the zona interna of the median eminence. A more complex organisation is seen in the paraventricular nucleus, which consists of magnocellular and parvicellular subdivisions (Figure 7.1.3; Swanson & Sawchenko, 1980). Most of the neurones in the magnocellular division of the paraventricular nucleus are vasopressinergic or oxytocinergic cells which project to the neurohypophysis. Separate cell groups within the parvicellular division give rise to projections to the median eminence, the brain stem and the spinal cord. Projections to the brain stem and the spinal cord are believed to be involved in regulating autonomic function. Most of the neurones which project to the zona externa of the median eminence are concentrated in the periventricular and medial parts of the parvicellular division of the paraventricular nucleus; these parvicellular neurones secrete their peptides into the first capillary bed of the hypothalamo-hypophysial portal system and they thus regulate the secretion of the anterior pituitary hormones (Figure 7.1.3; Swanson & Sawchenko, 1980). Afferent inputs from various hypothalamic regions and from the brain stem project to the paraventricular nucleus where the circadian, autonomic and other influences are coordinated into a coherent neuroendocrine response.

The distribution of catecholaminergic terminals within the supraoptic and paraventricular nuclei is heterogeneous and each group of peptidergic neurones receives a catecholaminergic innervation from a select group of neurones in the brain stem (McNeill & Sladek, 1980; Sawchenko & Swanson, 1981; Swanson et al, 1981). Approximately 70% of the noradrenergic innervation of the supraoptic and paraventricular nuclei is derived from the A1 region, 20% from the A2 region and only 5% from the locus coeruleus (see below for

further details; Sawchenko & Swanson, 1981). The A1 projection is predominantly directed to magnocellular neurones in the supraoptic and paraventricular nuclei, the A2 projection is predominantly to parvicellular neurones in the paraventricular nucleus and the small projection from A6 (locus coeruleus) is to the periventricular region (Figure 7.1.3; Sawchenko & Swanson, 1981; Cunningham & Sawchenko, 1988). These brain stem projections are both ipsilateral and contralateral (ie, both crossed and uncrossed; Sawchenko & Swanson, 1981 & 1982). Ultrastructural studies demonstrated that noradrenergic (DBH-immunoreactive) neurones form synaptic contacts with the dendrites of the neurones of the paraventricular nucleus (Olschowka et al, 1981).

In contrast to the noradrenergic innervation, the adrenergic (PNMT-immunoreactive) fibres are concentrated in the parvicellular divisions of the paraventricular nucleus; the magnocellular oxytocin and vasopressin neurones in the supraoptic and paraventricular nuclei are sparsely innervated by adrenergic nerve terminals (Figure 7.1.3; Swanson et al, 1981; Liposits et al, 1986a; Cunningham et al, 1990). Adrenergic neurones in each of the C1, C2 and C3 groups contribute to the innervation of the paraventricular nucleus (Sawchenko & Bohn, 1989).

The anatomical relationship of catecholaminergic nerve terminals to identified peptidergic neurones has been studied by the combined use of more than one antiserum or by simultaneously using catecholamine histofluorescence or autoradiographic techniques to identify catecholaminergic neurones. In the supraoptic nucleus, the densest noradrenergic innervation is in the region which contains vasopressin neurones, whereas the oxytocin neurones are much more sparsely innervated by noradrenergic nerve terminals (Figure 7.1.3; McNeill & Sladek, 1980; Swanson et al, 1981; Sladek & Zimmerman, 1982). In the paraventricular nucleus too, the magnocellular vasopressin neurones are densely innervated by noradrenergic nerve terminals whereas the oxytocin neurones are only sparsely innervated (Figure 7.1.3; Swanson et al, 1981; Sladek & Zimmerman, 1982).

The periventricular region and the regions of the paraventricular nucleus which contain parvicellular neurones are also innervated by catecholaminergic nerve terminals (McNeill & Sladek, 1980; Swanson et al, 1981). CRH neurones are present in many parts of the brain stem and the peptide is believed to be involved in mediating the autonomic responses to stressful stimuli (Swanson et al, 1983). In the hypothalamus, the cell bodies of CRH

neurones are located in the medial parvicellular parts of the paraventricular nucleus; the axons project to the zona externa of the median eminence where CRH is secreted into the hypothalamo-hypophysial portal system and stimulates the secretion of ACTH from the anterior pituitary corticotrophs (Figure 7.1.3; Bloom et al, 1982; Swanson et al, 1983). In a subset of these parvicellular CRH neurones, vasopressin is colocalised with CRH in the same neurosecretory vesicles; the proportion of these vasopressin-containing CRH neurones is increased by certain stressful stimuli, such as adrenalectomy (Roth et al, 1982; Tramu et al, 1983; Whitnall et al, 1985). Vasopressin is released into the portal circulation together with CRH, where the two peptides act synergistically in stimulating the secretion of ACTH. In colchicine-treated rats, CRH also appears in magnocellular oxytocin neurones (Dreyfus et al, 1984; Sawchenko et al, 1984). In light microscopic studies, adrenergic (PNMT-immunoreactive) and presumed noradrenergic (TH-immunoreactive) terminals were observed in close proximity to the parvicellular CRH neurones in the paraventricular nucleus (Mezey et al, 1984; Agnati et al, 1985; Liposits et al, 1986a & b). Electron microscopic studies demonstrated specialised synaptic connections between adrenergic or noradrenergic nerve terminals and the CRH neurones (Liposits et al, 1986a & b; Kitazawa et al, 1987).

TRH neurones are present in many parts of the hypothalamus and the brain stem, where the peptide presumably acts as a neurotransmitter or neuromodulator (Jackson et al, 1985). TRH neurones whose cell bodies are located in the periventricular region and in the medial parvocellular part of the paraventricular nucleus project to the zona externa of the median eminence where they secrete TRH into the portal circulation, thus stimulating the secretion of TSH from the thyrotroph cells of the pituitary (Figure 7.1.3; Lechan & Jackson, 1982). In light microscopic studies, these TRH neurones have been shown to be in close contact with PNMT- and DBH-immunoreactive nerve terminals and electron microscopic studies have demonstrated specialised synaptic connections between the TRH neurones or their dendrites and the adrenergic nerve terminals (Shioda et al, 1986; Liposits et al, 1987).

The periventricular region and the adjoining parts of the paraventricular nucleus also contain cell bodies of somatostatin neurones (Figure 7.1.3). Somatostatin functions as an inhibitory peptide in many regions of the nervous system; in addition, the peptide is secreted into the hypothalamo-hypophysial portal system where it inhibits the secretion of growth hormone from the somatotroph cells of the pituitary. In colchicine-treated rats, the cell bodies and the dendrites of the somatostatin neurones in the periventricular region were shown in light

microscopic studies to be in close contact with PNMT-immunoreactive nerve terminals, and electron microscopic studies demonstrated specialised synaptic connections between the somatostatin neurones or their dendrites and the PNMT-immunoreactive nerve terminals (Liposits et al, 1990). The locations of the cell bodies of these PNMT-immunoreactive terminals is unknown but are presumed to be in the brain stem (see above). Such synaptic contacts may form part of the anatomical basis for the influence of the central adrenergic system on the secretion of growth hormone.

The periventricular region of the hypothalamus and the adjoining parts of the paraventricular nucleus also contain cell bodies of dopaminergic neurones which will be discussed below.

The source of the adrenergic and noradrenergic fibres which innervate the paraventricular and supraoptic nuclei was studied in detail in the rat by Sawchenko & Swanson (1982). They used a method which allows the simultaneous localisation within single cells, of an antigen, DBH (using an antiserum to rat adrenal DBH) and a retrogradely transported fluorescent dye, true blue, which was injected in the region of the supraoptic or paraventricular nuclei. Cells which contained both the dye and DBH were considered to be adrenergic or noradrenergic neurones innervating the hypothalamic nuclei. Following identification of the noradrenergic cell groups which innervate the hypothalamic nuclei, an autoradiographic method was used to trace the pathways to the hypothalamus, and to assess the distribution of terminals within the paraventricular and supraoptic nuclei. The autoradiographic method consisted of injection of [^{3}H]proline and [^{3}H]leucine into the areas which had been identified to contain the cell bodies of the adrenergic or noradrenergic neurones by the double marker technique. The radiolabelled amino acids are accumulated by the neurones and are transported anterogradely along the axons, enabling detection of the projections of the neurones which are in the vicinity of the injection sites. Using these methods, the cell bodies of the neurones which innervate the paraventricular nucleus were found in the ventrolateral medulla (A1 and C1), in the medial part of the nucleus tractus solitarius of the dorsomedial medulla (A2 and C2) and in the rostral part of the locus coeruleus (A6). Following injection of true blue into the paraventricular nucleus, 98% of the doubly labelled cells were found within these cell groups. True blue was not found in any of the cells in the A4, A5, A7 (subcoeruleus) or area postrema, indicating that these areas did not contribute to the noradrenergic innervation of the nucleus. Following injection of true blue into the supraoptic nucleus, doubly labelled cells were found in the same areas and in a

similar distribution to the paraventricular nucleus.

The above findings were confirmed and extended in subsequent studies in which the radiolabelled amino acids were substituted with a plant lectin which could be detected immunocytochemically (Cunningham & Sawchenko, 1988; Cunningham et al, 1990). Figure 7.1.3 is a schematic illustration of the conclusions of those studies which can be summarised as follows:

1. The magnocellular vasopressin neurones of the supraoptic and paraventricular nuclei are predominantly innervated by the A1 collection of noradrenergic neurones in the ventrolateral medulla (Figure 7.1.3; Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988; Cunningham et al, 1990).
2. The above neuronal projections are predominantly ipsilateral, although a crossed projection with a similar distribution was also present; the decussation appears to take place within the medulla. The fibres ascend in the ventral noradrenergic bundle and they enter the hypothalamus via the medial forebrain bundle (Sawchenko & Swanson, 1982).
3. The oxytocinergic neurones of the supraoptic and paraventricular nuclei are very sparsely innervated with adrenergic and noradrenergic nerve terminals which are derived from the A2, C1, C2 and C3 areas (Figure 7.1.3; Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988; Cunningham et al, 1990).
4. The parvicellular CRH neurones (some of which also contain vasopressin; see above) are predominantly innervated by the A2 collection of noradrenergic neurones and by the C1, C2 and C3 collection of adrenergic (PNMT immunoreactive) neurones. These axonal projections are predominantly ipsilateral although a small contralateral projection is evident. The axons enter the hypothalamus via the medial forebrain bundle, together with the fibres from the A1 group (Figure 7.1.3; Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988; Cunningham et al, 1990).
5. The locus coeruleus (A6 area) provides the paraventricular nucleus with a small supply of terminals whose distribution is limited to the periventricular zone and the medial parvicellular subdivision of the nucleus which contain cell bodies of dopamine, somatostatin and TRH neurones. The projection is partly crossed, although the ipsilateral projection is denser. The fibres enter the paraventricular nucleus via the medial forebrain bundle (Figure 7.1.3; Sawchenko & Swanson, 1981 & 1982; Cunningham et al, 1990).
6. The locus coeruleus does not supply the supraoptic nucleus (Figure 7.1.3; Sawchenko & Swanson, 1981 & 1982; Cunningham & Sawchenko, 1988).

Much less is known of the dopaminergic innervation of the paraventricular nucleus. Liposits & Paull (1989) carried out unilateral knife cuts in the rostral midbrain, to reduce the ascending adrenergic and noradrenrgic neurones. They found fibres which immunostained for TH but not DBH, and assumed that these were dopaminergic fibres; such fibres were seen in the light microscope to be closely related to CRH neurones in the medial parvicellular part of the paraventricular nucleus. The findings were interpreted as demonstrating the presence of dopaminergic neurones arising in the diencephalon and innervating CRH neurones.

The supraoptic and paraventricular nuclei are some of the most vascular regions of the brain. A small proportion of the catecholaminergic nerve terminals in these nuclei appear to terminate in the regions of small blood vessels and may be involved in regulating the neuroendocrine responses to changes in plasma osmolality and blood pressure (Swanson et al, 1977; Silverman et al, 1985).

7.1.5 Catecholamine innervation of the medial preoptic area

In the rat, the gonadotrophin releasing hormone (GnRH) neurones are scattered in the ventral surface of the brain, from the region of the olfactory bulb rostrally to the arcuate nucleus caudally; the greatest density of these GnRH neurones is in the medial preoptic area in the ventral hypothalamus. The axons project to the zona externa of the median eminence where GnRH is secreted into the first capillary bed of the hypothalamo-hypophysial portal system and functions to stimulate the secretion of the gonadotrophins from the anterior pituitary (Hoffman et al, 1982). In the rat medial preoptic area, the GnRH neurones are in close relationship to nerve fibres which are immunoreactive to TH and are presumed to be noradrenergic neurones (Hoffman et al, 1982). The TH terminals innervate both the cell bodies and the dendrites of the GnRH neurones and specialised synaptic contacts have been observed by electron microscopy (Leranth et al, 1988; Chen et al, 1989). Retrograde studies demonstrated that the noradrenergic innervation of the neurones in the preoptic area is derived from the A1 and A2 noradrenergic areas of the brain stem and is predominantly ipsilateral (Day et al, 1980; Wright & Jennes, 1993). The GnRH neurones also appear to contact dopaminergic neurones of the arcuate nucleus (Hoffman et al, 1982; Leranth et al, 1988). Further, the dopaminergic neurones of the periventricular zone (area A14) project to the medial preoptic area and are presumably involved in regulating the secretion of GnRH in

the rat (Bjorklund et al, 1975; Day et al, 1980).

7.1.6 Catecholamines in the arcuate nucleus and the median eminence

The dopaminergic tuberoinfundibular tract has its cell bodies in the arcuate nucleus of the medial basal hypothalamus (Area A12) near the inferior end of the third ventricle (Figure 7.1.4; Bjorklund et al, 1970 & 1973; Bjorklund & Nobin, 1973). The axons project to the zona externa of the median eminence where they are in close proximity to the first capillary bed of the hypothalamo-hypophysial portal system, into which dopamine is secreted, and functions to inhibit the secretion of prolactin from the anterior pituitary. In addition, dopaminergic neurones with their cell bodies in the arcuate nucleus supply the posterior and intermediate lobes of the rat pituitary gland and appear to be involved in regulating the secretion of melanocyte stimulating hormone in the rat (Ungerstedt, 1971; Bjorklund et al, 1970 & 1973). The intermediate lobe is vestigial in adult humans; in the human fetus, the intermediate lobe is present but is not innervated by catecholamine neurones (Nobin & Bjorklund, 1973).

The dopaminergic neurones in the rat arcuate nucleus are innervated by PNMT-immunoreactive neurones (Hrabovsky & Liposits, 1994). In humans, activation of α_2 adrenoceptors which are located in the brain inhibits the prolactin response to certain stressful stimuli (reviewed in Al-Damluji, 1993); it is possible that such an inhibitory effect of α_2 adrenoceptors may be exerted by adrenaline acting on the dopaminergic neurones which inhibit the secretion of prolactin.

In addition to dopamine, the median eminence also contains a substantial amount of noradrenaline which is located predominantly in the axons of noradrenergic neurones which are in the zona interna (Bjorklund et al, 1970 & 1973). As can be expected, the median eminence also contains a substantial amount of DBH, confirming the presence of noradrenergic neurones (Palkovits & Brownstein, 1989). The cell bodies of the noradrenergic neurones which innervate the zona interna are in the brain stem but their exact location has not been identified; the axons ascend in the ventral noradrenergic bundle to the median eminence but their subsequent destination is unclear (Bjorklund et al, 1970 & 1973). Indirect evidence suggested that some of the noradrenergic innervation of the rat median eminence may be derived from the superior cervical ganglion (Brownstein et al, 1976;

Cardinali et al, 1981; Gallardo et al, 1984); however, detailed studies of the noradrenergic innervation of the median eminence provided no evidence for such innervation (Bjorklund et al, 1973). In any case, such a noradrenergic innervation would presumably be destined for the blood vessels of the median eminence and may be involved in vascular regulation, rather than in direct regulation of hormone secretion.

The arcuate nucleus also contains the cell bodies of growth hormone releasing hormone (GHRH) neurones whose axons project to the zona externa of the median eminence; GHRH secreted into the hypothalamo-hypophysial portal system then stimulates the secretion of growth hormone from the somatotroph cells of the anterior pituitary. The cell bodies and the dendrites of the GHRH neurones are innervated by PNMT-immunoreactive nerve terminals (Liposits et al, 1990). Such a synaptic contact may form part of the anatomical basis for the influence of the central adrenergic system on the secretion of growth hormone.

Most of the GHRH-immunoreactive neurones in the arcuate nucleus are also immunoreactive to TH, implying that they may be able to synthesise and release dopamine; other subsets of dopaminergic neurones have been shown to be immunoreactive to other peptides, such as neurotensin (Meister et al, 1986). The colocalisation of amine and peptide neurotransmitters appears to be a common feature and may serve synergistic or modulatory functions.

7.1.7 Catecholamines in the periventricular, dorsal and caudal hypothalamus

Cell bodies of dopaminergic neurones are scattered in the area which is adjacent to the third ventricle of the rat hypothalamus (Figures 7.1.3 & 7.1.4; Bjorklund & Nobin, 1973). These cells are contiguous to the dopaminergic neurones of the arcuate nucleus but they are distinguishable from the arcuate dopaminergic neurones in that their axons do not project to the median eminence; these periventricular dopaminergic neurones were therefore designated Area A14 (Bjorklund & Nobin, 1973; Bjorklund et al, 1973), to distinguish them from the dopaminergic neurones of the arcuate nucleus which had been designated A12 (Dahlstrom & Fuxe, 1965; Figure 7.1.4). Some of these A14 dopaminergic neurones project to the medial preoptic area which contains the cell bodies of GnRH neurones (Bjorklund et al, 1975). Swanson et al (1981) identified in the periventricular region cell bodies which are immunoreactive to tyrosine hydroxylase but not to DBH or PNMT, and it is possible that these may represent dopaminergic neurones of the A14 group. Some of these dopaminergic neurones were found to project to the medulla and the spinal cord and it is possible that they

may serve some regulatory autonomic function (Swanson et al, 1981). The periventricular dopaminergic neurones appear to be innervated by noradrenergic nerve terminals (Liposits et al, 1986b).

Cell bodies of dopaminergic neurones are also scattered in the posterior hypothalamus and in the zona incerta (Areas A11 and A13; Dahlstrom & Fuxe, 1965; Bjorklund & Nobin, 1973). The axons project diffusely to the anterior and dorsal hypothalamic areas; these are therefore relatively short neurones with a restricted regional distribution (Bjorklund et al, 1975).

The periventricular region and the adjoining parts of the paraventricular nucleus also contain cell bodies of somatostatin and TRH neurones which were discussed above.

7.1.8 Catecholamines in hypophysial portal blood

Dopamine is secreted into hypophysial portal plasma and its role in the tonic inhibition of prolactin secretion is established (Ben Jonathan et al, 1977). The cell bodies of these dopaminergic neurones are in the arcuate nucleus (A12 Area) and the axons project to the zona externa of the median eminence where they secrete dopamine into the first capillary bed of the hypothalamo-hypophysial portal system (see above; Figure 7.1.4).

There was controversy on whether adrenaline is secreted into hypophysial portal plasma in any significant quantities. In the original studies, it was found that concentrations of adrenaline and noradrenaline in rat hypophysial portal plasma were no greater than in peripheral plasma (Ben Jonathan et al, 1977 & 1980; Plotsky et al, 1978; Cramer et al, 1979; Gudelsky et al, 1979; Reymond & Porter, 1982). Subsequently, adrenaline concentrations in plasma from the transected pituitary stalk of anaesthetised rats were reported to be 50-100% greater than the adrenaline concentration in peripheral venous plasma and adrenaline was detectable in adrenalectomised animals. This was interpreted as indicating a central source of adrenaline secretion into the portal circulation (Johnston et al, 1983; Plotsky et al, 1985). The reason for the differences is unclear, but it is possible that the source of the adrenaline in the latter experiments may have been partly derived from the severed stalk nerves containing adrenergic fibres which innervate the posterior and intermediate lobes, as the concentration of adrenaline in plasma from a single portal vessel in the intact stalk is lower than that in peripheral plasma (Reymond & Porter, 1982). Gibbs (1985) suggested that adrenaline may be secreted into hypophysial portal blood during stress but not under basal conditions and

that most of the adrenaline in portal blood originates in the adrenal medulla. Experiments on conscious horses and sheep have shown that noradrenaline concentrations are greater in blood obtained from tributaries of the facial vein (Redekopp et al, 1986) and the lesioned pituitary stalk (Thomas et al, 1989) than in the jugular vein. The source of the noradrenaline is unclear, as it may have originated from sympathetic nerves derived from the superior cervical ganglion. This ganglion also appears to innervate some of the blood vessels in the median eminence (Gallardo et al, 1984). In humans undergoing pituitary surgery, catecholamine concentrations in blood from the lesioned pituitary stalk were greater than in the periphery, but mean concentrations did not exceed 1.5 nmol/l (Paradisi et al, 1989). Greater concentrations of noradrenaline are seen in peripheral blood during stressful stimuli, so it appears unlikely that the anterior pituitary is exposed to much greater concentrations of adrenaline and noradrenaline than are found in the periphery. In rats too, it was concluded that adrenaline in the hypophysial portal circulation is derived mainly from the adrenal glands, and that the hypothalamus makes no significant contribution to portal adrenaline concentration (Pesce et al, 1990). This is in accord with morphological studies showing that PNMT-immunoreactive nerve fibres are scarce in the median eminence, and are confined to the zona interna; PNMT fibres never abutted against the basal membrane of portal capillaries in the zona externa of the median eminence (Bosler et al, 1987).

7.1.9 Catecholamine innervation of the pituitary gland

The mammalian anterior pituitary is derived from the epithelium of the palate. Cajal-Golgi preparations demonstrated that the anterior pituitary is devoid of nerve fibres; the few fibres which were observed appeared to be associated with the blood vessels (Rasmussen, 1938). More recent studies confirmed that the mammalian pituitary is not innervated by catecholaminergic neurones (Bjorklund et al, 1967; Saavedra et al, 1975). In the human fetus, the intermediate lobe is not innervated by catecholamine neurones (Nobin & Bjorklund, 1973); the intermediate lobe becomes vestigial and is not present in adult humans. The human posterior pituitary is derived from the brain and is innervated by catecholamine nerve terminals (Nobin & Bjorklund, 1973).

The neural and intermediate lobes of the rat contain significant quantities of dopamine; noradrenaline is present in approximately 10% of the dopamine content, and adrenaline is either present in very small amounts or is undetectable (Bjorklund et al, 1967; Saavedra et al, 1975; Saavedra , 1985). The dopaminergic innervation of the rat intermediate and posterior

lobes is derived entirely from neurones of the arcuate nucleus (the tuberoinfundibular tract, ie A12 area in Figure 7.1.4). These dopaminergic neurones reach the posterior and intermediate lobes via the zona interna of the median eminence and the pituitary stalk (Bjorklund et al, 1970 & 1973; Saavedra, 1985). The dopaminergic nerve terminals were observed in the vicinity of the endocrine cells of the intermediate lobe and dopamine regulates the secretion of melanocyte-stimulating hormone in the rat (Bjorklund et al, 1973).

While the dopaminergic neurones appear to innervate the secretory cells of the intermediate lobe, the noradrenergic nerve terminals were observed only in the vicinity of blood vessels in the posterior lobe and in the border between the posterior and intermediate lobes (Bjorklund et al, 1973). The source of this noradrenergic innervation of the posterior pituitary has not been settled; in one detailed study, the conclusion was that most of the noradrenergic innervation of the posterior and intermediate lobes was derived from the superior cervical ganglion (Bjorklund et al, 1973); this would be in accord with the perivascular location of the noradrenergic nerve terminals, suggesting a sympathetic function. However, in other studies, the conclusion was that the noradrenergic innervation of the posterior pituitary is both from the brain and from the superior cervical ganglion (Alper et al, 1980; Saavedra, 1985). In any case, the noradrenergic innervation of the posterior pituitary and the bordering region of the intermediate lobe may be involved in regulating vascular tone, but it is unlikely to be important in directly influencing the secretion of pituitary hormones at the level of the pituitary.

Figure 7.1.1:

A. The condensation of adrenaline with sodium hydroxide to form a fluorescent isoquinoline derivative. This is probably the basis of the fluorescent assay for adrenaline which was developed by Gaddum & Schild (1934). These authors noted that noradrenaline was incapable of making a fluorescent derivative in this reaction; this is presumably due to absence of an N-alkyl group which is necessary for generation of isoquinoline.

B and C. The condensation of noradrenaline with formaldehyde (Falck, 1962; Falck et al, 1962) or with glyoxylic acid (Bjorklund et al, 1972; Lindvall et al, 1974) to form fluorescent isoquinoline derivatives. These reactions are the chemical basis of the histofluorescence methods which were used to map the distribution of catecholamines in the brain.

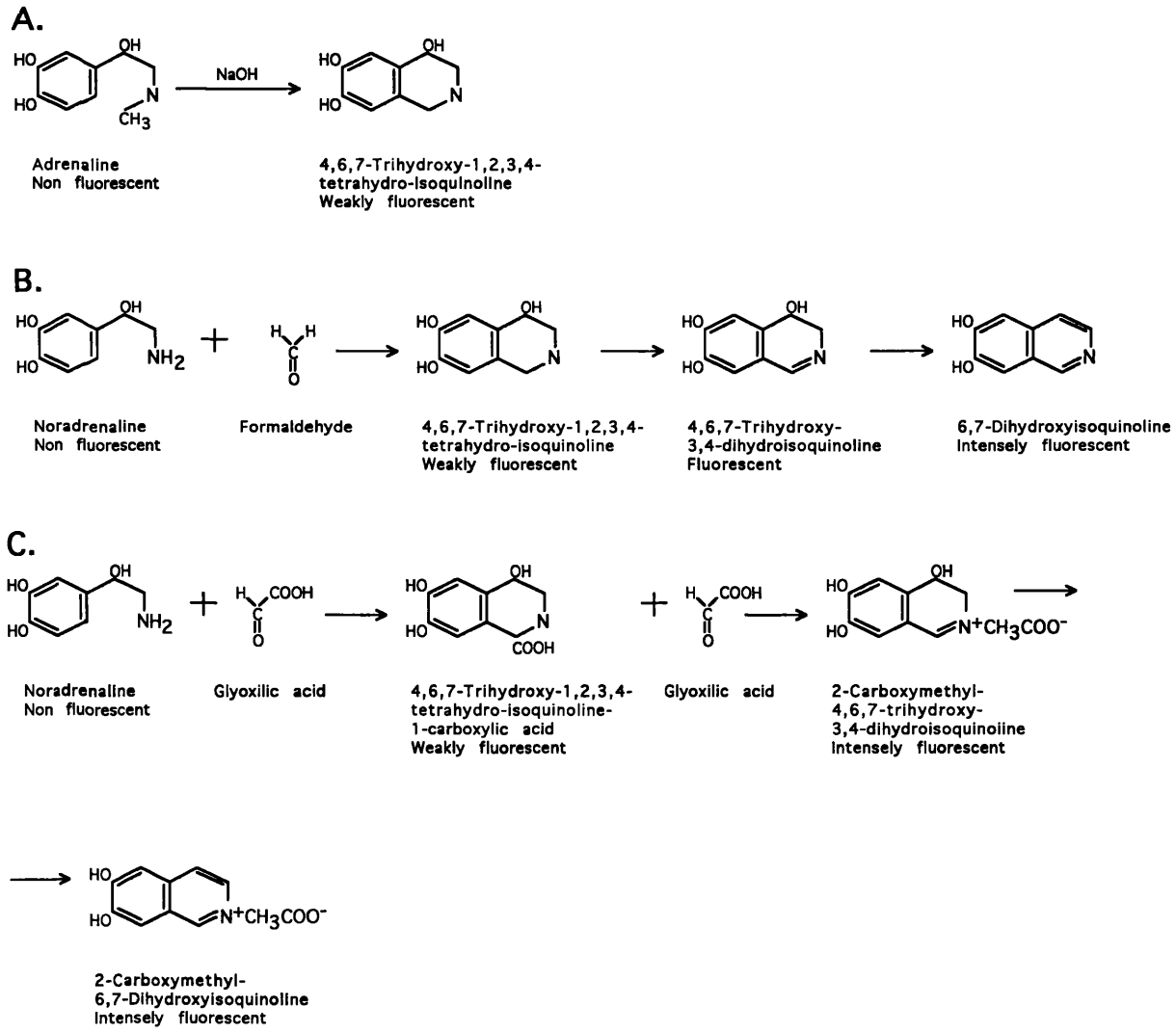
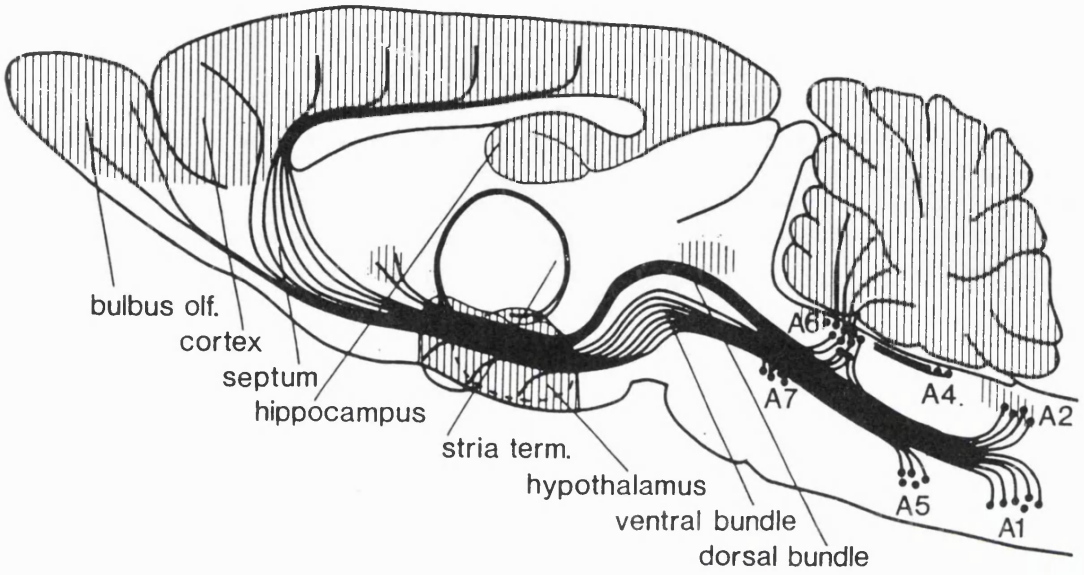
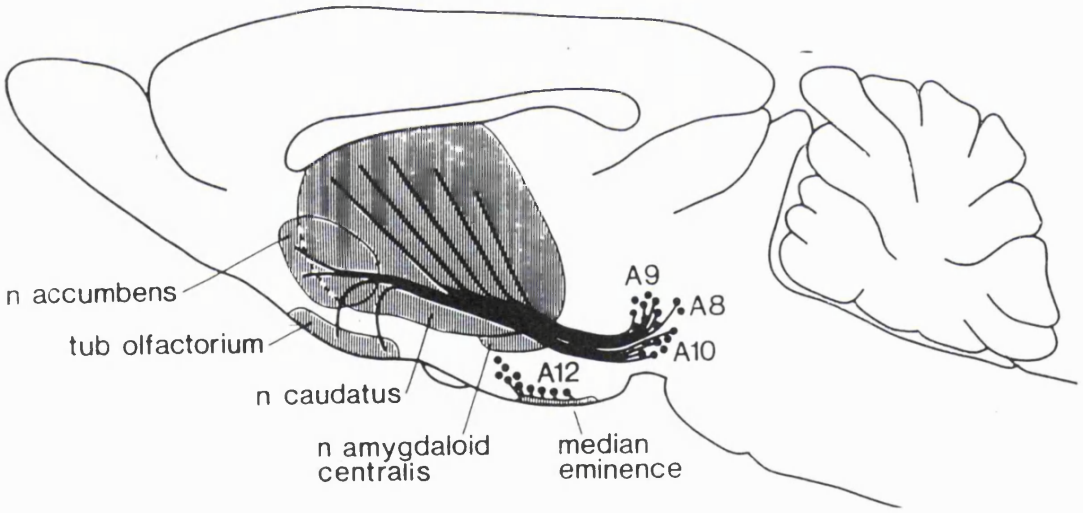


Figure 7.1.2:
A. The origin and distribution of noradrenaline in rat brain. From Ungerstedt, 1971.



B. The origin and distribution of dopamine in rat brain. From Ungerstedt, 1971.



C. Schematic representation of adrenaline in rat brain. Reproduced from Hokfelt et al, 1974

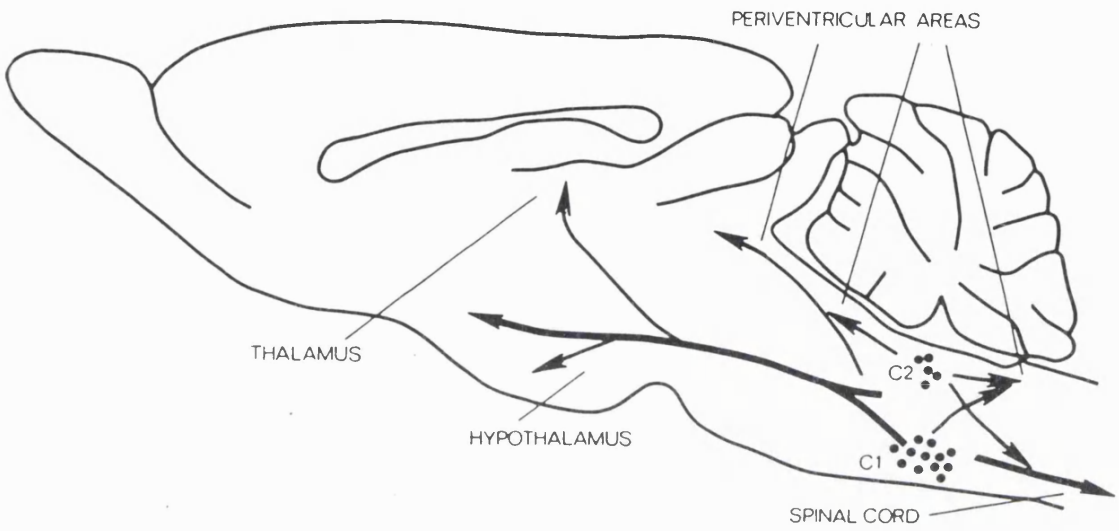


Figure 7.1.3:

The adrenergic and noradrenergic innervation of the hypothalamic paraventricular and supraoptic nuclei. Major innervations are represented by heavy lines and minor innervations by broken lines. In the rat brain, the C1 cell group is adjacent to the A1 group; it has been included with C2 and C3 in this Figure for convenience. For details, see text. Based on Cunningham & Sawchenko (1988) and Cunningham et al (1990).

Abbreviations: A1, A2 and A6 = noradrenergic neuronal groups of the ventrolateral medulla, the dorsomedial medulla and the locus coeruleus, respectively; AVP = magnocellular vasopressinergic neurones; C1, C2 and C3 = medullary groups of PNMT-immunoreactive neurones; crf = parvicellular CRH neurones; crf/avp = parvicellular neurones which contain both CRH and vasopressin; DA = dopaminergic neurones; OXY = magnocellular oxytocinergic neurones; PVN = paraventricular nucleus; SON = supraoptic nucleus; SS = somatostatinergic neurones; TRH = thyrotrophin-releasing hormone neurones.

Reproduced from Al-Damluji, 1993.

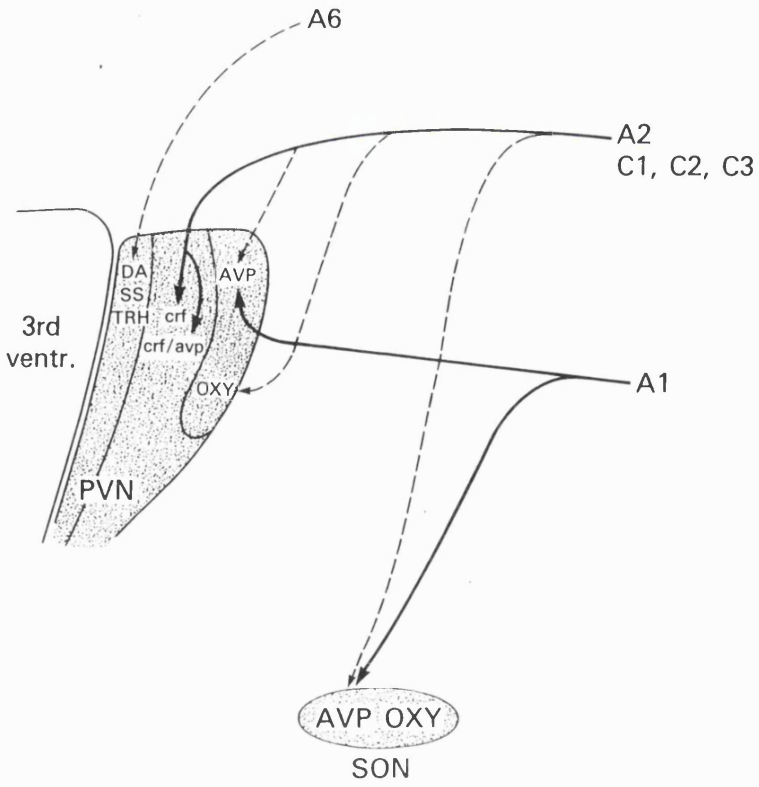
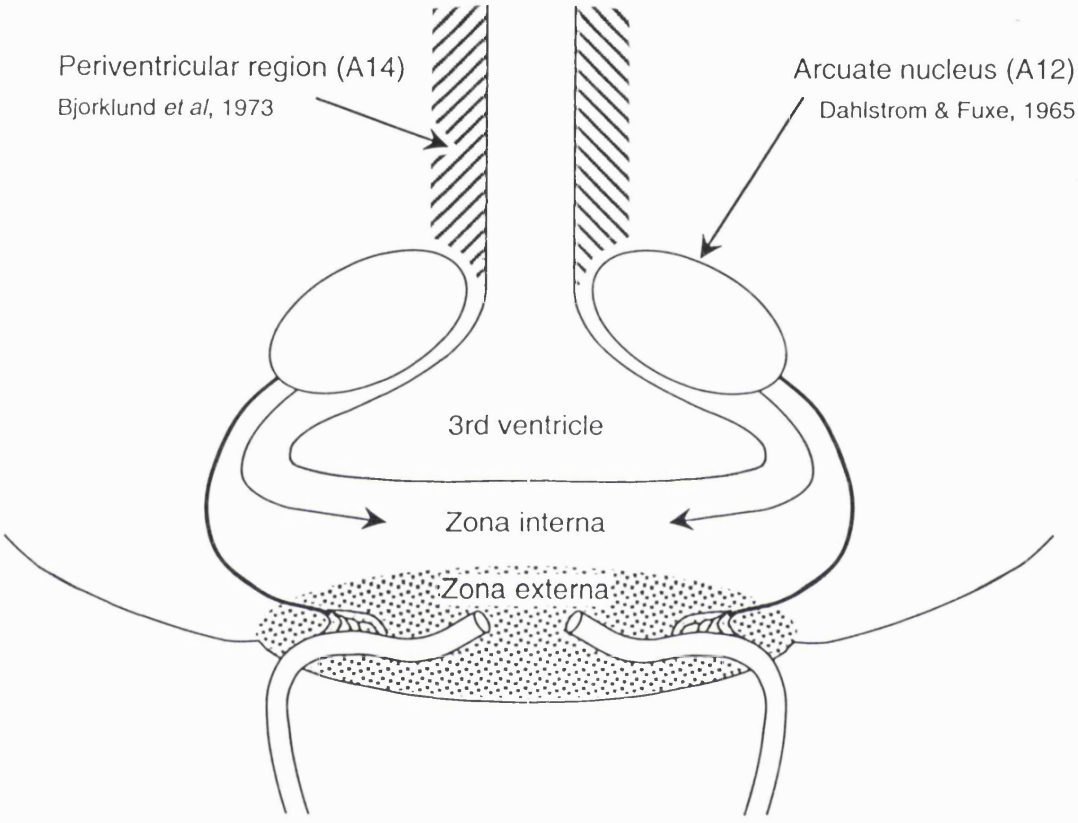


Figure 7.1.4:

Schematic representation of a coronal section through the rat medial basal hypothalamus, demonstrating the positions of the dopaminergic nuclei. Based on Bjorklund et al, 1973.



7.2 ALPHA-1 ADRENOCEPTORS IN PEPTIDERGIC NEURONES

7.2.1 Discovery of alpha-1 adrenoceptors

The concept of receptors was introduced in 1905 by J.N. Langley, Professor of Physiology in the University of Cambridge. Earlier, Langley had discovered that nicotine causes contraction of skeletal muscle and that the effect is antagonised by curari. He then discovered that "Degeneration of the nerves supplying the muscles leaves essentially unaltered the effects described above; but there is evidence of an increased responsiveness to nicotine. Since there is evidence that the axon-endings in skeletal muscle degenerate after section of the nerves supplying the muscle, I conclude that nicotine and curari do not act on the axon-endings but on the muscle itself. Further, since both nicotine and curari prevent nervous impulses from affecting the contractile substance, but do not prevent the muscle from contracting on direct stimulation, I conclude that the poisons do not act directly on the contractile substance, but on other substances in the muscle which may be called *receptive substances*. And as adrenalin, an internal secretion, acts upon receptive substance, it is probable that secretin, thyroidin, and the internal secretion formed by the generative organs, also act on receptive substances, although in these cases the cells may be unconnected with nerve fibres. The receptive substance of cells, even of the same class, varies considerably. This I consider is mainly due to an inherent tendency to variation in the chemical nature of the cells, so that even in the same class of cell the receptive substances formed are commonly not identical" (Langley, 1905).

In the above passage, Langley was introducing a coherent concept which we now recognise as the principle of receptors. He was describing post-synaptic receptors, the actions of agonists and antagonists, down-regulation and super-sensitivity of receptors, and the heterogeneity of receptors in different cell types. Langley's ideas had a profound impact both directly and via his students, TR Elliott, HH Dale, O Loewi, AV Hill and JH Burn. Elliott noted that "the reaction of any plain muscle to adrenalin is of a similar character to that following excitation of the sympathetic nerves supplying that muscle"; hence, "Adrenalin might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery" (Elliott, 1904 & 1905). This suggestion that sympathetic nerves release adrenaline which then acts on the muscles was the first formulation of the concept of chemical transmission.

During his studentship at Trinity College Cambridge, Hill carried out a mathematical analysis

of the contractile response to nicotine. He found that the data were consistent with a model in which the drugs combined with some constituent of muscle, which was presumably the receptive substances which had been postulated by Langley; gradual diffusion of these drugs into the cells could not account for the data (Hill, 1909). Hill's mathematical analysis (known as the Law of Mass Action) remains the mathematical basis for the quantitative study of the interaction of ligands with receptors (see below). The concept of receptors was developed further by Clark (1933) who considered that "the simplest probable conception of drug action is that potent drugs occupy certain specific receptors on the cell surfaces, and that these specific receptors only comprise a small fraction of the total cell surface". He postulated that the response to a drug such as adrenaline was proportional to the number of receptors occupied by the drug, and that this explains concentration-response curves (Clark, 1933).

Langley was aware of the heterogeneity of receptors (see above). Dale provided evidence for the existence of more than one type of adrenergic receptor; he observed that certain ergot alkaloids converted the vasopressor effect of adrenaline into a vasodepressor effect (Dale, 1906). He explained the effects of ergot by suggesting that the blood vessels contain "both motor and inhibitory" receptors and that the "inhibitor constituent is normally obscured by the preponderant motor effect". The modern interpretation of Dale's experiments is that adrenaline activates both α_1 adrenoceptors which are vasoconstrictor and β_2 adrenoceptors which are vasodilator; ergot alkaloids block α_1 adrenoceptors, allowing the vasodilator action of β_2 adrenoceptors to become apparent. The terminology which is currently used in the classification of adrenoceptors was initiated by Ahlquist (1948). He examined the effects of a series of six sympathomimetic amines on a number of tissues and found that these tissues could be divided into two groups on the basis of the order of potency of the sympathomimetic amines. He considered that there were at least two distinct general types of receptors which he named "alpha", mediating "excitatory functions" and "beta" which mediate "inhibitory functions" (Ahlquist, 1948).

The subdivision of α adrenoceptors was stimulated by the work of Brown & Gillespie at University College London. They found that α antagonists increased noradrenaline release elicited by nerve stimulation. They attributed this to occupancy of the tissue receptors by the

antagonist preventing the noradrenaline from combining with the receptor and resulting in overflow of the neurotransmitter (Brown & Gillespie, 1957). However, subsequent investigations demonstrated that noradrenaline release is regulated by a negative feedback mechanism mediated by α adrenoceptors which are situated on the noradrenergic neurones (Langer, 1977). The present interpretation of Brown & Gillespie's work is that α antagonists block pre-synaptic α adrenoceptors which inhibit noradrenaline release, and that this accounts for the increased release of noradrenaline during nerve stimulation. These "pre-synaptic" receptors therefore mediate a different function from the "post-synaptic" α adrenoceptors which mediate muscle contraction. In addition, the two types of receptors can be distinguished by the relative potencies of adrenergic drugs which act on them. In particular, phenoxybenzamine was a more potent antagonist of post-synaptic receptors than of pre-synaptic receptors; it was therefore suggested that the post-synaptic receptors should be designated "α_1" and the pre-synaptic receptors which are situated on the noradrenergic neurones "α_2" (Langer, 1974). Wikberg (1978) consolidated this classification by suggesting that α_1 adrenoceptors should be defined as those at which phenylephrine is more potent than clonidine, whereas these two agonists act in the reverse order of potency at α_2 adrenoceptors. It later became clear that α_2 adrenoceptors may also exist post-synaptically (Drew & Whiting, 1979; McGrath, 1982; Hieble et al, 1986a). Three subtypes of α_2 adrenoceptors have been cloned and their physiological significance in regulating noradrenaline release has been confirmed using gene knockout experiments in transgenic animals (Kobilka et al, 1987; Regan et al, 1988; Lomasney et al, 1990; Hein et al, 1999).

7.2.2 Binding studies for α_1 adrenoceptors

Binding studies on α adrenoceptors were carried out with the aim of identifying and isolating the receptors which had been postulated by Langley. The first attempts to carry out radioligand binding studies on α adrenoceptors were performed in the 1960s and were based on the experience which had been obtained in studying the active sites of enzymes (Moran et al, 1967). In those enzymatic studies, substantial progress had been made in the use of irreversibly acting substrate analogues which bind covalently to parts of the active centres of the enzymes. By analogy, alkylating α adrenergic antagonists such as [^{3}H]dibenamine, [^{3}H]SY28 and [^{14}C]phenoxybenzamine were used to label the α adrenoceptors in strips of various tissues (Dikstein & Sulman, 1965; Moran et al, 1967; Terner et al, 1971; Yong & Nickerson, 1973). However, the association of the radiolabel with the tissue strips was not saturable and pretreatment of the tissue strips with unlabelled adrenergic drugs was not always effective in preventing subsequent alkylation by the radiolabelled drug (Moran et al, 1967; Terner et al, 1971; Yong & Nickerson, 1973). These results were attributed to labelling of non-specific sites and to interference of the neuronal and extraneuronal noradrenaline uptake systems ($Uptake_1$ and $Uptake_2$) in these tissue strips. The specific radioactivity of the radioligands was very low (less than one mCi/mmole) so large amounts of radioligand had to be used, and this presumably increased non-specific binding (Terner et al, 1971).

Binding studies on cellular fractions were more successful. [^{3}H]Adrenaline and [^{3}H]noradrenaline bound to liver and spleen plasma membranes and were displaced by unlabelled adrenergic drugs; however, binding of the radioligand was still not saturable (Tomasi et al, 1970; Fiszer De Plaza & De Robertis, 1972). Subsequently, U'Prichard & Snyder (1977a&b) found that performing the binding assays in the presence of catechol reduced non-specific binding and improved the assay; under these conditions, [^{3}H]adrenaline and [^{3}H]noradrenaline bound to calf brain membranes in a saturable, stereoselective manner and the radioligands were displaced by appropriate α adrenergic drugs in the correct order of potency which is characteristic of α adrenoceptors, as determined in physiological assays. Binding to beta adrenoceptors could not be detected in

the presence of catechol (U'Prichard & Snyder, 1977a&b). Depletion of brain catecholamines by chronic treatment with reserpine or 6-hydroxydopamine increased the density of [^{3}H]adrenaline binding sites, suggesting that the [^{3}H]catecholamine binding assays were measuring physiologically relevant receptors which could be up-regulated by depletion of brain catecholamines (U'Prichard & Snyder, 1978; U'Prichard et al, 1979). Despite these improvements, the "specific" binding represented only 500 cpm out of "total" binding of approximately 1000 cpm (U'Prichard & Snyder, 1977a&b). The assays were therefore not sufficiently robust and further improvements were required.

The first successful binding assays for alpha adrenoceptors were reported in 1976. [^{3}H]Dihydroergocryptine ([^{3}H]DHE) was prepared at New England Nuclear by catalytic reduction of the α adrenergic antagonist ergocryptine (Figure 7.2.1). Tritium gas was used and the catalyst was paladium (Williams & Lefkowitz, 1976). The double bond in the 9,10 position was reduced, forming the dihydroergot alkaloid which is a more potent antagonist than ergocryptine. The resulting radioligand had a specific activity of 25 Ci/mmole (Williams & Lefkowitz, 1976). As the intrinsic activity of ^{3}H is 29 Ci/atom, this implied that on average, one atom of ^{3}H had been incorporated into each molecule of [^{3}H]DHE. Binding of [^{3}H]DHE to rabbit uterine membranes was rapid, reversible and saturable and the radioligand was displaced in a stereospecific manner by adrenaline and noradrenaline. [^{3}H]DHE (at 8 nM) was also displaced by α adrenergic agonists and antagonists but not by inactive catecholamine precursors or metabolites (Williams et al, 1976). The relative affinities of the α adrenergic agonists and antagonists for the [^{3}H]DHE binding site in rabbit uterine membranes were similar to their relative affinities as agonists or antagonists in physiological preparations. Further, the structural properties of ligands for the [^{3}H]DHE binding site in rabbit uterine membranes were similar to the structural properties of α adrenergic agonists and antagonists, as determined in physiological preparations (Williams et al, 1976). In rabbit uterine membranes, [^{3}H]DHE was displaced not only by drugs which act on α_1 adrenoceptors (eg, methoxamine, phenylephrine, phenoxybenzamine and prazosin) but also by compounds which act on α_2 adrenoceptors (eg, clonidine and yohimbine). This indicated

that [^{3}H]DHE binds both to α_1 and α_2 adrenoceptors (Williams et al, 1976; Hoffman et al, 1979).

The success of the [^{3}H]DHE method was attributed to the relatively high specific activity of [^{3}H]DHE (enabling use of relatively low concentrations of the radioligand with consequent reduction of non-specific binding) and to the use of membrane preparations rather than whole strips of tissues (Williams et al, 1976). However, [^{3}H]DHE was not an ideal radioligand for studying α adrenoceptors in the brain, as it bound non-selectively to α_1 and α_2 adrenergic as well as to dopaminergic and serotonergic receptors which may not be present in rabbit uterus (Williams et al, 1976; Hoffman et al, 1979). Subsequent investigators used lower concentrations of [^{3}H]DHE (0.3 nM) and were more successful in using this radioligand for labelling α adrenoceptors in brain membranes; however, it was not possible to abolish completely the binding of this compound to serotonergic and dopaminergic receptors (Greenberg & Snyder, 1978).

Also in 1976, [^{3}H]dihydroazapetine was prepared by catalytic reduction of the α_1 adrenoceptor antagonist azapetine. This radioligand had a specific activity of 49 Ci/mmole and was shown to bind to rat vas deferens membranes in a manner which was saturable and reversible with appropriate adrenergic drugs (Ruffolo et al, 1976). [^{3}H]Clonidine (specific activity 1.6 Ci/mmole) and [^{3}H]WB4101 (specific activity 7 Ci/mmole) were used to label α adrenoceptors in rat brain membranes in a saturable manner which was stereoselectively reversible with adrenergic drugs; it was thought initially that these two ligands preferentially labelled agonist and antagonist states of the same α adrenoceptor (Greenberg et al, 1976; U'Prichard et al, 1977). The current interpretation is that [^{3}H]WB4101 preferentially labels α_1 adrenoceptors whereas [^{3}H]clonidine preferentially labels α_2 adrenoceptors (U'Prichard et al, 1978a).

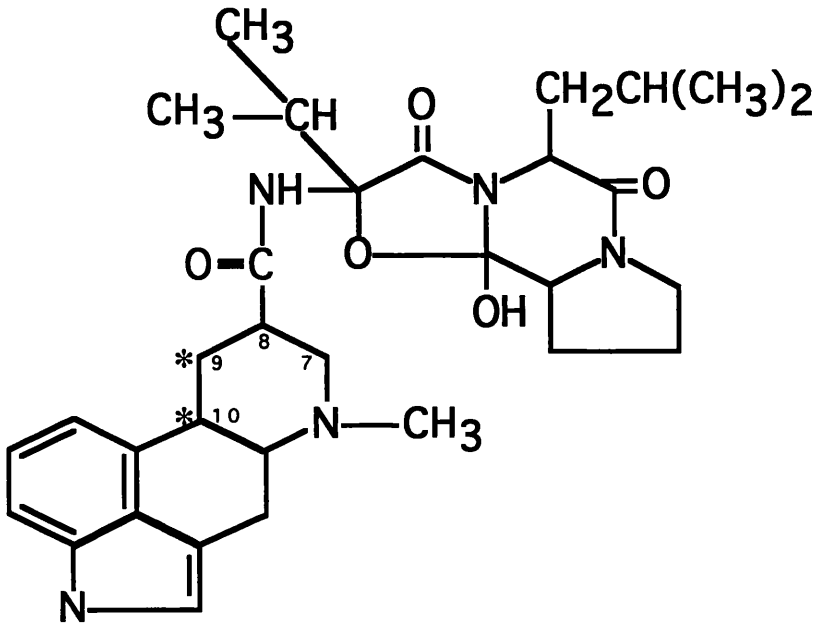
The first compound which was used in selective labelling of brain α_1 adrenoceptors was

[^{3}H]prazosin. Prazosin had been prepared at Pfizer as a phosphodiesterase inhibitor but it was subsequently found to be a highly selective α_1 adrenergic antagonist. In 1979, [^{3}H]prazosin was prepared at Amersham International by catalytic reduction of bromoprazosin with tritium gas (Greengrass & Bremner, 1979). The compound was labelled in the 7-methoxyl group and its specific activity was 75 Ci/mmole (Figure 7.2.1). This implied that on average, three atoms of ^{3}H (ie, one methoxyl group) had been incorporated into each molecule of [^{3}H]prazosin. Binding of [^{3}H]prazosin to rat brain membranes was rapid, saturable and reversible by the α antagonist phentolamine (Greengrass & Bremner, 1979). α_1 adrenergic antagonists were more potent than α_2 antagonists in displacing [^{3}H]prazosin from rat brain membranes. This indicated that rat brain membranes contain binding sites which have simlar characteristics to peripheral α_1 adrenoceptors (Greengrass & Bremner, 1979).

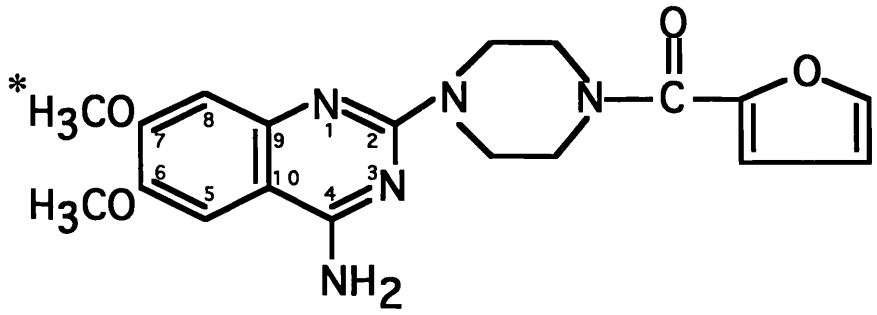
In 1981, a second selective α_1 adrenoceptor radioligand was prepared; it was originally designated [^{125}I]BE-2254 but is now generally known as [^{125}I]HEAT (Engel & Hoyer, 1981; Glossman et al, 1981). The parent compound (HEAT; 2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone) had been prepared at Beiersdorf in Germany as an α_1 adrenoceptor antagonist which was 100-fold more potent at α_1 than at α_2 adrenoceptors (Benthe et al, 1972; Heinz & Hofferber, 1980). As HEAT possesses a hydroxyl group in the *para* position of the phenyl ring, it was possible to label it with ^{125}I using the chloramine T method (Figure 7.2.1). As ^{125}I has much greater intrinsic activity than ^{3}H (2250 vs 29 Ci/atom, respectively), this enabled the production of a radioligand which had far greater specific activity than [^{3}H]DHE or [^{3}H]prazosin. Thus, the specific activity of [^{125}I]HEAT was 2175 Ci/mmole. [^{125}I]HEAT bound specifically to rat brain membranes; as in the case of [^{3}H]prazosin, binding of [^{125}I]HEAT was rapid, saturable and reversible with phentolamine, and α_1 adrenergic antagonists were more potent than α_2 antagonists in

displacing [^{125}I]HEAT from rat brain membranes (Engel & Hoyer, 1981; Glossman et al, 1981). However, [^{125}I]HEAT has some disadvantages: only 50% of [^{125}I]HEAT is available for binding to α_1 adrenoceptors, probably because this radioligand is prepared as a racemic mixture (Glossman et al, 1981), and [^{125}I]HEAT decomposes to [^{125}I]tyramine (Glossman et al, 1981). Further, despite its potency at α_1 adrenoceptors, HEAT has significant activity at α_2 adrenoceptors and dopaminergic receptors (Gothert et al, 1981; Clineschmidt et al, 1975; Williams et al, 1978). However, some investigators have felt that these disadvantages of [^{125}I]HEAT may be outweighed by its very high specific activity.

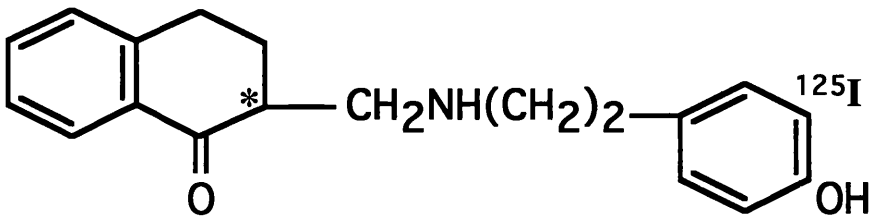
Figure 7.2.1:
Radioligands for α_1 adrenoceptors



[^{3}H]Dihydroergocryptine (asterisk is a position of ^{3}H)



[7-Methoxy-^{3}H]Prazosin (asterisk is position of ^{3}H)



[^{125}I]HEAT (asterisk is the asymmetrical carbon)

7.2.3 Subtypes of α_1 adrenoceptors

Using radioligand binding techniques, two methods were used to differentiate α_1 from α_2 adrenoceptors. In the earlier method, a non-selective radioligand such as [^{3}H]dihydroergocryptine ([^{3}H]DHE) was used to label both α_1 and α_2 adrenoceptors, and the two types of receptors were then distinguished by the relative potencies of a selection of unlabelled agonists and antagonists in displacing the radioligand. Unlabelled prazosin and yohimbine were usually used to define displacement from α_1 and α_2 adrenoceptors, respectively (Miach et al, 1978; Hoffman et al, 1979). In a later method, a radioligand which was selective for one of the two types of α adrenoceptors was used either to saturate the receptors, or in competition with a selection of unlabelled ligands. [^{3}H]WB4101, [^{3}H]Prazosin and [^{125}I]HEAT were used to label α_1 adrenoceptors (U'Prichard et al, 1978a; Greengrass & Bremner, 1979; Hornung et al, 1979; Engel & Hoyer, 1981; Glossman et al, 1981) and [^{3}H]clonidine, [^{3}H]yohimbine and [^{3}H]idazoxan were used to label α_2 adrenoceptors (U'Prichard et al, 1978a; Hoffman et al, 1981; Lane et al, 1983).

Radioligand binding studies which utilised non-selective α adrenoceptor ligands (such as [^{3}H]adrenaline, [^{3}H]noradrenaline, [^{3}H]dihydroergocryptine, [^{3}H]clonidine and [^{3}H]WB4101) indicated that there are two binding sites in membranes from rat brain and liver (Greenberg et al, 1976; Peroutka et al, 1978; El-Refai et al, 1979). Some of these binding sites presumably represented α_1 and α_2 adrenoceptors (Miach et al, 1978), although it was later shown using the antagonists prazosin and yohimbine that [^{3}H]adrenaline and [^{3}H]dihydroergocryptine bind predominantly to α_1 adrenoceptors in rat liver (Hoffman et al, 1979; El-Refai & Exton, 1980a). Subsequent studies using the selective α_1 adrenoceptor ligand [^{3}H]prazosin suggested that there may be two α_1 adrenoceptor binding sites in rat liver membranes. However, it was thought that these were different states of a single α_1 adrenoceptor whose affinity for the ligands may have been modulated by guanine nucleotide binding proteins (G-proteins; El-Refai et al, 1979; Geynet et al, 1981). One type of binding

site was thought to be the physiologically active receptor which binds agonists such as adrenaline with high affinity, and the other was thought to be an inactive binding site which has low affinity for adrenaline and which preferentially binds antagonists (Peroutka et al, 1978; El-Refai et al, 1979; El-Refai & Exton, 1980a; Battaglia et al, 1983a). Trypsin was thought to convert the inactive form of the receptor to the active form, so it was presumed that the inactive form of the receptor was a precursor for the physiologically active form (El-Refai & Exton, 1980b). However, other pieces of evidence did not fit this hypothesis; thus, the two binding sites existed in different proportions in various brain regions and the cerebellum appeared to possess only the agonist-preferring binding sites (Peroutka et al, 1978). Further, there was no evidence of cooperativity between the two binding sites, as the Hill coefficients for ligand binding were approximately 1.0; these findings were thought to be more consistent with the presence of discrete agonist and antagonist-preferring receptors which were not convertible (U'Prichard & Snyder, 1977b; U'Prichard et al, 1977; Greenberg & Snyder, 1978).

In the early 1980s, it became clear that the α adrenoceptors in some tissues could not be classified strictly as α_1 or α_2 on the basis of the potency of agonists and antagonists (Downing et al, 1980; Randriantsoa et al, 1981; Ruffolo et al, 1982; McGrath, 1982; Holck et al, 1983; Agrawal et al, 1984; Drew, 1985; Flavahan & Vanhoute, 1986; Hieble et al, 1986b). In 1982, Coates, Jahn & Wheetman provided evidence that the α_1 adrenoceptors in the rat anococcygeus muscle are a heterogeneous population. They suggested the designation "α_{1s}" adrenoceptors for a subtype which has high sensitivity for the agonist Sgd101/75 and to the alkylating antagonist phenoxybenzamine (Coates et al, 1982). They later confirmed this conclusion using another alkylating antagonist, benextramine (Coates & Wheetman, 1983). Subsequent investigators found that there were small, but consistent differences in the potencies of agonists which activate various pharmacological effects of α_1 adrenoceptors in rat liver and brain (Morgan et al, 1983; Johnson & Minneman, 1986); this was consistent with the suggestion of Coates et al (1982) that these agonists may act on more than one type of α_1 adrenoceptors which differ in their affinities for the agonists.

The current designations "α_{1A}" and "α_{1B}" for subtypes of α_1 adrenoceptors were suggested

by Morrow and Creese. They had found that [^{3}H]prazosin binds to rat brain membranes and is displaced by the antagonist WB4101 in a biphasic manner, suggesting the existence of two [^{3}H]prazosin binding sites with different affinities for WB4101. They concluded that rat brain contains two subtypes of α_1 adrenoceptors. In one subtype, the order of potency of antagonists in displacing [^{3}H]prazosin is WB4101>prazosin>phentolamine>indoramine> dihydroergocryptine. In the second subtype, the order is prazosin>indoramine> dihydroergocryptine>WB4101>phentolamine (Morrow et al, 1985). Those two binding sites were later designated "α_{1A}" and "α_{1B}", respectively (Morrow & Creese, 1986).

Chlorethylclonidine is an analogue of clonidine which possesses an aliphatic alkylating group in the *para* position of the aromatic ring (Figure 7.2.2). This alkylating function makes the compound a long-acting, irreversible adrenergic agonist (Leclerc et al, 1980). Johnson & Minneman (1987) found that chlorethylclonidine inactivated some, but not all α_1 adrenoceptor binding sites in membranes prepared from rat brain. Further studies demonstrated that the α_1 adrenoceptors in other tissues (such as liver and spleen) were also heterogeneous in their sensitivity to inactivation by chlorethylclonidine (Han et al, 1987a).

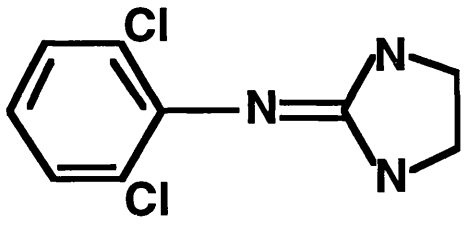
Other functional properties of α_1 adrenoceptor sub-types were described by Han et al (1987b) who reported that contraction of the spleen was mediated by receptors whose binding characteristics were of the α_{1B} subtype and that these receptors exerted their intracellular effects by mobilising intracellular calcium via hydrolysis of inositol phospholipids. In contrast, contraction of the rat vas deferens was mediated by α_{1A} adrenoceptors which opened dihydropyridine-sensitive plasma membrane calcium channels. Thus, the subtypes of α_1 adrenoceptors were distinguishable by their anatomical distribution, binding affinities and by the second messenger systems to which they are linked (Minneman, 1988; Minneman et al, 1988). These conclusions were confirmed by Tsujimoto et al (1989) who found that phosphorylation of glycogen in rat hepatocytes was activated by chlorethylclonidine-sensitive α_1 adrenoceptors which are linked to hydrolysis of

phosphatidyl inositol (α_{1B} subtype) whereas glycogen phosphorylation in rabbit aorta is activated by chlorethylclonidine insensitive α_1 adrenoceptors which are linked to entry of extracellular calcium (α_{1A}). Methoxamine was a more potent agonist at α_{1A} than at α_{1B} adrenoceptors (Tsujimoto et al, 1989). These studies formed the basis for the current classification of α_1 adrenoceptors which is summarised in Table 7.2.1 (Ford et al, 1994; Hieble et al, 1995a). This classification was subsequently underpinned by the molecular cloning of cDNAs which encode α_1 adrenoceptor subtypes, as described in the following Section.

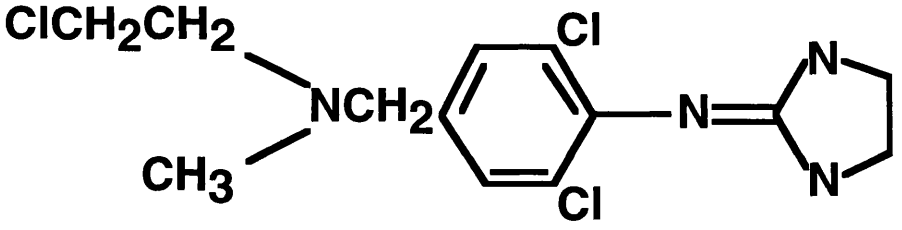
In summary, pharmacological studies which were performed in the 1980s indicated that there were at least two subtypes of α_1 adrenoceptors: α_{1A} adrenoceptors were the predominant type in the aorta; they were characterised by greater affinity for WB4101 than prazosin, weak inactivation by chlorethylclonidine and were believed to be linked to dihydropyridine-sensitive plasma membrane calcium channels. α_{1B} adrenoceptors predominated in the liver; they were characterised by greater affinity for prazosin than WB4101, strong inactivation by chlorethylclonidine and were believed to be linked to hydrolysis of inositol phospholipids.

The above subtypes of α_1 adrenoceptors have high affinity for [^{3}H]prazosin. In an alternative classification, α_1 adrenoceptors have been divided into classes which are based on their affinities for prazosin; receptors which have high or low affinity for prazosin are designated α_{1H} and α_{1L}, respectively (Muramatsu et al, 1994; Hieble et al, 1995b); however, cDNAs for α_1 adrenoceptors corresponding to these classes have not been cloned.

Figure 7.2.2:
Clonidine and its alkylating analogue chlorethylclonidine



Clonidine



Chlorethylclonidine

Table 7.2.1: Nomenclature for α1 adrenoceptor subtypes*

α_1 adrenoceptor subtype			Human chromosome location	Chlorethyl-clonidine sensitivity	Selective antagonist	Order of antagonist potency	Predominant second messenger
Native	Cloned new name	Cloned old name					
α_{1A}	α_{1a}	α_{1c}	C8	weak	SNAP-5089 (+)Niguldipine 5-Methylurapidil Indoramin	WB4101> prazosin> phentolamine> indoramine> dihydroergocryptine	Plasma membrane calcium channels
α_{1B}	α_{1b}	α_{1b}	C5	very strong		prazosin> indoramine> dihydroergocryptine> WB4101> phentolamine	Hydrolysis of inositol lipids
α_{1D}	α_{1d}	α_{1a}, α_{1d} $\alpha_{1a/d}$	C20	strong	BMY 7378 SK&F 105854	prazosin> WB4101> 5-methylurapidil> (+)niguldipine> phentolamine	

*Based on Hieble et al, 1995a and Perez et al, 1991.

7.2.4 Isolation of cDNA clones encoding α_1 adrenoceptors

The purification of α_1 adrenoceptor proteins was made possible by the development of photoaffinity labels for these receptors (Leeb-Lundberg et al, 1984; Dickinson et al, 1984). [^{3}H]Phenoxybenzamine was known to be an irreversible ligand for α_1 adrenoceptors. However, this compound binds to many other receptors and its affinity for α_1 adrenoceptors is poor (Yong & Nickerson, 1973). Therefore, new photoaffinity probes were developed, based on the selective α_1 adrenoceptor antagonist, prazosin. In these probes, the furan group in prazosin was substituted by an aryl azide which enables cross-linking following exposure to ultraviolet light, resulting in irreversible incorporation of the radioligand into the receptor. The aryl group can also be labelled with ^{125}I to enable detection of the radioligand with high specific radioactivity (Figure 7.2.3). These compounds bind to α_1 adrenoceptors with high affinity and with similar characteristics to prazosin (Leeb-Lundberg et al, 1984; Dickinson et al, 1984).

The first α_1 adrenoceptor to be cloned was the α_{1b} adrenoceptor (Cotecchia et al, 1988). DDT_1 MF-2 Syrian hamster smooth muscle cells had been shown to possess α_1 adrenoceptors (Cornett & Norris, 1982). The cells were grown in 10 litres of suspension cultures and cell membranes were prepared from 1600 litres of cultured cells in 100 litre batches. From those membranes, the α_1 adrenoceptor was solubilised in the detergent digitonin and one nmole of α_1 adrenoceptor was purified by affinity chromatography, wheat germ agarose chromatography and gel permeation HPLC (Lomasney et al, 1986). The affinity ligand in these experiments was A55453 coupled to Sepharose (Figure 7.2.3). The molecular weight of the purified peptide was 80 kDa (Cotecchia et al, 1988). The purified α_1 adrenoceptor was then cleaved with cyanogen bromide, the digest was fractionated by HPLC and the amino acid sequence was obtained by using an automated amino acid sequencer. An oligonucleotide was made, based on the sequence of one of the peptides. The oligonucleotide was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used as a probe to screen a hamster genomic library. One positive clone of 1.6 kb was identified, representing part of

the sequence of the α_1 adrenoceptor. This clone was labelled with [α-^{32}P]dCTP using random primers and used as a probe to screen a cDNA library which had been constructed from DDT_1 MF-2 cell RNA. A single clone containing an insert of 2 kb was identified and sequenced by Sanger's dideoxy chain termination method (Cotecchia et al, 1988). After a short 5' untranslated region, an initiator methionine was followed by a coding region of 1545 bp, followed by a 3' untranslated region of 543 bp. The deduced amino acid sequence is a peptide of 515 residues and a molecular weight of 56 kDa (Cotecchia et al, 1988). Hydrophobicity analysis revealed seven clusters of 20-25 hydrophobic residues separated by stretches of hydrophilic residues. The hydrophobic regions were thought to represent membrane-spanning domains which were connected by three extracellular and three cytoplasmic loops (Cotecchia et al, 1988). The amino terminus was thought to be extracellular and the carboxy terminus was thought to project into the cytoplasm. There were potential sites for asparagine-linked glycosylation, which was consistent with the fact that the α_1 adrenoceptor is a glycosylated peptide. These features were shared with rhodopsin and other G protein-coupled receptors. Indeed, this α_1 adrenoceptor had significant sequence homology with β adrenergic, α_2 adrenergic, serotonergic and muscarinic receptors. The regions of greatest sequence homology were in the membrane-spanning domains, whereas the extracellular and cytoplasmic loops and the amino and carboxy termini were more divergent (Cotecchia et al, 1988). The sequence of the α_1 adrenoceptor contained several serine and threonine residues which may be sites for phosphorylation by protein kinase A and which may represent mechanisms for regulation of the function of the receptor. The cDNA encoding the α_1 adrenoceptor was then inserted into a mammalian expression plasmid which was transfected into COS-7 cells using the DEAE dextran technique. Transfected cells were able to bind the α_1 adrenergic antagonist [^{125}I]HEAT with high affinity and the radioligand was displaced by α_1 adrenergic agonists and antagonists with the appropriate order of affinities (Cotecchia et al, 1988). The expressed receptor had low affinity for the antagonist WB4101, suggesting that it corresponds to the native α_{1B} subtype of α_1 adrenoceptors. In transfected COS-7 cells, exposure to noradrenaline caused a marked increase in the phosphorylation of phosphatidyl-inositol, confirming that the expressed

receptor is functional (Cotecchia et al, 1988). This clone is now designated the α_{1b} cDNA and is believed to correspond to the native α_{1B} adrenoceptor (Hieble et al, 1995a).

In 1990, a second subtype of α_1 adrenoceptor was cloned; it was originally designated "α_{1c}" but is now known as "α_{1a}". This clone was obtained by screening a human genomic library with an oligonucleotide probe which had been labelled with ^{32}P using T4 polynucleotide kinase. The sequence of the probe was derived from the hamster α_{1b} cDNA (Schwinn et al, 1990a). A single positive clone was obtained, the sequence of which included an open reading frame which encoded a peptide whose sequence was 67% homologous to the hamster α_{1b} sequence. This incomplete clone was then used as a probe to screen a bovine brain cDNA library. A single positive clone was obtained, containing an insert of 3.1 kb. The open reading frame encoded a peptide of 466 amino acids with a molecular mass of 51 kDa. 72% of the amino acids in the membrane-spanning domains of this receptor were identical to the corresponding regions of the hamster α_{1b} adrenoceptor; this degree of homology is similar to the homologies of the membrane-spanning domains of subtypes of β adrenoceptors and α_2 adrenoceptors (Schwinn et al, 1990a). The most divergent regions of these two α_1 adrenoceptor subtypes were the amino terminus (27% amino acid identity) and the carboxy terminus (12% identity). As in the hamster α_{1b} sequence, there were asparagine, serine and threonine residues which are presumably sites for glycosylation and phosphorylation. Probes derived from the two receptors were then used for human somatic cell hybridisation; this showed that the gene corresponding to the hamster α_{1b} receptor was located on human chromosome 5, whereas the gene corresponding to the bovine receptor was located on human chromosome 8; this confirmed that these two clones represented separate receptors, rather than species homologues of the same receptor. A fragment of the clone representing the coding and untranslated regions was inserted into a mammalian expression plasmid and COS-7 cells were transfected with the construct using the DEAE dextran method. The bovine receptor had 10-fold greater affinity than the hamster α_{1b}

receptor for the antagonist WB4101 and was relatively resistant to inactivation by chlorethylclonidine, confirming that these two clones encoded two different receptors (Schwinn et al, 1990a). The relative insensitivity to inactivation by chlorethylclonidine and greater affinity for WB4101 suggested that the bovine clone may correspond to the α_{1A} adrenoceptor; however, the tissue distribution of RNA in this initial study appeared to be different from the expected tissue distribution of α_{1A} adrenoceptors, so this new bovine clone was initially thought to represent a new subtype of adrenoceptors which was designated α_{1c} (Schwinn et al, 1990a). Subsequent studies using Northern blots and RNAse protection assays have demonstrated that the RNA corresponding to the bovine clone is in fact expressed in tissues which are appropriate to α_{1A} adrenoceptors (Faure et al, 1994; Price et al, 1994a); therefore, it is now thought that this bovine clone in fact corresponds to the native α_{1A} adrenoceptor, and has consequently been re-designated the α_{1a} adrenoceptor cDNA (Ford et al, 1994; Hieble et al, 1995a).

The third clone encoding an α_1 adrenoceptor was isolated in 1991 (Lomasney et al, 1991). A rat cerebral cortex cDNA library was screened at low stringency with a cDNA probe derived from the hamster α_{1b} adrenoceptor cDNA. Two clones were isolated, one of which represented the rat homologue of the hamster α_{1b} adrenoceptor cDNA. The other clone contained a 1680 bp open reading frame encoding a protein of 560 amino acids. This protein also contained seven hydrophobic regions which are presumed to be membrane-spanning domains. 73% of the amino acids in these hydrophobic regions were identical to the amino acids in the corresponding regions of the α_{1b} clone, and 65% were identical to the corresponding regions of the bovine α_{1a} receptor. In situ hybridisation in human cells localised the gene encoding this third receptor to chromosome 5, regions q23-q32. This region also contains the genes which encode the α_{1b} and β_2 adrenoceptors, suggesting that this family of proteins may have arisen by gene duplication. The genes encoding the three subtypes of α_1 adrenoceptors were unique in the adrenoceptor family in that they contained

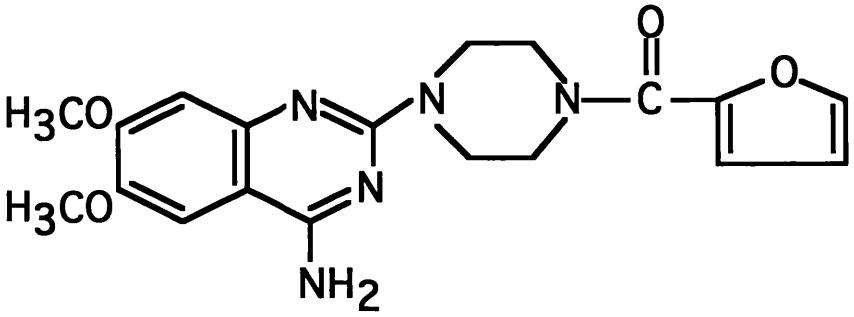
introns; the genes encoding the other six α_2 and β receptors are intronless (Lomasney et al, 1991). The cDNA of this third receptor was then inserted in a mammalian expression plasmid and transfected into COS-7 cells using the DEAE dextran method. The α_1 adrenergic ligand [^{125}I]HEAT bound to transfected cells with high affinity and was displaced by unlabelled α_1 adrenergic agonists and antagonists. The potencies of these compounds at the three subtypes of adrenergic receptors expressed in COS-7 cells are given in Table 7.2.2. When this third clone was expressed in COS-7 cells, it appeared to encode a receptor which had high affinity for the antagonist WB4101 and was relatively resistant to inactivation by chlorethylclonidine. This cDNA was therefore originally designated the "α_{1A}" adrenoceptor clone (Lomasney et al, 1991). However, Perez et al (1991) independently isolated the same cDNA from a rat hippocampus cDNA library, and they recognised that several pharmacological features of the expressed receptor were different from the α_{1A} adrenoceptor which had been characterised in native tissues; they therefore suggested that the cDNA may encode a novel receptor which they designated the "α_{1D}-adrenergic receptor". Subsequent investigators gave this third clone the compromise designation " $\alpha_{1A/D}$". However, more recent work demonstrated that the clone which corresponds to the native α_{1A} receptor is in fact the bovine cDNA (see above), so the third " $\alpha_{1A/D}$" rat clone was re-designated the α_{1d} adrenoceptor and is now recognised as encoding a receptor which had not been identified in tissue membrane preparations (Ford et al, 1994; Hieble et al, 1995a).

In a comparative study which used ribonuclease protection assays, most rat tissues which were examined contained RNA encoding all three subtypes of α_1 adrenoceptors (Price et al, 1994a). However, RNA for α_{1A} adrenoceptors was found in greatest abundance in vas deferens, cerebral cortex, hippocampus, submaxillary gland and the heart; RNA for α_{1B} adrenoceptors was in greatest abundance in the heart, liver, cerebral cortex and kidney; RNA for α_{1D} adrenoceptors was in greatest abundance in hippocampus, vas deferens and cerebral cortex (Price et al, 1994a). A similar distribution of RNA for these adrenoceptor subtypes

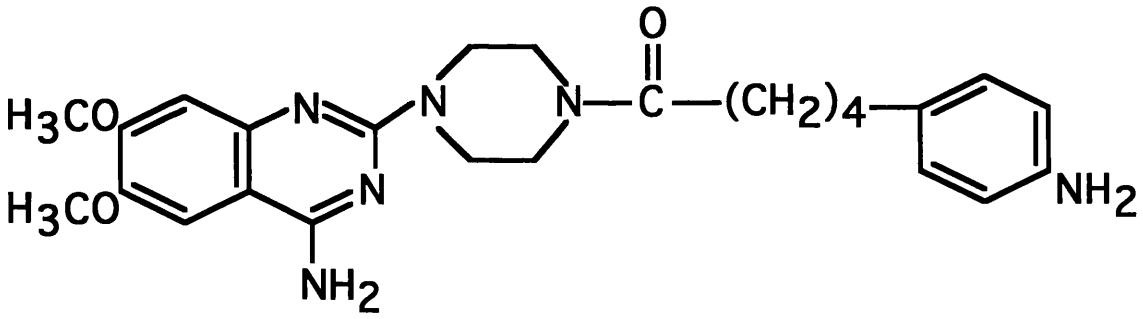
was reported in a study which utilised Northern blot analysis of rat tissues (Faure et al, 1994). This distribution is similar to the distribution in rat tissues of the α_1 adrenoceptor subtypes which had been identified by pharmacological methods (Minneman, 1988). *In situ* hybridisation studies on rat brain using oligonucleotide and ribonucleotide probes demonstrated that RNA for the α_{1B} and α_{1D} subtypes were present in discrete regions of the cerebral cortex, thalamus, amygdala, hippocampus, olfactory system and various regions of the brain stem (Nicholas et al, 1991; McCune et al, 1993; Day et al, 1997a). In a detailed *in situ* hybridisation study on rat brain using ribonucleotide probes, RNA encoding the α_{1A} subtype was found in greatest density in the olfactory system, hypothalamus, brain stem and the spinal cord, particularly in regions which are related to motor function (Day et al, 1997a). RNA encoding α_{1D} adrenoceptors was the least widespread and was undetectable in many regions of the rat brain (Day et al, 1997a).

Clones have been isolated representing the human homologues for all three subtypes of α_1 adrenoceptors (Weinberg et al, 1994). RNAse protection assays revealed that the distribution of α_1 adrenoceptor subtype RNA in human tissues is different from the rat; in humans, RNA for the α_{1A} subtype is most abundant in the liver, heart, cerebral cortex and cerebellum (Price et al, 1994b; Weinberg et al, 1994). In the human prostate, the predominant subtype is the α_{1A} adrenoceptor and these receptors may be involved in benign prostatic hyperplasia (Price et al, 1993). RNA for α_{1B} adrenoceptors is most abundant in human spleen, kidney and cerebellum, whereas RNA for α_{1D} adrenoceptors is most abundant in human cerebral cortex, adrenal, heart and spleen (Price et al, 1994b; Weinberg et al, 1994).

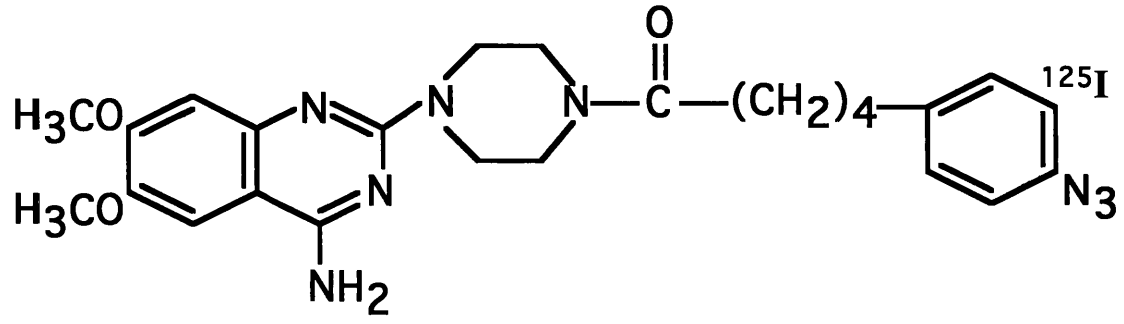
Figure 7.2.3:
Affinity ligands for alpha-1 adrenoceptors



Prazosin



A-55453



[^{125}I]APDQ

Table 7.2.2

Pharmacological profile of adrenergic agonists and antagonists at expressed α_1 adrenoceptor clones. The data represent KD (nM) values for displacement of [^{125}I]HEAT from membranes of COS-7 cells transfected with clones containing the three cDNAs encoding α_1 adrenoceptors. (Reproduced from Lomasney et al, 1991).

Compound	α_{1a} (originally α_{1C})	α_{1b} (originally α_{1B})	α_{1d} (originally α_{1A})
Agonists:			
(-)Adrenaline	6,250	4,690	546
(-)Noradrenaline	9,730	10,500	100
Methoxamine	203,000	1,610,000	110,000
Phenylephrine	478,800	23,900	1,440
Oxymetazoline	114	824	2,140
Antagonists:			
Prazosin	0.37	0.56	0.33
Phentolamine	15.30	340.00	111.00
Indoramine	Not done	226.00	611.00
Corynanthine	142.00	517.00	253.00
WB4101	0.68	28.60	2.10

7.2.5 α_1 adrenergic receptors in the hypothalamus

The first studies which examined the presence of α_1 adrenoceptors in the hypothalamus were performed by binding of [^{3}H]dihydroergocryptine to homogenised rat hypothalamic membranes; specific binding was detectable and appeared to be sensitive to manipulations which affected the status of gonadal steroids (Wilkinson et al, 1979a & b). Administration of the noradrenergic neurotoxin 6-hydroxydopamine was followed by a modest increase in the density of [^{3}H]WB4101 binding sites in membranes of rat hypothalamus, suggesting that the α_1 adrenoceptors can be up-regulated by depletion of brain catecholamines (U'Prichard et al, 1980). α_1 adrenoceptor binding sites were also detected in the rat hypothalamus by autoradiography of slide-mounted sections, using [^{3}H]WB4101 as the radioligand; however, the sensitivity of the early methods was low because of the relatively low specific activity of the radioligand, necessitating exposure of the tissue sections for three months (Young & Kuhar, 1980). In subsequent autoradiographic studies, [^{125}I]HEAT was used as the radioligand; the greater specific activity of this compound provided increased sensitivity and the sections required exposure for only three days (Jones et al, 1985). Autoradiographic studies which utilised [^{3}H]prazosin as the radioligand demonstrated that α_1 adrenoceptor binding sites were present in all nuclei of the hypothalamus (Rainbow & Biegon, 1983). The regional distribution of α_1 adrenoceptor binding sites within the hypothalamus was also studied using the punch biopsy microdissection technique which had been developed by Palkovits; [^{3}H]WB4101 binding sites were present in most hypothalamic nuclei but were lowest in the median eminence (Leibowitz et al, 1982).

In the rat hypothalamus, approximately 30% of the α_1 adrenoceptor binding sites can be inactivated by chlorethylclonidine and are resistant to WB4101, and are therefore presumed to be α_{1B} adrenoceptors (Johnson & Minneman, 1987; Blendy et al, 1990; Petitti et al, 1992). *In situ* hybridisation studies using ribonucleotide probes confirmed that the hypothalamus has a greater density of RNA encoding α_{1A} than α_{1B} adrenoceptors; RNA encoding α_{1D} adrenoceptors was scarce and was undetectable in most regions of the rat

hypothalamus (Day et al, 1997a).

7.2.5.1 The hypothalamic preoptic area

In the adult rat, the gonadotrophin-releasing hormone (GnRH) neurones are located in the pre-optic area of the hypothalamus. This area has been shown to possess binding sites for [^{3}H]WB4101 and [^{3}H]prazosin (Leibowitz et al, 1982; Petitti et al, 1992). Approximately 30% of the [^{3}H]prazosin binding sites in the pre-optic area can be inactivated by chlorethylclonidine and are therefore presumed to be α_{1B} adrenoceptors (Petitti et al, 1992). These chlorethylclonidine-sensitive binding sites have low affinity for the antagonist WB4101, which is consistent with the suggestion that they are α_{1B} adrenoceptors (Petitti et al, 1992). mRNA encoding α_{1B} adrenoceptors was detected in the pre-optic area using reverse-transcriptase (RNA) polymerase chain reactions (Karkanias et al, 1996). *In situ* hybridisation studies using ribonucleotide probes demonstrated that the pre-optic area contains RNA encoding both α_{1A} and α_{1B} adrenoceptors but RNA encoding α_{1D} adrenoceptors was not detectable in the rat pre-optic area (Day et al, 1997a).

The α_1 adrenergic agonist phenylephrine has no effect on the production of c-AMP in the pre-optic area of ovariectomised rats. However, phenylephrine augments the c-AMP response to the β adrenergic agonist isoprenaline in the pre-optic area (Petitti et al, 1992). Oestradiol enhances this augmenting effect of phenylephrine whereas progesterone diminishes the effect of phenylephrine in these rats (Petitti et al, 1992). Chlorethylclonidine abolished the ability of phenylephrine to augment the c-AMP response to isoprenaline in the pre-optic area, suggesting that the effect of phenylephrine is mediated by α_{1B} adrenoceptors (Petitti et al, 1992). Oestradiol increases the density of these chlorethylclonidine-sensitive binding sites but has no effect on the chlorethylclonidine-insensitive sites (presumed to represent α_{1A} adrenoceptors; Petitti et al, 1992). Oestradiol also increases the density of mRNA encoding α_{1B} adrenoceptors in the pre-optic area, suggesting that the effects of this hormone may be exerted on the transcription of the α_{1B} adrenoceptor gene (Karkanias et al,

1996). Progesterone had no effect on the density of either sub-type of α_1 adrenoceptors and neither hormone had any effect on the affinity of prazosin for these binding sites. Progesterone reduced the inositol phosphate response to activation of α_1 adrenoceptors, suggesting that the inhibitory effect of progesterone may be exerted on the second messenger systems which mediate the effects of α_1 adrenoceptors (Karkanias et al, 1995).

Taken together, the data suggest that in ovariectomised rats, activation of α_{1B} adrenoceptors in the hypothalamic pre-optic area activates phospholipase-C which leads to hydrolysis of phosphatidylinositol, generation of inositol trisphosphate and consequent augmentation of the c-AMP response to activation of β adrenoceptors. Oestrogens enhance this interaction by increasing the density of α_{1B} adrenoceptors, whereas progestogens diminish the interaction by interfering with the second messenger systems which mediate the effects of the α_{1B} receptors (Petitti et al, 1992; Karkanias et al, 1996). These effects of the gonadal steroids may be important in regulating reproductive function in the rat.

7.2.5.2 The hypothalamic paraventricular nucleus

α_1 adrenergic binding sites are present in high density in the paraventricular nucleus of the rat hypothalamus (Leibowitz et al, 1982), including the regions which are immunoreactive to corticotrophin-releasing hormone and vasopressin neurophysin (Cummings & Seybold, 1988). *In situ* hybridisation studies on rat brain using ribonucleotide probes demonstrated that the paraventricular nucleus contains predominantly RNA encoding α_{1A} adrenoceptors and smaller quantities of RNA encoding α_{1B} adrenoceptors; RNA encoding α_{1D} adrenoceptors was not detectable in the rat paraventricular nucleus (Day et al, 1997a). RNA for α_{1A} adrenoceptors is located predominantly in magnocellular neurones, whereas RNA for α_{1B} adrenoceptors is predominantly in the parvicellular neurones (Day et al, 1997a). Studies on the colocalisation of RNA for α_1 adrenoceptor subtypes and hypothalamic peptides have been performed, using the dual probe *in situ* hybridisation technique. This

revealed that RNA for corticotrophin-releasing hormone was colocalised with both α_{1A} and α_{1B} adrenoceptors (Day et al, 1997b). This suggests that these adrenoceptor subtypes may be involved in the central adrenergic regulation of the hypothalamo-pituitary adrenal axis in the rat. The role of α_1 adrenoceptors in the control of the hypothalamo-pituitary adrenal axis will be discussed in detail in following sections.

7.2.5.3 The median eminence of the hypothalamus

α_1 adrenoceptor binding sites are present in the median eminence but their density is lower than in other hypothalamic regions (Leibowitz et al, 1982). Some of these α_1 adrenoceptors appear to be innervated by the superior cervical ganglion, as destruction of this ganglion results in an increase in the density of the binding sites in the median eminence, presumably indicating up-regulation of receptors on denervated cells (Cardinali et al, 1981). The function of these receptors is unknown, although they may be involved in vascular regulation in the median eminence.

7.2.5.4 The pituitary gland

The pituitary gland does not contain RNA for any of the three subtypes of α_1 adrenoceptors (Lomasney et al, 1991; Schwinn et al, 1990b). α_1 adrenoceptor binding sites are undetectable in homogenates of the porcine anterior pituitary (Battaglia et al, 1983b), and autoradiographic studies on fixed sections of rat pituitary did not demonstrate α_1 adrenoceptors in the anterior lobe (DeSouza & Kuyatt, 1987). α_1 adrenoceptor binding sites are detectable in homogenates of the porcine neurointermediate lobe (Battaglia et al, 1983a), and autoradiographic studies on rat pituitaries have shown α_1 adrenoceptor binding sites only in the posterior lobe; these appear to be innervated by the superior cervical ganglion rather than from the brain (DeSouza & Kuyatt, 1987). Surprisingly, α_1 adrenoceptor binding sites become detectable in anterior pituitary cells in primary culture (Giguere et al, 1981; Peters et al, 1983). Appearance of the α_1 adrenoceptors in cultured cells may be due to up-regulation of the receptors, in the absence of catecholamines in the culture medium.

7.3 THE ADRENERGIC CONTROL OF ACTH SECRETION

7.3.1 Hypothalamic control of secretion of the adrenocorticotrophic hormone

The hypothalamus secretes a mixture of peptides which act synergistically with each other to stimulate the secretion of the adrenocorticotrophic hormone (ACTH). These peptides are referred to as the 'corticotrophin-releasing factor complex' (Gillies et al, 1982). Two of the main constituents of this complex are corticotrophin-releasing hormone (CRH) and vasopressin. The synergistic action of these two peptides has been shown to be important in the physiological control of ACTH secretion (Linton et al, 1985). Several other hypothalamic peptides can stimulate the secretion of ACTH but their physiological significance has not been established. It is possible that the hypothalamus may release different ACTH secretagogues in response to different stimuli (Plotsky et al, 1989). Although CRH is much more potent than vasopressin *in vitro* (Vale et al, 1983), the two peptides are equipotent in stimulating ACTH secretion when injected into rats *in vivo* (Rivier & Vale, 1983). This difference is likely to be due to exogenous vasopressin interacting synergistically with endogenous CRH, when the former peptide is injected *in vivo* (Rivier & Vale, 1983).

The cell bodies of the CRH neurones are in the parvicellular part of the paraventricular nucleus, and the axons project to the zona externa of the median eminence where the hormone is secreted into portal plasma. Under basal conditions, approximately half the CRH neurones also contain vasopressin, but following adrenalectomy, vasopressin appears in most of the CRH neurones (Whitnall et al, 1987). This indicates the ability of these peptidergic neurones to respond to perturbations of homeostasis by appropriately altering the identity of their neurosecretory products. Vasopressin is also present in the magnocellular neurones of the supraoptic and paraventricular nuclei, whose axons project to the posterior pituitary. The secretory products of the posterior pituitary can reach the anterior lobe via the short portal vessels which connect these two lobes (Oliver et al, 1977). CRH is also present in some of the magnocellular oxytocinergic neurones (Dreyfuss et al, 1984; Sawchenko et al, 1984) which may play a role in the control of ACTH secretion.

7.3.2 Background

The adrenergic control of secretion of the adrenocorticotrophic hormone was of particular interest, as this was a controversial subject in neuroendocrinology. The historical development of this field has been described (Al-Damluji, 1988). Briefly, Selye (1936) reported that adrenaline injections in rats were followed by enlargement of the adrenal cortex

and involution of the thymus, and that the effect was much reduced by prior hypophysectomy. He described this phenomenon as an 'alarm reaction' to a toxic substance. However, Long believed that circulating adrenaline which is derived from the adrenal glands was a major physiological stimulus to ACTH secretion; he stated that "stimulation of the elements of the autonomic nervous system with concomitant release of epinephrine that occurs under a variety of conditions appears to be a major factor in the activation of the adrenocorticotrophic secretion from the anterior lobe" of the pituitary (Long, 1947). Marthe Vogt examined the effect of denervation of the adrenals on the adrenocortical response to a variety of stressful stimuli. She reported that adrenocortical activity was independent of adrenal innervation; conversely, she found that in some circumstances, massive adrenaline secretion can take place without causing adrenocortical activation. She concluded that "stress did not require release of adrenaline to bring about the action of the suprarenal cortex" (Vogt, 1947). Geoffrey Harris, reflecting on the conflict of views before his 'neurovascular hypothesis' had been widely accepted, wrote: "Shortly after these studies I made my first visit to the United States, where I had the pleasure and distinction of being able to meet workers who, with their colleagues, had put forward three different views of the mechanisms controlling ACTH secretion. First, in the University of Utah, Salt Lake City, I met Dr George Sayers who had proposed that the rate of secretion of ACTH was primarily governed by the circulating blood level of adrenal steroids. Then in the University of Yale, New Haven, I met Dr CNH Long who believed that an increased secretion of adrenaline under conditions of stress, was a primary factor in mediating increased ACTH discharge. And thirdly, at Harvard, I met Dr Hume who, independently from De Groot and myself, had produced evidence from experiments in dogs that stimulation of the hypothalamus evoked ACTH secretion. For some time then, three views on the ACTH control mechanism were discussed-the feedback theory, adrenaline theory and the hypothalamic (CNS) control theory" (Harris, 1970). Harris did not spend much time on the 'adrenaline theory', as he had found, like Vogt, that denervation of the adrenal glands did not alter the glucocorticoid response to stress, so he concluded that the adrenal medulla played little part in the pituitary adrenal response to stress (Colfer et al 1950). However, many investigators continued to pursue the hypothesis that circulating catecholamines may directly stimulate ACTH secretion or (more recently) may enhance the action of hypothalamic corticotrophin-releasing factors. Following the discovery of catecholamines in the brain (von Euler, 1946; Vogt, 1954), it was suggested that central catecholamines inhibit ACTH secretion (Weiner & Ganong, 1978), and this was the dominant view until the 1980s. However, it had also been proposed

that activation of central catecholamine systems may stimulate the secretion of ACTH in the rat (Saffran & Schally, 1955) and in man (Besser et al, 1969). Despite the considerable interest in this field, the plethora of apparently contradictory hypotheses made this a particularly confusing area of neuroendocrinology. However, studies carried out in the 1980s utilising improvements in knowledge of the anatomy and physiology of the catecholamine systems, and of experimental techniques and pharmacological tools, made it possible to offer a clearer picture of the relationships of catecholamines to the activity of the hypothalamo-pituitary adrenal axis, and to offer alternative interpretations of apparently contradictory data.

7.3.3 α_1 adrenoceptors in the control of ACTH secretion

In humans and in rats, activation of α_1 adrenergic receptors which are located in the brain stimulated the secretion of ACTH. The evidence for this was as follows:

1. In humans, the secretion of ACTH can be stimulated by intravenous infusions of methoxamine, an α_1 adrenergic agonist which crosses the blood-brain barrier, but not by an equipotent dose of noradrenaline, an α_1 agonist which reaches the pituitary gland and the median eminence following an intravenous infusion but does not cross the blood-brain barrier (Al-Damluji et al, 1985 & 1987a; Figure 7.3.1). Further work demonstrated that the α_2 and β agonist properties of noradrenaline did not account for the differences from methoxamine (Al-Damluji et al, 1987a). This provided the first evidence that activation of α_1 adrenoceptors which are located in the brain could stimulate the secretion of ACTH. Prior to that work, it had been known that certain drugs such as amphetamines (Rees et al, 1970) and methoxamine (Nakai et al, 1973) could stimulate the secretion of ACTH, but the location of the responsible receptors was unknown.
2. In patients with hypothalamic disease but with responsive pituitary corticotroph cells, intravenous infusions of methoxamine had no stimulant action on ACTH secretion (Al-Damluji & Rees, 1987; Al-Damluji & Francis, 1993; Figure 7.3.2). This confirmed that the stimulant α_1 adrenoceptors are not located in the pituitary gland.
3. In rats, intracerebroventricular infusion of α_1 agonists also stimulated the secretion of ACTH (Szafarczyk et al, 1987); methoxamine was more potent in stimulating ACTH

secretion when it was administered intracerebroventricularly than intravenously (Al-Damluji et al, 1990a; Figure 7.3.3).

In all the above studies, the effects of methoxamine on the secretion of ACTH could be abolished by selective α_1 antagonists, such as prazosin and thymoxamine (Al-Damluji et al, 1987a & 1990a).

The identity of the hypothalamic peptides which mediate the effects of the α_1 adrenoceptors was the subject of further studies. The hypothalamus secretes a complex of peptides which act in a synergistic manner to stimulate the secretion of ACTH. In rats, activation of α_1 adrenoceptors stimulates the secretion of several hypothalamic peptides, including CRH and vasopressin (Hiwatari & Johnston, 1985; Plotsky, 1987; Szafarczyk et al, 1987; Calogero et al, 1988; Hillhouse & Milton, 1989). It appears that the effect of the α_1 adrenoceptors is exerted primarily on neurones secreting vasopressin, which in turn stimulates the secretion of ACTH, acting synergistically with CRH. The evidence for this was obtained in rats: the ACTH response to an intracerebroventricular infusion of methoxamine could be reduced by a vasopressin antagonist but not by an equipotent dose of a CRH antagonist (Al-Damluji et al, 1990a). However, the combination of the two antagonists caused a reduction in the ACTH response to methoxamine that was greater than that of the vasopressin antagonist alone. This suggested that CRH plays some role in this response, possibly by enhancing the activity of vasopressin in a synergistic manner (Al-Damluji et al, 1990a). Confirmation of the essential role of vasopressin was obtained from the finding that Brattleboro rats, which are deficient in bioactive vasopressin but not in other hypothalamic peptides, had no ACTH response to intracerebroventricular infusion of methoxamine (Al-Damluji et al, 1990b). Further evidence for the primary role of vasopressin in mediating the ACTH response to activation of central α_1 adrenoceptors was obtained using immunocytochemical studies in rats: cerebroventricular infusion of methoxamine caused depletion of neurosecretory vesicles from parvicellular neurones which contain both vasopressin and CRH, but not from the parvicellular neurones which contain CRH without vasopressin (Whitnall et al, 1993). Figure 7.3.4 is a representation of the hypothetical model of the complex interaction between the noradrenergic neurones and the hypothalamic peptidergic neurones which influence the secretion of ACTH.

The physiological significance of the stimulant α_1 adrenoceptors was demonstrated in two situations in humans: the cortisol secretory pattern during waking hours and the ACTH and cortisol responses to feeding could be enhanced by intravenous infusions of α_1 adrenergic agonists and diminished by α_1 antagonists which cross the blood-brain barrier (Al-Damluji et al, 1987b,c; Figures 7.3.5 and 7.3.6). This indicated that the secretion of ACTH in these physiological situations was mediated by α_1 adrenoceptors. However, catecholamines do not mediate all stimuli to ACTH secretion in humans, as the nocturnal cortisol surge and the response to hypoglycaemia are unaffected by α_1 adrenoceptor blockade (Al-Damluji et al, 1987b; Cuneo et al, 1987). Although physiological stimuli have not been studied in the rat, the corticosterone response to immobilisation stress, the ACTH response to ether and the CRH response to haemorrhage can be reduced by α_1 adrenergic antagonists (Gibson et al, 1986; Plotsky, 1987; Szafarczyk et al, 1987; Kiss & Aguilera, 1992).

7.3.4 α_2 adrenoceptors in the control of ACTH secretion

In the brain, as in the periphery, α_2 adrenoceptors are believed to be located both pre-synaptically (on the noradrenergic neurones) and post-synaptically (on the target cells). Activation of the pre-synaptic receptors is believed to inhibit the discharge of the neurones on which they are located, and this acts as a negative feedback mechanism which limits the rate of synthesis and release of noradrenaline (Langer, 1977). In dogs, activation of α_2 adrenoceptors which are located in the brain inhibits the secretion of ACTH in response to stress (Ganong et al, 1982). In humans, the α_2 adrenoceptors are not activated under basal conditions (Al-Damluji et al, 1988 & 1990c), ie, with the subjects lying supine in a darkened room, with no auditory or visual stimulation. Under such conditions, administration of an α_2 antagonist has no effect on the secretion of noradrenaline or ACTH (Al-Damluji et al, 1988 & 1990c). This is presumably because noradrenaline release from the neurones is minimal under such circumstances, so the pre-synaptic α_2 adrenoceptors are not occupied by noradrenaline to a significant extent. In contrast, when ACTH secretion is stimulated by an

agent which exerts its action on central noradrenergic neurones, administration of an α_2 antagonist enhances the ACTH response to that stimulus (Al-Damluji et al, 1988 & 1990c). It seems likely that central α_2 adrenoceptors provide a central negative feedback mechanism which prevents excessive glucocorticoid responses to stress. This effect presumably complements the inhibitory effect of glucocorticoids on the secretion of ACTH. In addition to this pre-synaptic α_2 adrenergic inhibition, there is evidence in rats that, under some circumstances, activation of post-synaptic α_2 adrenoceptors may stimulate the secretion of corticotrophin-releasing hormone (Calogero et al, 1988; Assenmacher et al, 1992).

7.3.5 β adrenoceptors in the control of ACTH secretion

In humans, activation of β adrenoceptors with intravenous infusions of selective agonists does not stimulate ACTH secretion (Al-Damluji et al, 1987a). It was not possible to study the effects of activation of central β adrenoceptors in humans due to the lack of agonists with adequate penetration of the blood-brain barrier. Most investigators have found that β blockade has no effect on the adrenocortical response to hypoglycaemia in humans (reviewed in Al-Damluji, 1988) and no evidence has been presented as yet for a physiological role for these receptors in the control of ACTH secretion in humans. The situation in the rat is less clear. Some investigators found that activation of central β adrenoceptors stimulates the secretion of CRH or ACTH, whereas others found an inhibitory effect (Plotsky, 1987; Szafarczyk et al, 1987; Takao et al, 1988; Tsagarakis et al, 1988; Widmaier et al, 1989). The role (if any) of β adrenoceptors in the control of ACTH secretion in the rat remains ambiguous.

7.3.6 Circulating catecholamines

As described above, Long (1947) proposed that circulating adrenaline, derived from the adrenal medulla, stimulated the secretion of ACTH by a direct action on the pituitary corticotrophs. Some investigators subsequently reported that adrenaline enhanced the action of CRH on cultured rat adenohypophysial cells in vitro (reviewed in Al-Damluji, 1988). However, in normal human volunteers, intravenous infusions of adrenaline and noradrenaline which increase plasma catecholamine concentrations to the upper limit of the

physiological range do not stimulate ACTH secretion, nor do they enhance the stimulant effect of CRH or vasopressin on ACTH secretion (Milsom et al, 1986; Al-Damluji et al, 1987d; Jackson et al, 1987; Figure 7.3.7). It is therefore clear that in humans, peripheral circulating catecholamines have no stimulant effect on ACTH secretion, and the ACTH response to stress in humans is unlikely to be mediated by the concomitant sympathoadrenal response. In contrast, a stimulant action of circulating catecholamines acting on β_2 adrenoceptors in the rat intermediate lobe may be physiologically relevant in the responses of that lobe to some stressful stimuli (Berkenbosch et al, 1983). The intermediate lobe is vestigial in adult humans.

7.3.7 The role of dietary tyrosine

Tyrosine is a dietary amino acid which is also the precursor in the catecholamine synthesis pathway. The large neutral amino acids, including tyrosine, share a common, competitive mechanism for transport across the blood-brain barrier and into the neurones (Pardridge & Oldendorf, 1977; Morre & Wurtman, 1981). The possible effects of dietary intake of tyrosine on the central noradrenergic system have been the subject of considerable interest and some controversy. In the course of my MD thesis studies, I investigated the effect of dietary tyrosine on the secretion of ACTH and I applied the findings to the treatment of depressive illness.

In the early studies following the discovery of tyrosine hydroxylase, it was reported that the products of the catecholamine synthesis pathway (eg, noradrenaline) exerted an inhibitory effect on the activity of this enzyme (Nagatsu et al, 1964; Udenfriend et al, 1965). This was thought to act as a negative feedback process, whereby noradrenaline limits its own rate of synthesis. Administration of tyrosine was therefore not expected to increase the rate of turnover of noradrenaline unless the animals were stressed, as stress increases the neuronal discharge rate, resulting in increased activity of tyrosine hydroxylase (Gordon et al, 1966). However, it was later proposed that the synthesis of catecholamines may be dependent on the availability of tyrosine (Wurtman et al, 1974; Carlsson & Lindqvist, 1978). This proposal was based on experiments in which tyrosine was administered to animals which had been treated with a dihydroxyphenylalanine (DOPA) decarboxylase inhibitor to inhibit the conversion of DOPA to dopamine and noradrenaline. In view of the confusion regarding the role of tyrosine, I examined the effect of tyrosine in humans, using the secretion of ACTH as an index of the release of noradrenaline in the hypothalamus (see below).

Tyrosine was administered in doses which were similar to the estimated tyrosine content of an average meal and also in supraphysiological doses. The secretion of ACTH and the release of noradrenaline into plasma were unaffected by administration of any of the doses of tyrosine (Al-Damluji et al, 1988). The reasons for the lack of effect of tyrosine were then investigated. We postulated that this may have been due to activation of one of the negative feedback processes which control the rate of synthesis and release of the catecholamines. Whereas tyrosine on its own had no effect on the release of noradrenaline or the secretion of ACTH, following pre-treatment with an α_2 antagonist, idazoxan, administration of tyrosine caused a significant increase in the release of noradrenaline and the secretion of ACTH (Al-Damluji et al, 1988). It therefore seems that the lack of effect of tyrosine under basal conditions is due in part to activation of pre-synaptic α_2 adrenoceptors which inhibit the release of noradrenaline.

In conclusion, administration of tyrosine under basal conditions has no effect on the release of noradrenaline. The lack of effect of tyrosine is due to two main factors: inhibition of tyrosine hydroxylase by intracellular noradrenaline (Nagatsu et al, 1964; Udenfriend et al, 1965) and activation of pre-synaptic α_2 adrenoceptors which inhibit noradrenaline release (Al-Damluji et al, 1988). The likely explanation for the stimulant effect of tyrosine following blockade of the DOPA decarboxylase enzyme (Wurtman et al, 1974; Carlsson & Lindqvist, 1978) is that blockade of the enzyme prevented the increase in intracellular noradrenaline concentrations which would inhibit tyrosine hydroxylase under physiological conditions. The clinical application of this knowledge for the treatment of depressive illness is described below.

7.3.8 The ACTH response to adrenergic drugs as an index of activation of the hypothalamic noradrenergic system

An important problem in neuropharmacology is the absence of a method for assessing the activation of adrenergic and serotonergic receptors in the human brain. For example, it is not possible currently to assess whether a drug in development as a potential antidepressant is capable of increasing the release of noradrenaline or serotonin in the human brain. It is therefore difficult to screen a series of drugs or to determine suitable doses in humans without carrying out long term, placebo controlled therapeutic trials, which consume much

time and resources. Based on the findings described above, it was proposed that the ACTH response to adrenergic drugs might serve as an *in vivo* marker of the release of endogenous catecholamines and of activation of α_1 adrenoceptors in the hypothalamus (Al-Damluji, 1991). This would represent a rapid, inexpensive method which would enable the screening of compounds for their ability to activate the central adrenergic system in humans.

The available antidepressants are believed to exert their therapeutic effects by inhibiting the pre-synaptic re-uptake of noradrenaline or serotonin. The effects of these antidepressant compounds therefore require the presence of intact pre-synaptic noradrenergic or serotonergic nerve terminal. However, it was not known whether a hypothalamic noradrenergic lesion might result in denervation hypersensitivity of the post-synaptic α_1 adrenoceptors, which might restore the ACTH response to the release of endogenous catecholamines. Rats with disruption of noradrenergic nerve terminals induced by 6-hydroxydopamine were examined for their responsiveness to stimuli which evoke ACTH release, either via release of endogenous catecholamines or with the selective α_1 adrenergic agonist methoxamine which is not accumulated by $Uptake_1$ in pre-synaptic noradrenergic neurones (Al-Damluji & White, 1992). In the 6-hydroxydopamine pre-treated animals, the post-synaptic α_1 adrenoceptors which modulate ACTH secretion did not undergo denervation hypersensitivity, and were therefore unable to compensate for the loss of noradrenergic nerve terminals. The ACTH response to the combination of catecholamine precursor and α_2 antagonist was reduced, enabling detection of the hypothalamic noradrenergic lesion in the rats *in vivo* (Al-Damluji & White, 1992). The ACTH response to release of endogenous catecholamines in humans promises to be useful for screening drugs (such as antidepressants) which are intended to increase the release of noradrenaline in the human brain.

The central noradrenergic system is involved in various diseases such as Parkinson's disease and Alzheimer's disease (see Holets, 1990). At present, there is no clinical method for detecting the central noradrenergic lesion in such diseases. The methods which are described above can be adapted for use in humans, to enable detection of a central noradrenergic lesion in patients who suffer from these disease (Al-Damluji & White, 1992).

7.3.9 Activation of endogenous catecholamines as treatment for depressive illness

The role of brain monoamines in the aetiology of depressive illness is discussed later in this thesis. According to the biogenic amine hypothesis of depressive illness, depression is caused by a deficiency of the action of noradrenaline or serotonin in the extracellular synaptic space; drugs which improve the clinical features of depressive illness are believed to exert their therapeutic effects by increasing the availability of noradrenaline or serotonin in the extracellular synaptic space. In the 1980s, α_2 adrenoceptor antagonists were suggested as treatment for depressive illness. The rationale for this is that α_2 antagonists increase noradrenaline release by competing with endogenous noradrenaline for the pre-synaptic α_2 adrenoceptors, and thus counteracting the negative feedback action of noradrenaline on its own release. However, as α_2 antagonists increase noradrenaline release by competing with endogenous noradrenaline, they can only be expected to exert their effects in the presence of an ambient concentration of noradrenaline. This has been demonstrated *in vitro*, as it has been shown that these drugs are more effective in releasing [^{3}H]noradrenaline in the presence of cocaine, which increases the extracellular concentrations of noradrenaline (Enero, 1984). Conversely, when tissue noradrenaline concentrations are reduced by reserpine, α_2 antagonists are much less effective in enhancing noradrenaline release (Enero & Langer, 1973). As has been discussed already, α_2 antagonists have minimal effects in normal subjects under basal conditions, when noradrenaline release is very low (Al-Damluji et al, 1988 & 1990c). Two methods were therefore developed for enhancing the actions of α_2 antagonists:

1. Combined administration of an α_2 antagonist and a catecholamine precursor:

   In human volunteers under basal conditions, the α_2 antagonist idazoxan had no effect on the release of noradrenaline or the secretion of ACTH; however, the combination of idazoxan with a catecholamine precursor, tyrosine, resulted in an increase in the release of noradrenaline and the secretion of ACTH (Al-Damluji et al, 1988). This indicated that it was possible to enhance the action of an α_2 antagonist in humans by combining it with a catecholamine precursor. In rats, the combination of idazoxan with L-DOPA increases the

release of noradrenaline and stimulates the secretion of ACTH (Al-Damluji & White, 1992). L-DOPA may be more effective than tyrosine in enhancing the effects of α_2 antagonists as it bypasses the block in catecholamine synthesis at the tyrosine hydroxylase step, which is caused by inhibition of this enzyme by the products of the catecholamine synthesis pathway (see above).

2. Combined blockade of α_2 and opioid receptors:

In humans, the central opioid system exerts a tonic inhibition on the release of noradrenaline and the secretion of ACTH (Grossman & Besser, 1982). This effect of the opioid system was utilised to enhance the action of the α_2 antagonist idazoxan. While idazoxan had no effect on the secretion of ACTH in human volunteers under basal conditions, the combination of idazoxan with the opioid antagonist naloxone caused a significant increase in the secretion of these hormones, presumably indicating an increase in the release of noradrenaline in the brain (Al-Damluji et al, 1990c). The mechanism of this interaction is likely to be that removal of the tonic opioid inhibition on the central noradrenergic system increases the availability of noradrenaline at pre-synaptic α_2 adrenoceptors; activation of these pre-synaptic α_2 adrenoceptors would normally limit the effectiveness of the opioid antagonist in increasing noradrenaline release. The combination of idazoxan with naloxone was therefore more effective in activating the central noradrenergic system than naloxone alone.

Idazoxan was developed as a selective α_2 adrenoceptor antagonist by Reckitt & Colman plc for treatment of depressive illness. During the course of my studies on the effects of α_2 antagonists on the secretion of ACTH, I pointed out to the pharmacologists at Reckitt & Colman that it is possible that in depressed patients, in whom noradrenaline turnover and the extracellular concentrations of noradrenaline are presumably very low, administration of the α_2 antagonist alone may be ineffective in significantly increasing noradrenaline turnover. Subsequent clinical trials on idazoxan and other selective α_2 antagonists demonstrated that these drugs had very poor efficacy in the treatment of depressive illness. Reckitt & Colman encouraged me to apply for patents to protect the methods which I had developed for

enhancing the actions of α_2 antagonists. My patent applications were acquired by the British Technology Group (National Research Development Corporation) and the patents were granted internationally, confirming the originality of the work.

Figure 7.3.1:

Comparison of the effects in humans of the α_1 agonists methoxamine and noradrenaline (NA) on plasma cortisol and systolic blood pressure (expressed as percentage change from mean control ± SEM). Infusions of noradrenaline were given to elevate systolic blood pressure by approximately 10% and 25% of the mean control value, similar to the changes after methoxamine. ACTH and cortisol secretion are stimulated by methoxamine, which crosses the blood-brain barrier, but not by equipotent doses of noradrenaline which reaches the pituitary gland and the median eminence but does not cross the blood brain barrier. This is consistent with the hypothesis that secretion of ACTH is stimulated by α_1 adrenoceptors which are located in the brain. Reproduced from Al-Damluji et al, 1987a.

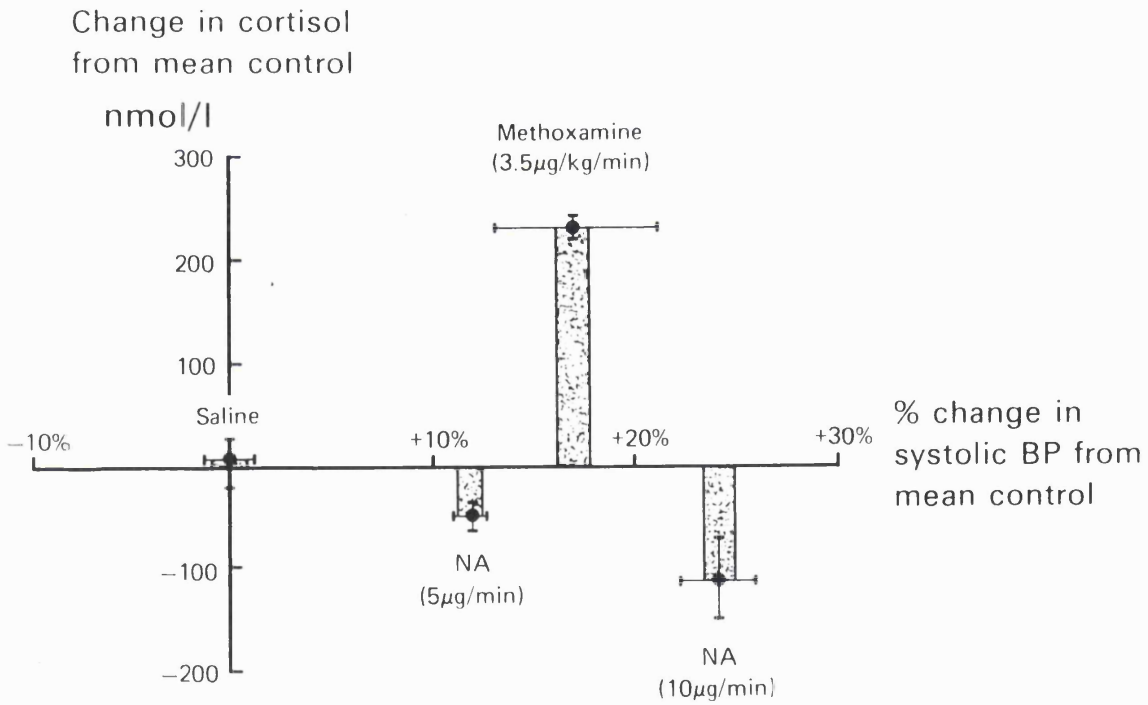


Figure 7.3.2:

Effects of synthetic ovine corticotrophin releasing hormone (CRF; 100 ug intravenously over one minute) and methoxamine (20 mg intravenously from 0 to 90 minutes) on plasma ACTH concentrations in four patients with hypopituitarism due to hypothalamic dysfunction (upper panels) and in six normal volunteers (lower panels). Data from the volunteers are expressed as means ± SEM, and those from the patients are plotted individually. Each symbol refers to an individual patient. Methoxamine does not stimulate the secretion of ACTH in these patients who have intact pituitaries; this is consistent with the hypothesis that the stimulant α_1 adrenoceptors are located in the brain, and not directly on the pituitary. Reproduced from Al-Damluji & Francis, 1993.

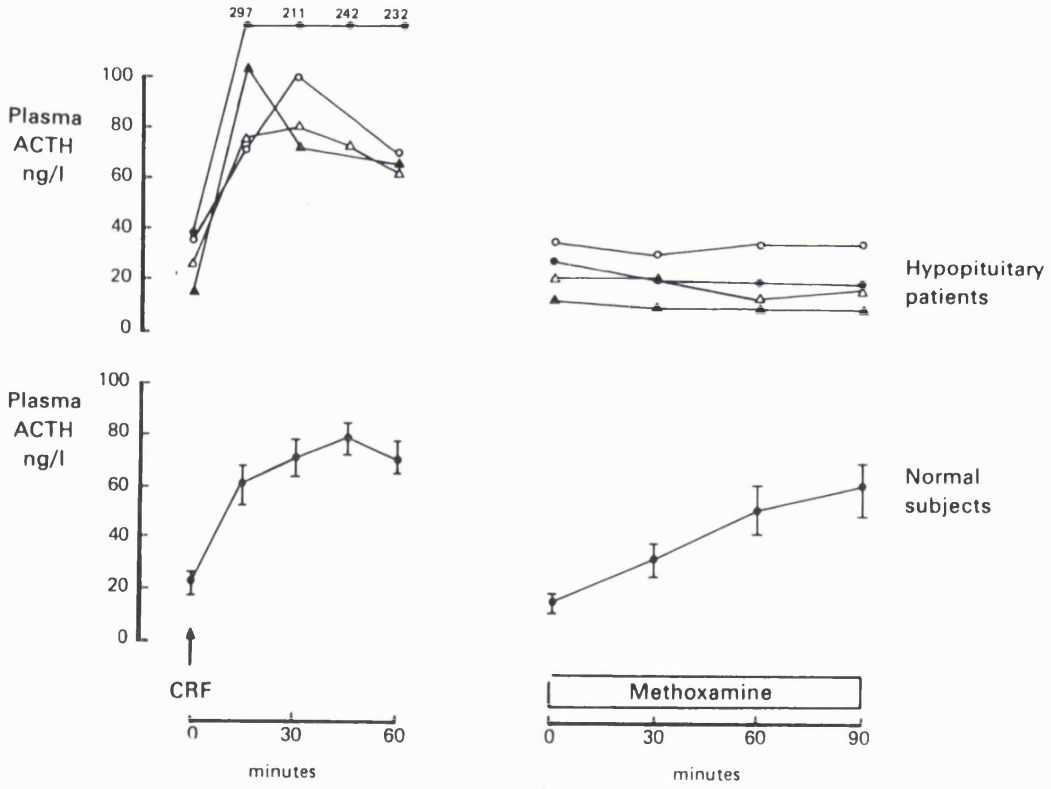


Figure 7.3.3:

The relationship of methoxamine dose to plasma ACTH response, expressed as the mean ± SEM change in plasma ACTH concentrations (15 minutes - baseline) in groups of four male rats. The injections were administered in the left lateral cerebral ventricle (icv) or intravenously (iv). Methoxamine was more potent by the icv route than by the iv route, which is consistent with the hypothesis that the stimulant α_1 adrenoceptors are located in the brain. Reproduced from Al-Damluji et al, 1990a.

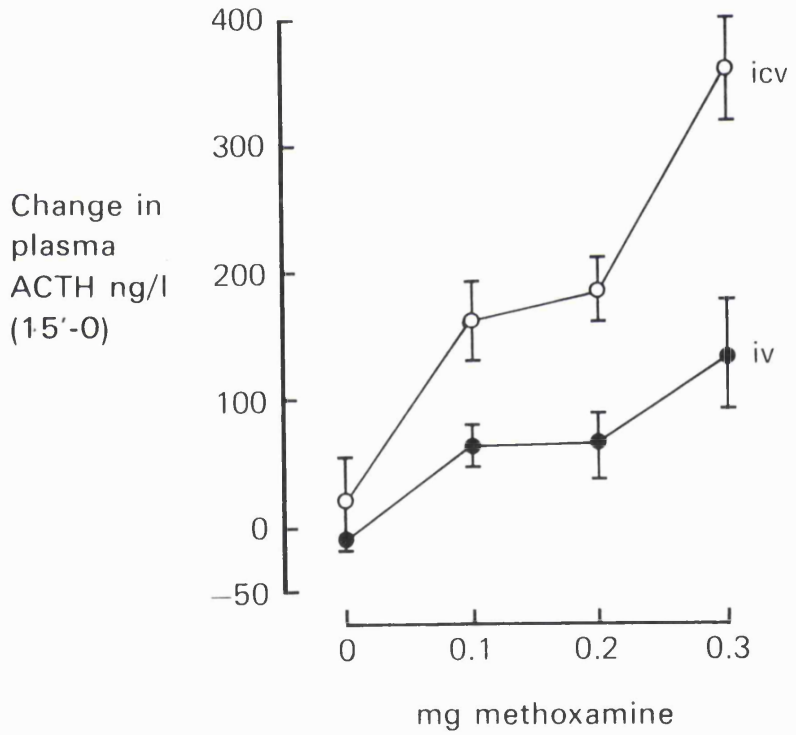


Figure 7.3.4:

Proposed model of the relationship between the α_1 adrenoceptors and vasopressin and CRH neurones which stimulate the secretion of ACTH. α_1 adrenoceptors are located on neurones which secrete CRH, vasopressin, and CRH and vasopressin. The findings reported in our studies make it likely that activation of α_1 adrenoceptors results in dynamic changes in the secretion of vasopressin, but that the presence of CRH in portal plasma is important for amplifying the effects of these changes on the secretion of ACTH. Reproduced from Al-Damluji et al, 1990b.

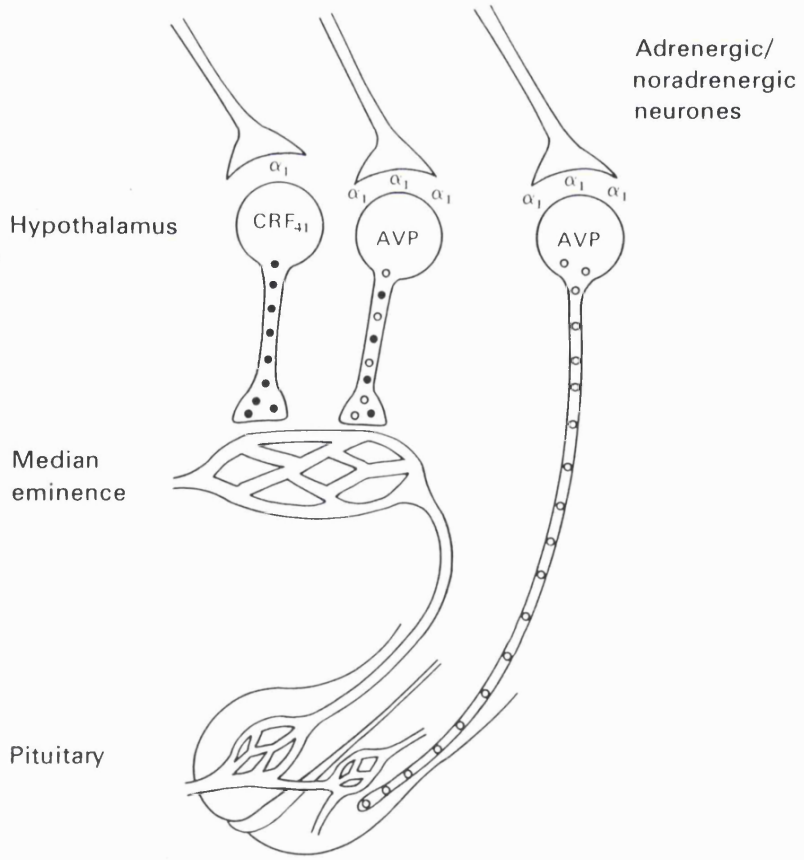


Figure 7.3.5:

Effects of continuous 24-hour intravenous infusions of the α_1 adrenergic agonist methoxamine (closed triangles), the α_1 antagonist thymoxamine (open circles) and saline (placebo control; closed circles) on the circadian pattern of plasma cortisol in six normal human subjects. C: coffee; L: lunch; T: tea; S: supper; D: milk drink; B: breakfast. Reproduced from Al-Damluji et al, 1987b.

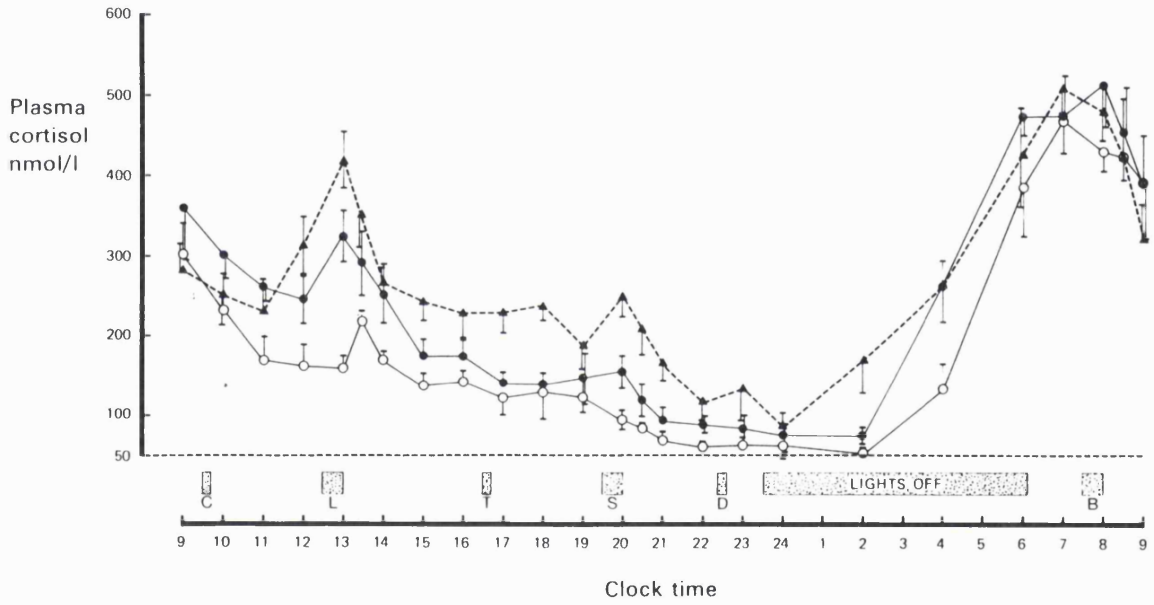


Figure 7.3.6:

Effects of intravenous infusions of the α_1 adrenergic agonist methoxamine (closed triangles), the α_1 antagonist thymoxamine (open circles) and saline (placebo control; closed circles) on the ACTH and cortisol responses to food ingestion in six normal human subjects. The infusions were given continuously throughout the study and lunch was given at 60 minutes. Values are mean ± SEM. Reproduced from Al-Damluji et al, 1987c.

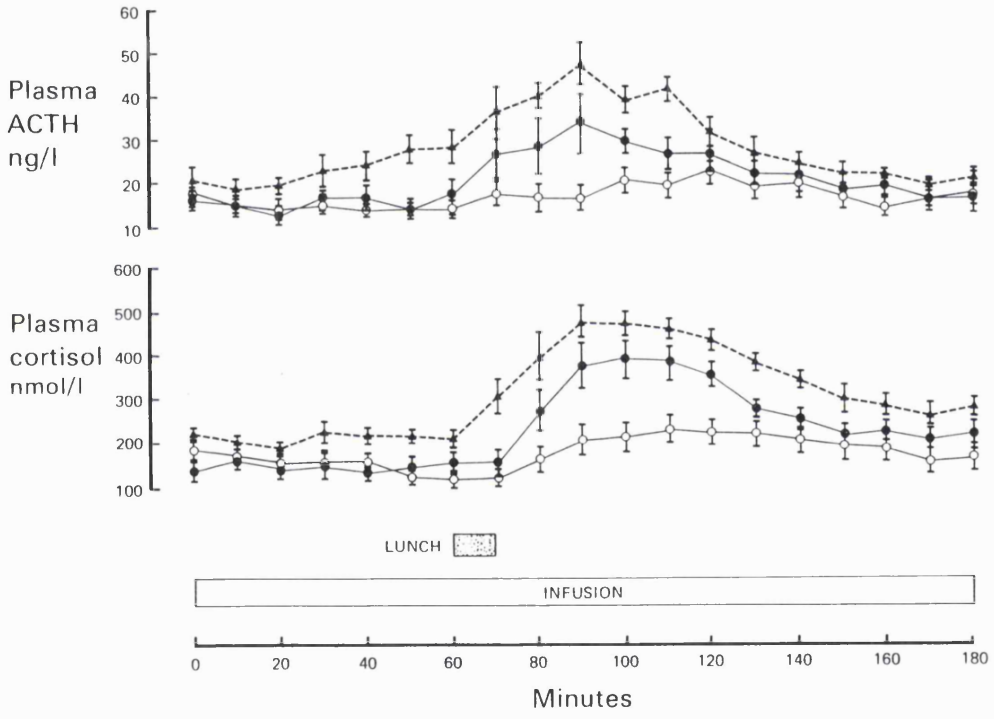
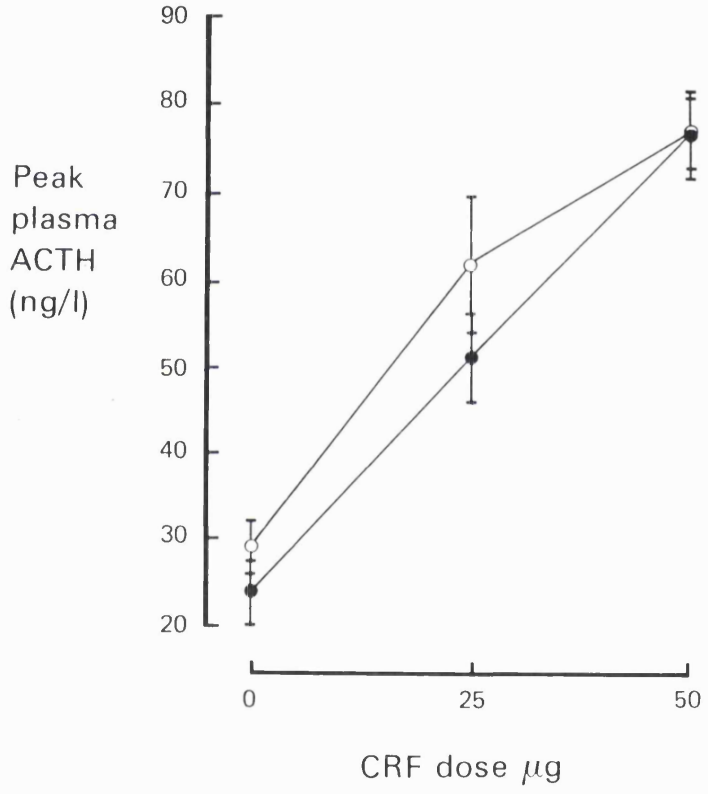


Figure 7.3.7:

Dose-response relationship of plasma ACTH to bolus doses of synthetic ovine corticotrophin-releasing hormone (CRF) during infusions of saline (open circles) and adrenaline (closed circles) in six normal human subjects. Values are mean ± SEM. Adrenaline does not enhance the stimulant effect of CRF on the secretion of ACTH in humans. Reproduced from Al-Damluji et al, 1987d.



7.4 DISCOVERY AND DEFINITION OF TRANSPORT-P

While studying the cellular and biochemical mechanisms of the actions of catecholamines on hypothalamic peptidergic neurones, I analysed the properties of α_1 adrenoceptors in cultured neonatal hypothalamic cells and in immortalised gonadotrophin-releasing hormone (GnRH) neurones (Al-Damluji et al, 1993). The latter cells possess many of the characteristics of differentiated hypothalamic neurones: they extend neurites; they secrete the peptide neurotransmitter GnRH in a characteristic pulsatile manner; and they possess fast sodium channels, synaptic membrane proteins and other neuronal (but not glial cell) markers (Mellon et al, 1990; see Section 10.3.1). As expected from the physiological studies (see above), α_1 adrenoceptors were present in both the cultured neonatal cells and in the GnRH cell line. However, I also detected a novel high affinity uptake process for amines in these post-synaptic peptidergic neurones (Al-Damluji et al, 1993). Preliminary studies also suggested that the uptake process operated *in vivo*. Presence of an uptake process for amines in post-synaptic neurones was a surprising finding which had not been suspected.

7.4.1 Binding of prazosin in peptidergic neurones

[^{3}H]Prazosin (3 to 18x10^{-10} M) bound to both the cultured neonatal cells and the GnRH cell line. Time course studies showed that apparent equilibrium was reached by 60 minutes. Displacement studies at equilibrium showed that [^{3}H]prazosin was displaced by unlabelled prazosin in concentrations of 10^{-10} to 10^{-7} M, suggesting the presence of α_1 adrenoceptor binding sites in both the neonatal cells and the GnRH cell line (Figure 7.4.1). However, at concentrations of unlabelled prazosin greater than 10^{-7} M, there was a paradoxical increase in apparent [^{3}H]prazosin binding (Figure 7.4.1; Al-Damluji et al, 1993).

Further experiments examined the hypothesis that this paradoxical increase in apparent [^{3}H]prazosin binding could be due to an uptake process which becomes apparent at greater concentrations of the ligand. Desipramine is a tricyclic antidepressant which inhibits neuronal uptake (Glowinski & Axelrod, 1964). Desipramine (10^{-5} M) reduced the total amount of [^{3}H]prazosin associated with the cells (B_0) by 40% (Figure 7.4.2A). In the presence of desipramine, unlabelled prazosin displaced [^{3}H]prazosin as before but no increase in binding

was seen above 10^{-7} M. This was consistent with the hypothesis that the paradoxical increase in binding of [^{3}H]prazosin was due to cellular uptake of the radioligand.

Although desipramine 10^{-5} M reduced the total amount of [^{3}H]prazosin associated with the cells, it had no effect on the specific binding of [^{3}H]prazosin (Figure 7.4.2B), indicating that at this concentration, desipramine did not interfere with the binding of [^{3}H]prazosin to these α_1 adrenoceptors.

Figure 7.4.2C demonstrates radioligand binding curves for [^{3}H]prazosin and its displacement by unlabelled prazosin, in the presence of desipramine 10^{-5} M. The density (B_{max}) of the binding sites was 150 fmol/mg in the neonatal cells and 126 fmol/mg in the GnRH cell line (Al-Damluji et al, 1993). The affinity K_d of prazosin was 4.4×10^{-9} M in the neonatal cells and 11×10^{-9} M in the GnRH cell line (Al-Damluji et al, 1993). This is similar to the affinity of prazosin for the cloned α_{1b} adrenoceptors expressed in cultured cells (K_i 2.5×10^{-9} M; Cotecchia et al, 1988).

As prazosin may bind with lower affinity to α_{2B} adrenoceptors (K_i 41 nM; Regan et al, 1988), we further investigated the identity of the adrenoceptors in the cell cultures. The selective α_1 adrenoceptor ligand [^{125}I]HEAT bound to the cells with high affinity (at a concentration of 3×10^{-11} M, maximum binding [B_0] was 26% of total counts and NSB was 1.9%; Al-Damluji et al, 1993). The α_1 adrenoceptor agonist methoxamine displaced 63% of the bound [^{125}I]HEAT; this confirmed the presence of α_1 adrenoceptors (Al-Damluji et al, 1993). We also examined the cells for α_2 adrenoceptors using [^{3}H]idazoxan which binds to α_{2B} adrenoceptors (K_i 17 nM; Regan et al, 1988). In two separate experiments in the neonatal cells, [^{3}H]idazoxan (1.8×10^{-9} M) binding did not exceed 1% of total counts, and this was not displaced with the α_2 agonist clonidine (Al-Damluji et al, 1993). Similarly, in

the GnRH cells, binding of [^{3}H]idazoxan (at concentrations up to 10^{-7} M) did not exceed 1% of total counts and was not displaced by clonidine (Al-Damluji et al, 1993). These experiments confirmed the identity of the [^{3}H]prazosin binding sites as α_1 adrenoceptors.

In broken cell (membrane) preparations, [^{3}H]prazosin was displaced by unlabelled prazosin in the concentration range 10^{-9} to 10^{-6} M, and there was no paradoxical increase in apparent binding of [^{3}H]prazosin at concentrations of unlabelled prazosin greater than 10^{-7} M (Figure 7.4.2D; Al-Damluji et al, 1993). This indicated that the paradoxical increase requires the presence of intact cells or storage organelles, and is unlikely to be due to some non-specific association of prazosin with cell membranes.

Further experiments examined the possible contribution of ionic changes to the paradoxical increase in [^{3}H]prazosin binding at concentrations of unlabelled prazosin greater than 10^{-7} M. Increasing concentrations of unlabelled prazosin had no effect on the pH of Krebs-Ringer solutions buffered with either 25 mM HEPES or 25 mM Tris (Figure 7.4.3; Al-Damluji et al, 1993). An identical pattern of displacement of [^{3}H]prazosin by unlabelled prazosin (10^{-10} to 10^{-7} M) was observed in the two buffers. At greater concentrations of unlabelled prazosin, there was an identical increase in the apparent binding of [^{3}H]prazosin up to 10^{-6} M; saturation was evident above this concentration in both buffers (Figure 7.4.3; Al-Damluji et al, 1993). These experiments demonstrated that the paradoxical increase is not due to some effects of prazosin on ionic strength or buffering capacity.

We postulated that the decline in radioactivity at unlabelled prazosin concentrations up to 10^{-7} M was due to displacement of [^{3}H]prazosin by unlabelled prazosin from α_1 adrenoceptors in the peptidergic neurones; the paradoxical increase in radioactivity was due to a cellular uptake process which is activated by prazosin (Al-Damluji & Krsmanovic, 1992). The affinity of prazosin for the uptake process is lower than its affinity for the receptors; this would explain why displacement of [^{3}H]prazosin from the receptors is observed at low concentrations of unlabelled prazosin, whereas uptake is evident at prazosin concentrations greater than 10^{-7} M (Figure 7.4.1). Uptake of prazosin is demonstrable at low

concentrations of the drug, as desipramine reduced the association of nanomolar concentrations of prazosin with the cells (Figure 7.4.2A). Prazosin therefore both binds to α_1 adrenoceptors and is subject to cellular uptake. The proportion of prazosin which is bound to α_1 adrenoceptors would be expected to be dependent on the concentration of prazosin in the assay; at low concentrations, a greater proportion of prazosin will presumably be bound to receptors than at high concentrations, when uptake becomes more prominent due to saturation of the receptors and activation of the uptake process (Al-Damluji et al, 1993).

7.4.2 Definition of Transport-P

Transport-P is an antidepressant-sensitive uptake process for prazosin in peptidergic neurones of the hypothalamus. It is distinguishable from other uptake processes by its anatomical location in post-synaptic neurones and by its functional properties, most notably the prazosin paradox: increasing extracellular concentrations of unlabelled prazosin greater than 10^{-7} M cause a paradoxical increase in accumulation of [^{3}H]prazosin. This property of Transport-P has not been described for any other membrane transport process.

7.4.3 Uptake of noradrenaline in peptidergic neurones

The hypothalamic peptidergic neurones, including GnRH, CRH and vasopressin neurones, are densely innervated by noradrenergic nerve terminals (Section 7.1). We therefore determined whether the peptidergic neurones accumulate the physiological neurotransmitter, noradrenaline. Uptake of noradrenaline was known to take place in pre-synaptic nerve terminals and in non-neuronal cells, including glial cells. In pre-synaptic nerve terminals, the plasma membrane noradrenaline transporter ($Uptake_1$) can be blocked by tricyclic antidepressants such as desipramine and by exclusion of sodium from the extracellular space (Glowinski & Axelrod, 1964; Iversen & Kravitz, 1966; Bogdanski & Brodi, 1966; Pacholczyk et al, 1991). Uptake of noradrenaline in non-neuronal cells ($Uptake_2$) is insensitive to desipramine and absence of sodium but is blocked by certain steroid hormones such as testosterone and corticosterone (Iversen & Salt, 1970; Salt, 1972; Russ et al, 1996; Grundemann et al, 1998).

(±)[^{3}H]Noradrenaline in nanomolar concentrations was accumulated in a time-dependent

manner by the neonatal hypothalamic cells ($38x10^{-9}$ M) and by the GnRH cells ($80x10^{-9}$ M; Figure 7.4.4; Al-Damluji et al, 1993). The amount of accumulated noradrenaline was far smaller than the amount of prazosin which was accumulated by either type of cells (approximately 1/1000; Al-Damluji et al, 1993). At 15 minutes, uptake of (±)[^{3}H]noradrenaline in both types of cells was reduced by approximately 30% either by desipramine or by exclusion of sodium from the incubation medium (Figure 7.4.4; Al-Damluji et al, 1993). The remainder of the (±)[^{3}H]noradrenaline associated with the cells appeared to be bound to α_1 adrenoceptors, as the combination of desipramine (which blocks uptake) and methoxamine (which blocks binding to α_1 adrenoceptors) almost abolished the association of (±)[^{3}H]noradrenaline with the cells (Al-Damluji et al, 1993).

Although blockade of uptake by desipramine and by exclusion of sodium was only partial, the findings were interpreted to indicate that uptake of noradrenaline in peptidergic neurones shares some of the features of the pre-synaptic neuronal uptake process ($Uptake_1$; Al-Damluji et al, 1993). Uptake of (±)[^{3}H]noradrenaline in GnRH cells was unaffected by testosterone ($6x10^{-6}$ M; Figure 7.4.5A; Al-Damluji et al, 1993) which distinguished the uptake of noradrenaline in peptidergic neurones from $Uptake_2$. However, in the neonatal cells, both testosterone and corticosterone reduced (±)[^{3}H]noradrenaline uptake (Figure 7.4.5B; Al-Damluji et al, 1993). This was taken to reflect the heterogeneous nature of the primary cell cultures which contain both neuronal and glial elements, and the likelihood that the hypothalamus may possess more than one uptake process for noradrenaline.

The measured uptake of (-)[^{3}H]noradrenaline in the GnRH cells was extremely low (Al-Damluji et al, 1993). As previous investigators had found different rates of metabolism of (+) and (-) noradrenaline in various tissues (Blaschko et al, 1937; for review, see Graefe & Bonisch, 1988), further experiments examined the effects of enzyme inhibitors on the apparent uptake of (-)[^{3}H]noradrenaline in the GnRH cells. The catechol-O-methyl transferase (COMT) inhibitor OR-611 (10^{-6} M) markedly increased the measurable uptake of (-)[^{3}H]noradrenaline in the GnRH cells. In contrast, the combination of clorgyline (10^{-4} M) and pargyline (10^{-4} M) which inhibit monoamine oxidase (MAO) A and B, respectively, had

no effect on measurable (-)[^{3}H]noradrenaline uptake. However, these two drugs caused a further increase in the measurable uptake of (-)[^{3}H]noradrenaline in the GnRH cells when combined with OR-611 (Figure 7.4.5C; Al-Damluji et al, 1993). The findings were interpreted as suggesting that peptidergic neurones accumulate both (-) and (+) noradrenaline, but that the (-) isomer is more rapidly metabolised (Al-Damluji et al, 1993); a similar mechanism had been proposed for the uptake of noradrenaline in pre-synaptic nerve terminals (Iversen, 1963; for review see Graefe & Bonisch, 1988). As the MAO inhibitors were ineffective on their own, the metabolic pathway for noradrenaline in peptidergic neurones was thought to differ from pre-synaptic nerve terminals; it was proposed that the peptidergic neurones metabolise noradrenaline predominantly via COMT to lipophilic compounds which can cross the plasma membrane into the larger volume of distribution of the incubation and wash buffers. A likely metabolic path would involve COMT to produce normetanephrine, followed by MAO and aldehyde reductase to produce 3-methoxy-4-hydroxy-phenylethylene-glycol (MHPG), a lipophilic compound which has a high efflux rate from cells (Figure 7.5.2; Graefe & Henseling, 1983; Kopin, 1985). Such a rapid efflux rate of lipophilic metabolites may have explained the extremely low measurable uptake of (-)[^{3}H]noradrenaline in the GnRH cells (Al-Damluji et al, 1993).

The kinetics of (-)noradrenaline uptake were studied in the GnRH cell line in the presence of OR-611 and in the neonatal cells in the absence of any enzyme inhibitor. In both cases, the data fitted the Michaelis-Menten model (Figure 7.4.6; Al-Damluji et al, 1993). In the neonatal cells, the K_m for (-)noradrenaline was 3.3×10^{-7} M and the V_{max} was 143 fmol/min/mg protein. In the GnRH cells, the K_m and the V_{max} were 2×10^{-6} M and 400 fmol/min/mg, respectively (Figure 7.4.6; Al-Damluji et al, 1993). These affinity constants were similar to the kinetic constants for the high-affinity $Uptake_1$ noradrenaline transporter in pre-synaptic nerve terminals (Iversen, 1963; Pacholczyk et al, 1991). In contrast, the K_m of (-)noradrenaline for $Uptake_2$ in non-neuronal cells is 250 uM (Burgen & Iversen, 1965).

7.4.4 Effect of 6-hydroxydopamine on post-synaptic peptidergic neurones *in vivo*:

Further experiments aimed to determine if peptidergic neurones are capable of accumulating amines *in vivo*. The neurotoxin 6-hydroxydopamine is an isomer of noradrenaline which is

accumulated by the pre-synaptic plasma membrane $Uptake_1$ noradrenaline transporter in the rat hypothalamus (Iversen, 1970). It is then oxidised to toxic products, resulting in destruction of the noradrenergic nerve terminals (Kostrzewa & Jacobowitz, 1974; Kopin, 1985). The uptake of 6-hydroxydopamine and its neurotoxic effects can be blocked by desipramine (Stone et al, 1964; Jonsson & Sachs, 1970). Administration of the α_1 adrenergic agonist methoxamine to rats activates α_1 adrenoceptors which are located post-synaptically on vasopressin and CRH neurones, whose secretory products in turn stimulate the secretion of ACTH (Section 7.3.3). We investigated the possibility that the vasopressin and CRH neurones may be able to accumulate 6-hydroxydopamine (Al-Damluji et al, 1993). Methoxamine is not subject to significant uptake by the pre-synaptic $Uptake_1$ noradrenaline transporter so its bioavailability is not influenced by destruction of pre-synaptic nerve terminals (Burgen & Iversen, 1965; Trendelenburg et al, 1970).

In rats which had been pre-treated with intracerebroventricular (icv) cerebrospinal fluid as a control, icv administration of methoxamine stimulated the secretion of ACTH, as previously described (Al-Damluji et al, 1990a). Pre-treatment with a single dose of 6-hydroxydopamine (250 ug icv) reduced the hypothalamic content of noradrenaline significantly, indicating uptake into and destruction of the hypothalamic noradrenergic nerve terminals (Al-Damluji et al, 1993). This dose of 6-hydroxydopamine had no effect on the ACTH response to methoxamine, indicating sparing of the post-synaptic hypothalamic peptidergic neurones which mediate the effects of methoxamine on ACTH secretion (Figure 7.4.7; Al-Damluji et al, 1993). In contrast, rats which had been pre-treated with two doses of 6-hydroxydopamine (three days apart) showed a similar reduction in hypothalamic noradrenaline content but in addition, the ACTH response to methoxamine was significantly reduced (Figure 7.4.7; Al-Damluji et al, 1993). This suggested destruction of the hypothalamic vasopressin and CRH neurones which mediate the effects of methoxamine. It seems likely that 6-hydroxydopamine has greater affinity for the pre-synaptic uptake process than for post-synaptic uptake. This would explain why a single dose of this drug spared the post-synaptic neurones; only when the noradrenergic nerve terminals have been destroyed can 6-hydroxydopamine exert its full effect on the post-synaptic neurones. These preliminary findings suggest that hypothalamic vasopressin and CRH neurones possess an amine uptake process, and provide an experimental model which should be useful for further studies in this field.

In conclusion, the peptidergic neurones of the hypothalamus, including the GnRH, vasopressin and CRH neurones, are capable of accumulating noradrenaline. This was a surprising finding as uptake of neurotransmitters had not been described in post-synaptic neurones. The affinities of (-)-noradrenaline for the post-synaptic uptake process (apparent K_m $0.33x10^{-6}$ M in neonatal cells, $2x10^{-6}$ M in GnRH cells) and for the post-synaptic α_{1B} adrenoceptors (K_d $3.2x10^{-6}$ M; Cotecchia et al, 1988) make it likely that at concentrations that activate the post-synaptic receptor, noradrenaline will be accumulated by the post-synaptic uptake process. Exposure of α_1 adrenoceptors to noradrenaline leads to desensitisation of the receptors (Wikberg et al, 1983; Bobik et al, 1984; Awaji et al, 1998). A post-synaptically located high affinity uptake process may serve the important function of removing noradrenaline from the vicinity of the receptors, thus maintaining the responsiveness of the post-synaptic receptors to repeated bursts of neurotransmitter released from the noradrenergic nerve teminals (Figure 7.4.8; Al-Damluji et al, 1993). In contrast, the pre-synaptic $Uptake_1$ noradrenaline transporter would presumably be less effective in removing noradrenaline from the vicinity of post-synaptic receptors, as it would have to rely on the diffusion of molecules of noradrenaline back across the synapse, against their concentration gradient. Clearly, a concentration gradient of transmitter molecules must exist across the synapse, otherwise forward diffusion would not take place; diffusion against a concentration gradient is energetically and kinetically unfavourable.

Figure 7.4.1:

Effect of unlabelled prazosin on the binding of [^{3}H]prazosin in cultured neonatal hypothalamic cells (left) and a GnRH neuronal cell line. The data represent total bound cpm. Reproduced from Al-Damluji et al, 1993.

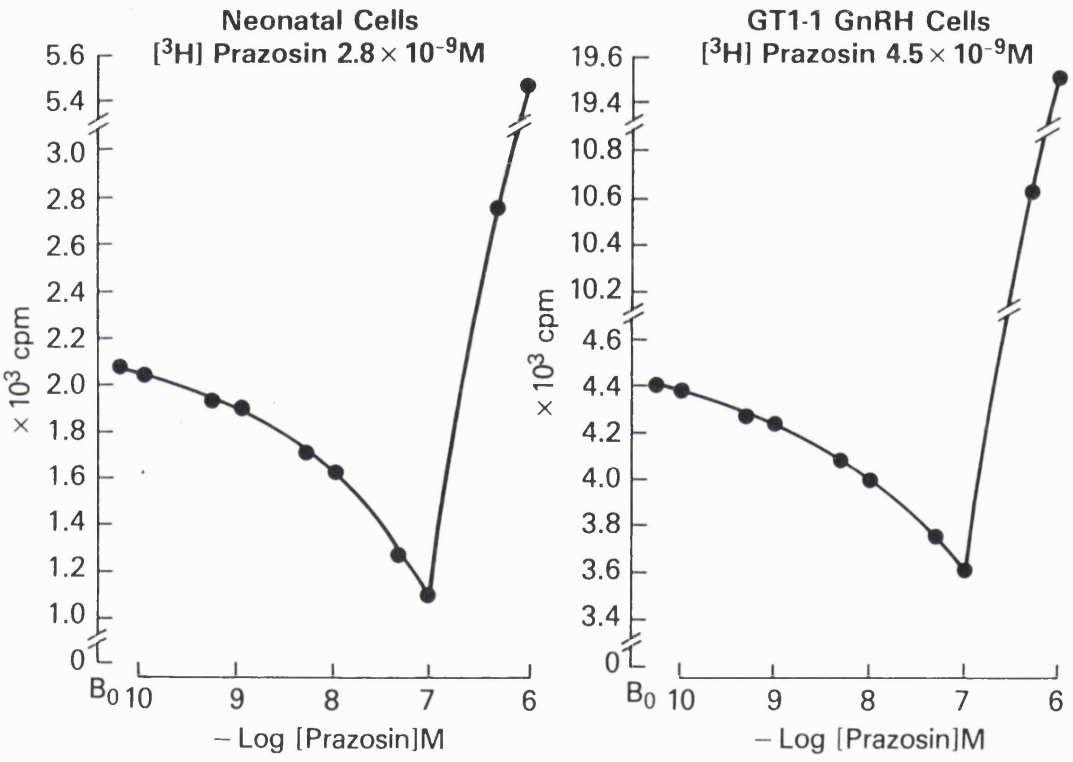


Figure 7.4.2:

A. Effects of desipramine (at 10^{-5} M) on [^{3}H]prazosin (at 1.8×10^{-9} M) binding and its displacement by unlabelled prazosin in intact GT1-1 GnRH cells. The data represent total bound cpm. Open circles: control; closed circles: desipramine.

B. Specific binding of prazosin with (closed circles) or without (open circles) desipramine 10^{-5} M. Non-specific binding was defined as cpm at 10^{-6} M unlabelled prazosin in the presence of desipramine 10^{-5} M. Specific binding was obtained by subtracting non-specific from total binding.

C: Binding of [^{3}H]prazosin and its displacement by unlabelled prazosin in intact neonatal hypothalamic cells (open circles) and in GT1-1 GnRH cells (closed circles).

D: Binding of [^{3}H]prazosin and its displacement by unlabelled prazosin in membrane preparations from broken GT1-1 GnRH cells. The data represent total bound cpm. Reproduced from Al-Damluji et al, 1993.

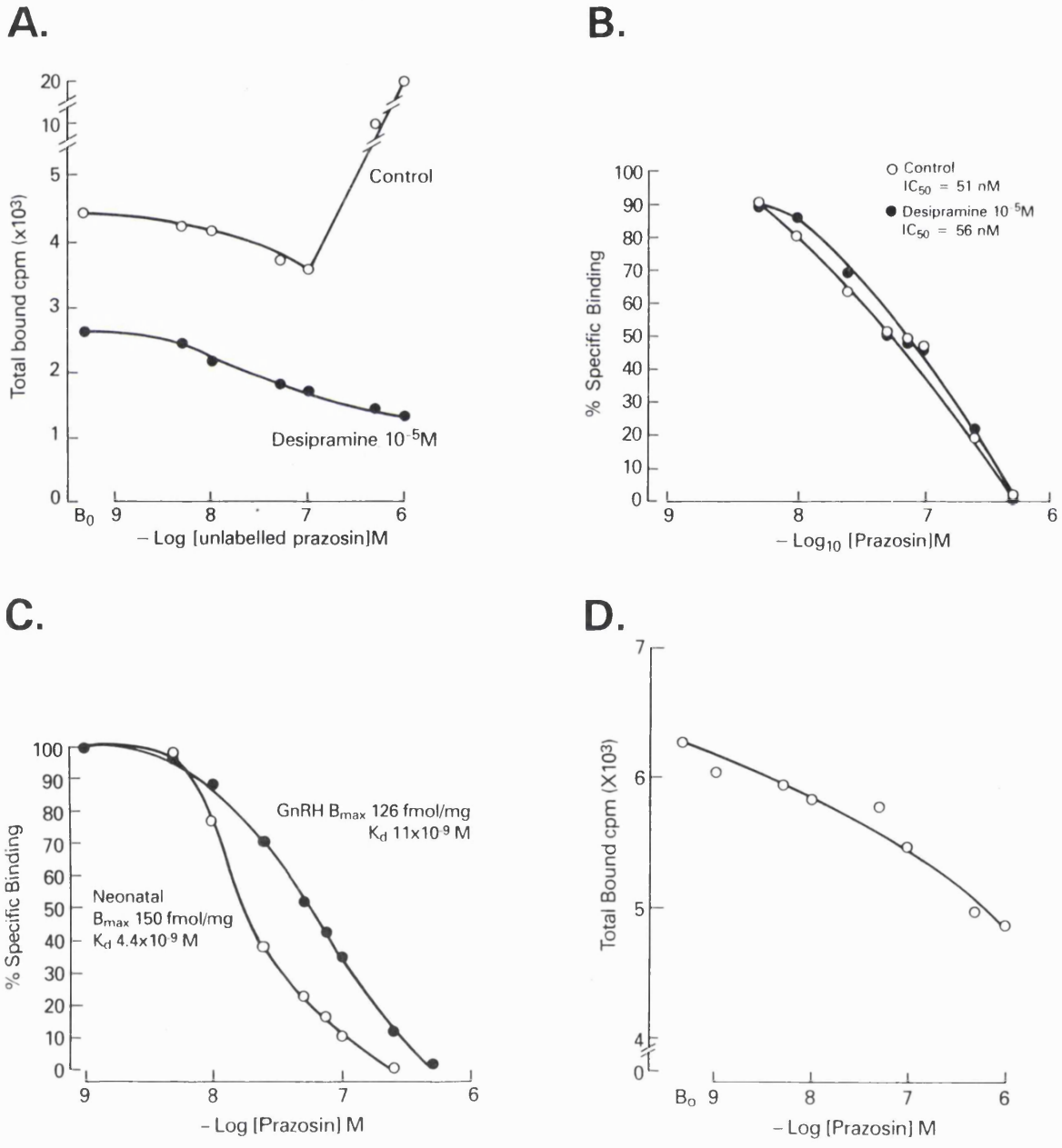


Figure 7.4.3:
Effect of unlabelled prazosin on the binding of [^{3}H]prazosin in intact GT1-1 GnRH cells, and the effect of prazosin on the pH of the solutions. The experiments were carried out in Krebs-Ringer buffered with 25 mM HEPES (upper panels) or 25 mM Tris (lower panels). The data represent total bound cpm. In the left panels, the data are presented as log-linear plots, in order to include the full range of concentrations of prazosin which were used. In the right panels, linear-linear scales were used to present the data from unlabelled prazosin concentrations of 10^{-7} M to $3x10^{-6}$ M. Reproduced from Al-Damluji et al, 1993.

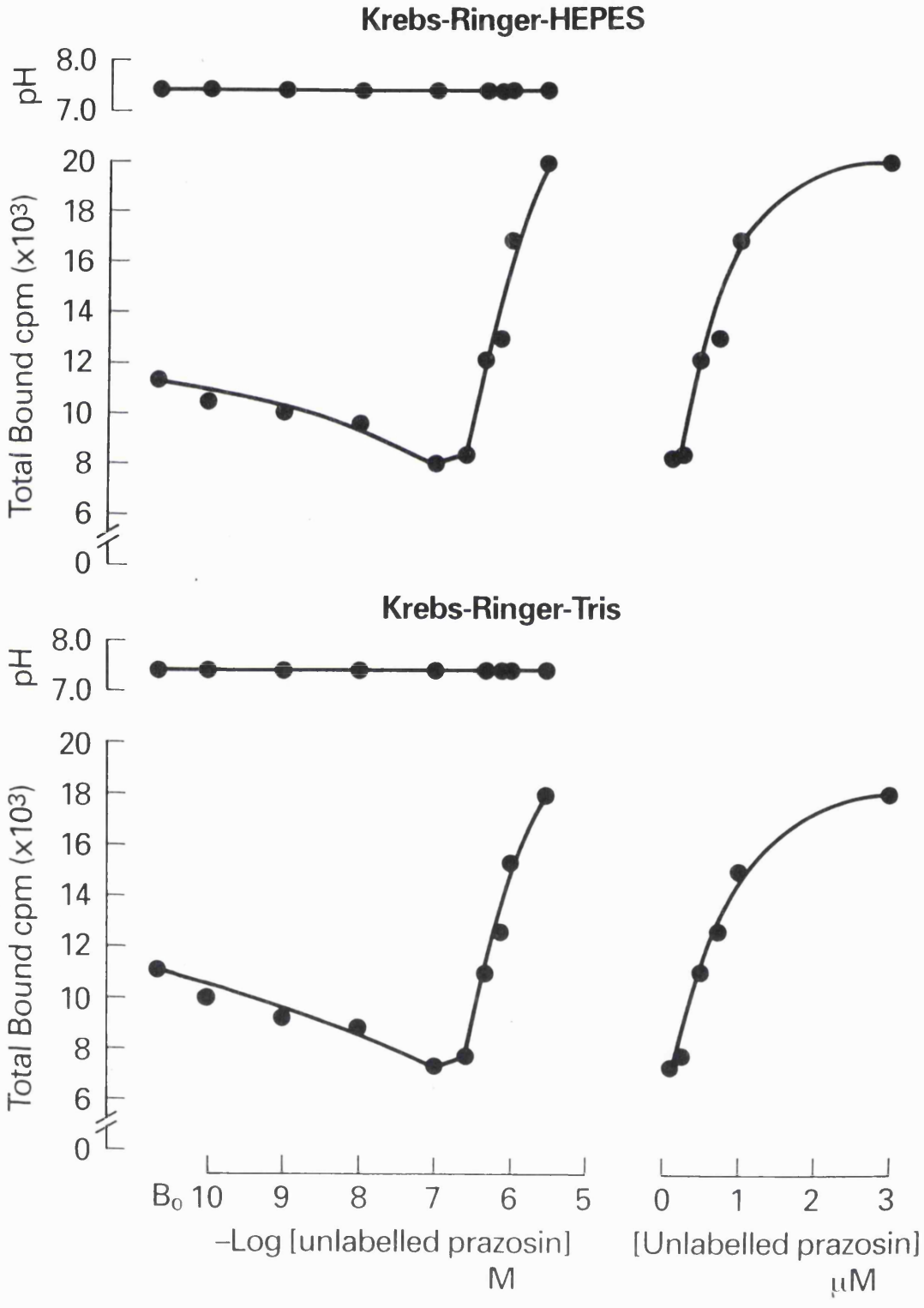


Figure 7.4.4:

Uptake of (±)[^{3}H]noradrenaline by GT1-1 GnRH cells (A) and neonatal hypothalamic cells (B): effects of desipramine (at 10^{-5} M) and omission of sodium from the incubation medium. Reproduced from Al-Damluji et al, 1993.

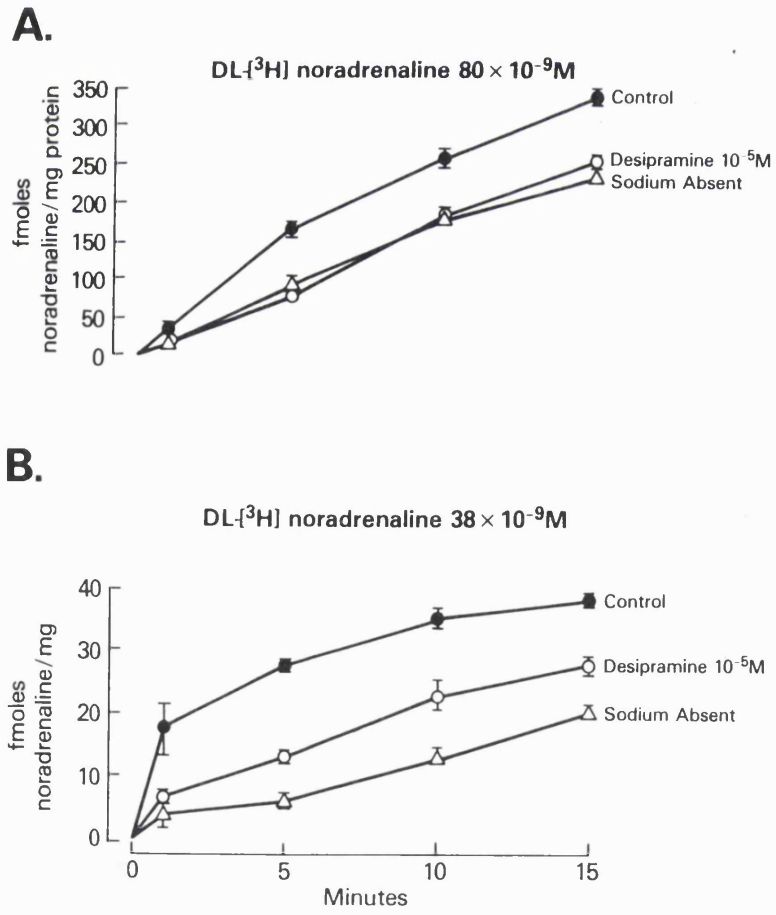


Figure 7.4.5:

A and B. Effects of steroids on the uptake of (±)[^{3}H]noradrenaline by GT1-1 GnRH cells (A) and neonatal hypothalamic cells (B).
C: Effects of inhibition of catechol-O-methyltransferase and monoamine oxidase A and B with OR-611, clorgyline and pargyline, respectively, on the measurable uptake of (-)noradrenaline in GT1-1 GnRH cells. Closed circles: control; open circles: OR-611, 10^{-6} M; triangles: OR-611, 10^{-6} M + clorgyline 10^{-4} M + pargyline 10^{-4} M. Reproduced from Al-Damluji et al, 1993.

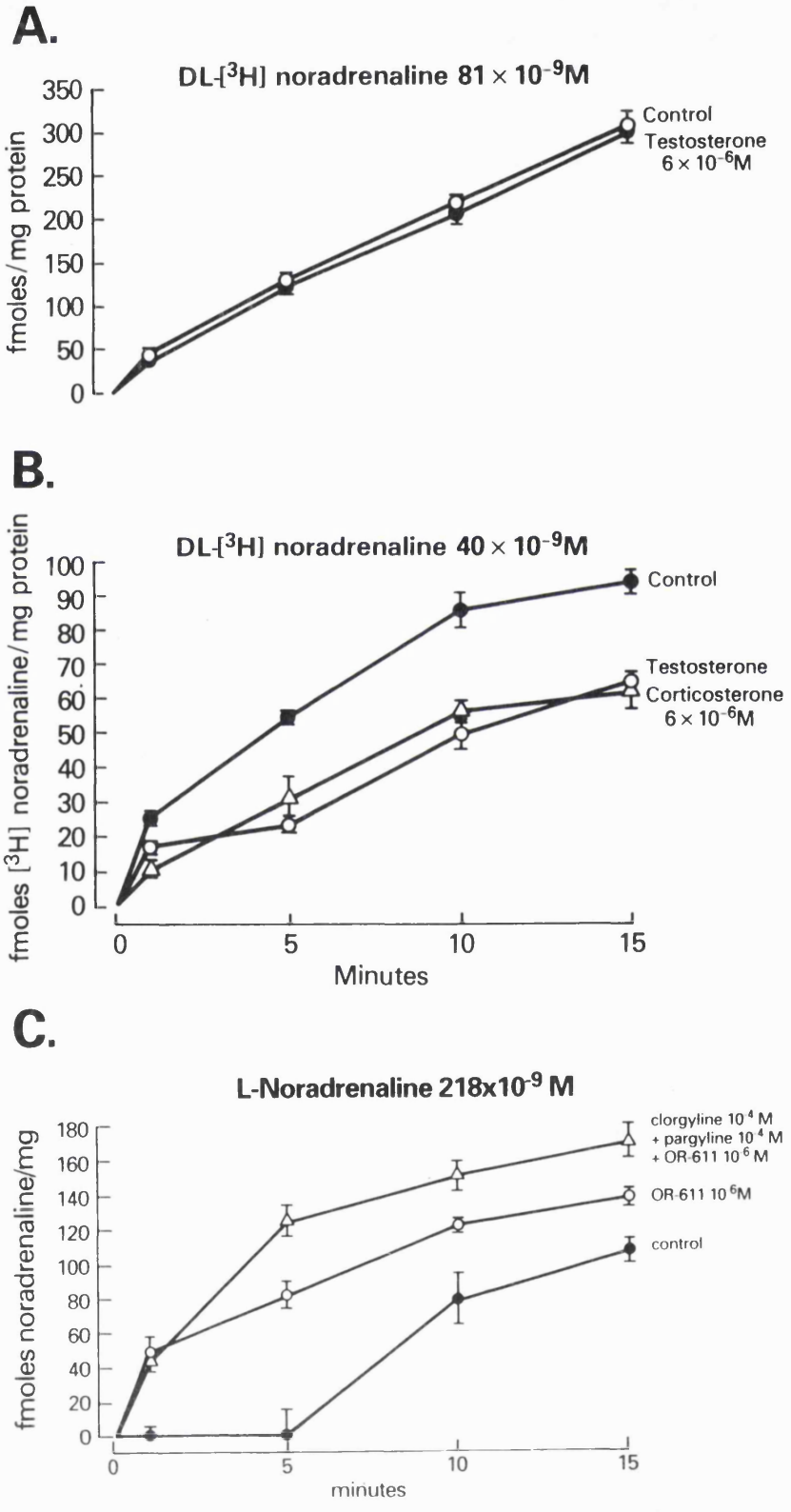


Figure 7.4.6:

Kinetic analysis of the uptake of (-)-noradrenaline in neonatal hypothalamic cells (left) and GT1-1 GnRH cells (right). The GT1-1 cells were studied in the presence of OR-611, 10^{-6} M to inhibit catechol-O-methyltransferase. Incubation was for 5 minutes at 37^0C. The data are presented as plots of concentration *vs* initial velocity in the upper panels, and as Lineweaver-Burk plots in the lower panels. Reproduced from Al-Damluji et al, 1993.

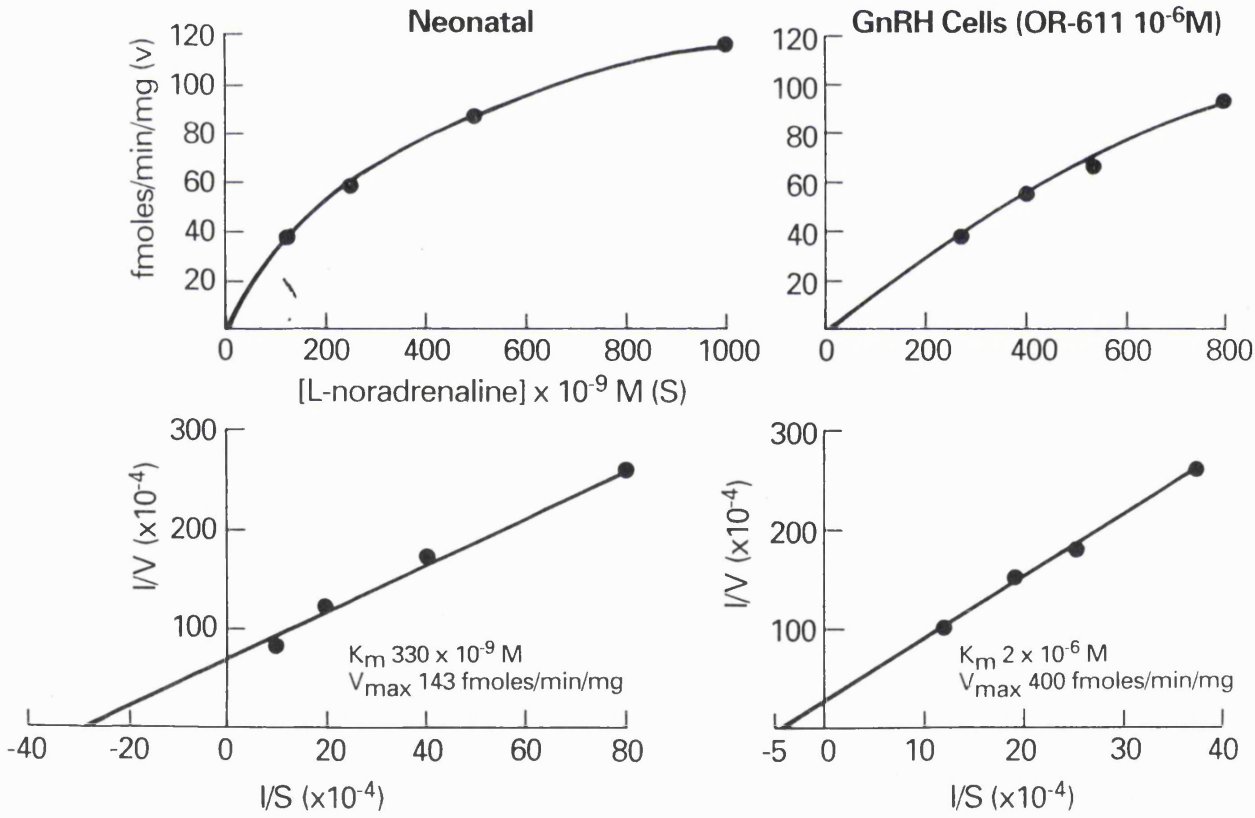


Figure 7.4.7:

Effect of intracerebroventricular (icv) 6-hydroxydopamine (6-OHDA) given as a single dose of 250 ug or as two 250 ug doses three days apart, on hypothalamic noradrenaline content (B) and the plasma ACTH response (A) to icv methoxamine in conscious, cannulated rats. Methoxamine or artificial cerebrospinal fluid (CSF) were administered seven days after the first dose of 6-OHDA. Each group consisted of four rats. Reproduced from Al-Damluji et al, 1993.

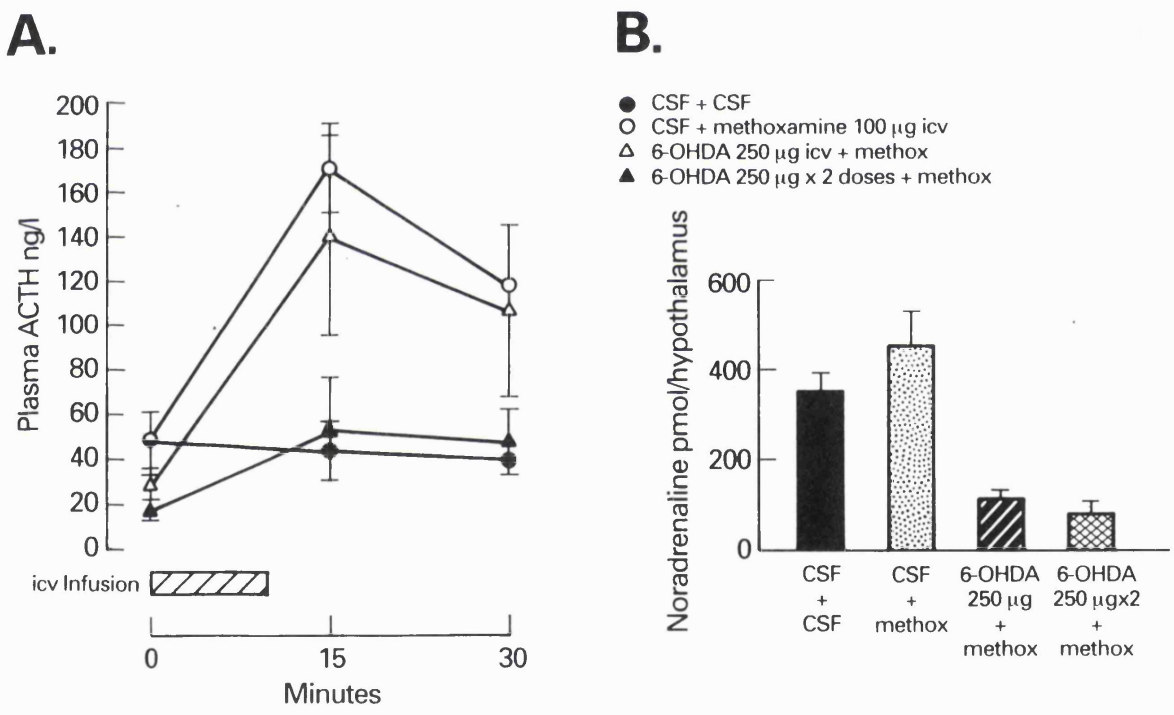
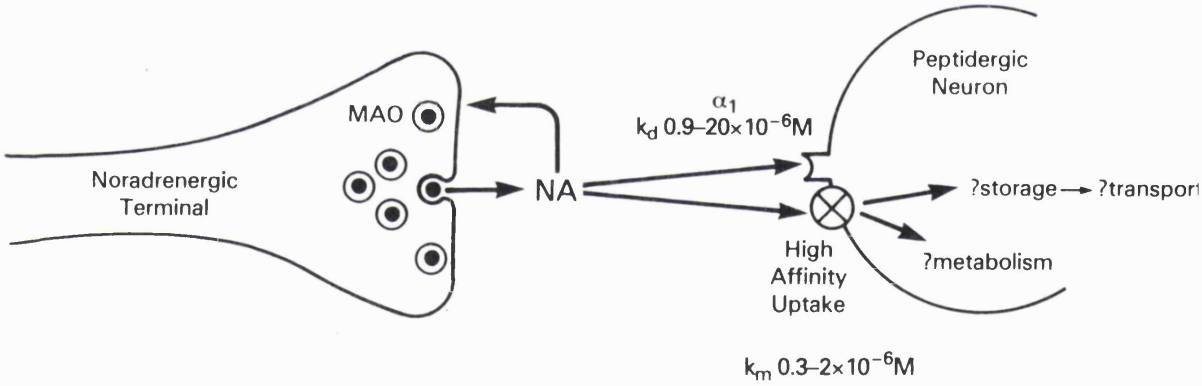


Figure 7.4.8:

Proposed model of the action of the post-synaptic uptake process. The affinities of (-)-noradrenaline (NA) for the post-synaptic uptake process (K_m 0.3-2x10^{-6} M) and for the post-synaptic α_{1B} adrenoceptors (K_d 3.2x10^{-6} M) make it likely that at concentrations that activate the post-synaptic receptor, noradrenaline will be accumulated by the post-synaptic uptake process. The post-synaptic uptake process may remove noradrenaline from the vicinity of the receptors, thus preventing desensitisation and maintaining the responsiveness of post-synaptic receptors to bursts of neurotransmitter released from the noradrenergic nerve teminals. In contrast, the pre-synaptic $Uptake_1$ noradrenaline transporter would presumably be less effective in removing noradrenaline from the vicinity of post-synaptic receptors, as it would have to rely on diffusion of molecules of noradrenaline back across the synapse, against their concentration gradient. Reproduced from Al-Damluji et al, 1993.



7.5 UPTAKE OF AMINES IN PRE-SYNAPTIC NEURONES AND IN GLIA

7.5.1 Discovery of mechanisms which terminate the actions of the neurotransmitters

In 1914, Sir Henry Dale discovered the activity of acetylcholine esterase. In that study, he identified acetylcholine as a constituent of ergot and he defined the two components of the cholinergic system as follows: "choline-esters have, then, two quite distinct types of action on the heart and circulation-the depressor, cardioinhibitor "muscarine" type of action, unaltered by nicotine, but abolished by atropine, and a pressor action of the nicotine type, unaffected by atropine, but abolished by large doses of nicotine" (Dale, 1914). Dale was describing the actions of acetylcholine on distinct muscarinic and nicotinic receptors. In the same seminal article, Dale noted that the actions of acetylcholine were very transient; he postulated correctly that the evanescence of the effect of acetylcholine was due to "the readiness with which the ester is hydrolysed into its relatively inert constituents, choline and acetic acid" (Dale, 1914). Otto Loewi demonstrated that the vagus nerve liberated a substance whose effects were indistinguishable from acetyl choline (Loewi, 1921). Acetylcholine was subsequently identified by chemical analysis in the spleen of horses and oxen, and it was found to "reproduce the effects of parasympathetic nerves, in an intense and evanescent form, with a fidelity rivalling that with which adrenaline reproduces effects of true sympathetic nerves" (Dale & Dudley, 1929). As physostigmine (eserine) inhibited the action of the esterase, it was suggested that parasympathetic nerves release a chemical transmitter, acetylcholine, whose action is rapidly terminated by an acetylcholine esterase (reviewed by Dale, 1934). Acetylcholine esterase is now known to be located in the synaptic cleft, where it hydrolyses released acetylcholine. Acetylcholine esterase is a member of a family of hydrolytic enzymes and the amino acid sequence of mammalian acetylcholine esterase is also homologous to that of some non-hydrolytic proteins such as thyroglobulin. The catalytic core consists of 543 amino acids whose crystal structure has been resolved, and some of its biochemical actions are understood at the atomic level (reviewed by Taylor & Radic, 1994).

Oliver and Schafer (1895a) discovered that adrenaline undergoes auto-oxidation in aqueous solutions; they commented that extracts of the adrenal medulla "assume a rose-red colour on exposure to the air or to oxidizing agents". However, adrenaline released from the adrenal glands exists in a relatively stabilised form; this was explained by Heard & Welch (1935) who proposed that presence of ascorbic acid in the adrenal glands "is involved in a system which maintains adrenaline or adrenaline-like substances in a reduced state". They proposed

that the oxidation of adrenaline to a quinone can be prevented by ascorbic acid, as shown in Figure 7.5.1.

In 1937, Blaschko and his colleagues in Cambridge discovered the oxidative deamination of adrenaline and they distinguished this enzymatic activity from non-specific auto-oxidation. They prepared liver extracts "which were free from cells but contained granular cell fragments", presumably including mitochondria. They found that the oxygen consumption of these liver extracts was greatly increased by addition of adrenaline, and they calculated that each molecule of adrenaline consumed one atom of oxygen; this oxidation process was accompanied by inactivation of adrenaline (Blaschko et al, 1937). They purified an enzyme which oxidised adrenaline stereospecifically, the L-isomer being oxidised at twice the rate of the D-isomer (Blaschko et al, 1937). Thus, the enzymatic oxidation of adrenaline had different properties from auto-oxidation which is non-specific. The monoamine oxidase (MAO) enzymes are widely distributed in most tissues, where they are located predominantly in the outer membranes of mitochondria; they catalyse the conversion of primary, secondary and tertiary amines to aldehydes as follows:

$RCH_2NH_2 + O_2 + H_2O \Rightarrow RCHO + NH_3 + H_2O_2$

Some of the aldehydes are then oxidised to carboxylic acids (Figure 7.5.2). Quaternary amines are not oxidised, nor are neutral amines such as aniline. Compounds which possess a methyl group on the α carbon (such as amphetamine and ephedrine) cannot be deaminated by this enzyme and may act as inhibitors (Blaschko, 1952). Two forms of MAO are now known to exist (designated A and B) which are 70% similar in their amino acid sequences. They are encoded by separate genes which are closely linked on the X chromosome; the two genes have identical exon-intron organisation, suggesting that they may have arisen by duplication of an ancestral gene (Shih et al, 1999). In the brain, MAO A is expressed predominantly in catecholaminergic neurones, whereas MAO B is in serotonergic and histaminergic neurones and in glial cells. Administration of MAO inhibitor drugs or disruption of the MAO A gene in transgenic animals results in aggressive behaviour and moderate increases in the concentrations of serotonin, noradrenaline and dopamine in the brain, confirming the physiological significance of this enzyme in metabolising the neurotransmitter amines (Cases et al, 1995). In contrast, disruption of the MAO B gene is not associated with aggressive behaviour, but the animals are resistant to the Parkinsonian effects of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is

activated by oxidative deamination (Shih et al, 1999).

Catecholamines are also degraded by a separate enzyme which was discovered by Axelrod in the Laboratory of Clinical Science at the National Institute of Mental Health in Bethesda. The clue for the existence of this enzyme came from the discovery in Salt Lake City that human urine contained 3-methoxy-4-hydroxymandelic acid (vanillyl mandelic acid; VMA; Armstrong et al, 1957). These investigators reported that urinary excretion of VMA was increased following administration of noradrenaline; patients with phaeochromocytomas excreted large amounts of VMA preoperatively and normal amounts postoperatively; administration of 3,4-dihydroxymandelic acid was followed by increased excretion of VMA. They therefore concluded correctly that VMA "might be expected to be formed by the action of amine oxidase upon norepinephrine or epinephrine, followed by methylation of the resulting 3,4-dihydroxymandelic acid" (Armstrong et al, 1957; Figure 7.5.2). Subsequently, Axelrod et al (1958) detected in rat urine and tissues the 3-O-methyl analogues of adrenaline and noradrenaline which they named "metanephrine" and "normetanephrine", respectively (Figure 7.5.2). They also showed that the methoxylated analogues appeared in increased amounts in the urine when the rats were given adrenaline, noradrenaline or dopamine (Axelrod et al, 1958). The methylating enzyme, catechol O-methyl transferase (COMT) was extracted from rat liver and found to require for its activity the methyl group donor S-adenosylmethionine (Axelrod & Tomchick, 1958). This enzyme also required divalent cations such as magnesium or manganese but calcium was inactive. Substrates for this enzyme must possess two phenolic hydroxyl groups and monophenols were inactive. A side chain was not required for activity of the enzyme. In the case of catecholamines, the enzyme transferred the methyl group to the phenolic hydroxyl group which is in the *meta* position. In the original work, it was thought that the intracellular distribution of COMT was confined to the cytoplasm (Axelrod & Tomchick, 1958). In later studies, it became clear that a proportion of COMT is membrane-bound; it is anchored to the cytoplasmic surface of internal cellular membranes such as the endoplasmic reticulum, so it is still capable of exerting its catalytic effect in the cytoplasm (Kopin, 1985; Mannisto & Kaakkola, 1999). In general, COMT is an enzyme of non-neuronal cells; in peripheral organs, it is detectable in the vascular endothelium of most tissues, particularly the liver, kidneys and heart. In the brain, COMT is probably confined to non-neuronal cells such as glia, ventricular ependymal cells, choroid plexus and the circumventricular organs (Kopin, 1985; Mannisto & Kaakkola, 1999).

In humans, a single gene on chromosome 22 encodes both the soluble and the membrane-bound enzymes. The soluble enzyme consists of 221 amino acids and the membrane-bound enzyme consists of 271 amino acids; the additional 50 residues are mostly hydrophobic amino acids which anchor the enzyme to the membranes. The two peptides are expressed from the same gene by utilising alternative start sites. The enzyme crystal structure has been resolved and some aspects of its catalytic function are understood at the atomic level (Mannisto & Kaakkola, 1999). Disruption of the COMT gene in transgenic mice is accompanied by no significant changes in the concentrations of adrenaline, noradrenaline or serotonin under basal conditions, and the concentrations of dopamine are also unaltered, except in the frontal cortex of male mice, where there was a moderate increase in dopamine content; this absence of a major effect is consistent with the results of pharmacological blockade of COMT, suggesting that these neurotransmitters are largely protected against enzymatic degradation under basal conditions (Gogos et al, 1998).

Following the discovery of the inactivating enzymes, it was thought that enzymatic degradation was the general mechanism for terminating the actions of neurotransmitters, although it was recognised that other factors were likely to be involved. However, in subsequent years, it became clear that acetylcholine was the only neurotransmitter which was inactivated predominantly by enzymatic degradation; the actions of the amine and amino acid neurotransmitters are terminated predominantly by removal of the intact molecules from the extracellular space by specific transporter molecules, followed by accumulation and storage of the neurotransmitter molecules in vesicles within the pre-synaptic neurones. The vesicular location of the neurotransmitters protects them against degradation by the metabolising enzymes.

7.5.2 Discovery of the uptake of amines in pre-synaptic neurones

Remarkably, Oliver & Schafer (1895a) commented that following injection of the extract of the adrenal medulla, "it is possible that the active principle (ie, adrenaline) may be for a time at least stored away within the skeletal muscles". Their speculation was based on their observation that the action of adrenaline "upon the muscles appears to be prolonged beyond that upon the heart and blood vessels". More direct evidence for uptake of adrenaline by tissues was provided by Burn (1932) during his investigations on the mechanism of action of the sympathomimetic amines. He found that the sympathomimetic amines "tyramine and

ephedrine have no appreciable constrictor effect on the vessels of the cat's fore-limb if the post-ganglionic sympathetic fibres have degenerated. It is concluded that tyramine and ephedrine normally stimulate the sympathetic nerve endings, whereas adrenaline stimulates the myo-neural junction which survives degeneration of the sympathetic nerve fibre". Further, "if adrenaline is added to the circulating blood, the constrictor action of tyramine and ephedrine is greatly increased". Therefore, "it may be supposed that the magazine or store at the end of each sympathetic nerve is replenished (by the adrenaline infusion), so that when an injection of tyramine or ephedrine is given to stimulate the sympathetic nerve endings, a large amount of adrenaline is liberated, and the constrictor effect is much greater than before" (Burn, 1932). Burn's students extended this work to cocaine (Macmillan, 1959; Trendelenburg, 1959). It was concluded that "cocaine may prevent the uptake of noradrenaline by the tissue stores" (Macmillan, 1959). Thus, it was clear to Burn and his colleagues that sympathetic nerves can remove catecholamines from the circulation, and that the neurotransmitters are then stored in the nerve terminals.

Subsequent investigators confirmed that following peripheral administration of large doses of adrenaline or noradrenaline, these amines accumulate in the heart, as detected by bioassays and colorimetric assays (Nickerson et al, 1950; Raab & Gigee, 1955). Peripheral administration of [^{14}C]adrenaline was followed by appearance of the radiolabelled compound in the heart (Schayer, 1951).

[^{3}H]Adrenaline was synthesised at New England Nuclear in 1958 at the instigation of S.S. Ketty of the National Institute of Mental Health in Bethesda (LaBrosse et al, 1958). The availability of radiolabelled catecholamines represented a major technical advance; it was no longer necessary to administer large doses of catecholamines, as the radiolabelled hormones could be detected by sensitive scintillation counting. Detailed studies were performed on the tissue distribution of [^{3}H]adrenaline following intravenous infusion in animals; the greatest accumulation of both adrenaline and its metabolite metanephrine was in the heart, spleen, adrenal glands and the sympathetic chain, but the brain was almost devoid of uptake; this was attributed to the inability of adrenaline to penetrate the blood-brain barrier (Axelrod et al, 1959). Following intravenous injection of [^{3}H]noradrenaline, the distribution of [^{3}H]noradrenaline and its metabolite [^{3}H]normetanephrine were similar to the distribution of [^{3}H]adrenaline and [^{3}H]metanephrine (Whitby et al, 1961). Thus, accumulation of injected

catecholamines was greatest in tissues which had the greatest concentrations of endogenous catecholamines (Axelrod et al, 1959). This suggested that accumulation of the injected catecholamines was mostly into sympathetic nerve endings. Further evidence for this hypothesis was provided by the observation that surgical sympathetic denervation almost abolished the ability of tissues to accumulate injected catecholamines (Hertting et al, 1961a; Stromblad & Nickerson, 1961). Injection of antiserum against nerve growth factor into 3-day old rats or mice causes atrophy of noradrenergic neurones in certain parts of the peripheral sympathetic nervous system; in such animals, the reduction in the ability to accumulate [^{3}H]noradrenaline in various tissues paralleled the reduction in the content of endogenous noradrenaline in these tissues (Iversen, 1965; Iversen et al, 1966). This provided further evidence that the accumulation of noradrenaline is into the sympathetic neurones in these tissues. Visual evidence for the neuronal location of [^{3}H]noradrenaline was provided by autoradiographic studies of organs which are densely innervated with noradrenergic nerve terminals, such as the pineal gland and the heart; autoradiographic grains were seen only over non-myelinated axons which contained granulated vesicles (Wolfe et al, 1962; Marks et al, 1962).

Much of the [^{3}H]noradrenaline was retained in the tissues in unchanged form, long after its physiological effects had ceased (Whitby et al, 1961). Further, following uptake into the sympathetic nerves, [^{3}H]noradrenaline could be released upon electrical stimulation of the nerve endings; this suggested that following release from the nerve terminals, some endogenous noradrenaline diffuses towards post-synaptic cells, some is metabolised, and the remainder is re-accumulated back into the pre-synaptic nerve terminals, where it is stored in unchanged form, ready for release in response to the next stimulus (Hertting & Axelrod, 1961).

The above studies suggested that uptake of catecholamines into tissues may be an important mechanism of inactivation of these neurotransmitters. As a substantial proportion of [^{3}H]noradrenaline was stored in unchanged form, it was suggested that in normal tissues, nerve uptake is the major inactivation mechanism, and this process competes with the metabolising enzymes for extracellular noradrenaline molecules. However, in immunosympathectomised or surgically denervated tissues, the proportion of [^{3}H]-O-methylated metabolites was greatly increased, suggesting that in the absence of sympathetic

nerve terminals, the major inactivating mechanism becomes catechol-O-methyltransferase which exists extra-neuronally (Iversen et al, 1966). The observation that surgical denervation reduced the uptake of noradrenaline provided a partial explanation for the denervation hypersensitivity which is observed in sympathetically innervated organs (Hertting et al, 1961a). Hypersensitivity of such organs is also due in part to up-regulation of post-synaptic receptors, as had been explained by Langley (see above).

In view of the impenetrability of the blood brain barrier to catecholamines, [^{3}H]adrenaline and [^{3}H]noradrenaline were either injected into the cerebral ventricles or brain slices were incubated in the presence of [^{3}H]catecholamines (Dengler et al, 1961; Glowinski et al, 1965). Such studies demonstrated that as in the periphery, [^{3}H]catecholamines accumulated in greatest amounts in the brain regions which contained the greatest concentrations of endogenous catecholamines (Glowinski & Iversen, 1966). Thus, following cerebroventricular injection of [^{3}H]noradrenaline, the hypothalamus, which has the greatest concentration of endogenous noradrenaline, also contained the greatest concentration of [^{3}H]noradrenaline (Glowinski & Iversen, 1966). These findings were consistent with the view that in the brain, as in the periphery, catecholamines were accumulated and stored in noradrenergic nerve terminals. The striatum, which is rich in dopaminergic neurones, accumulated large amounts of [^{3}H]dopamine and [^{3}H]noradrenaline, indicating that the pre-synaptic uptake process for dopamine is capable of accumulating noradrenaline (Glowinski & Iversen, 1966). Brain regions which contained a dense noradrenergic innervation also had a high proportion of unchanged [^{3}H]noradrenaline, whereas the cerebral cortex and the cerebellum which have few noradrenergic nerve terminals contained a greater proportion of [^{3}H]metabolites of [^{3}H]noradrenaline (Glowinski & Iversen, 1966). This was consistent with the view that noradrenergic neurones store noradrenaline in a compartment which is inaccessible to metabolising enzymes (see below). In all brain regions, the major metabolites were deaminated O-methylated products, indicating that in the brain, unstored noradrenaline is degraded by both monoamine oxidase and catechol-O-methyltransferase (Glowinski & Iversen, 1966). Subsequent autoradiographic studies in which [^{3}H]noradrenaline had been injected into the rat cerebral ventricles confirmed that the greatest accumulation of [^{3}H]noradrenaline was in the hypothalamus; the autoradiographic activity was over nerve

endings which contained dense core vesicles, presumably representing pre-synaptic noradrenergic nerve terminals (Aghajanian & Bloom, 1967). Within the hypothalamus, the greatest autoradiographic densities were in the median eminence, the periventricular regions and the lateral basal hypothalamus, which receive the densest innervation of catecholamine nerve terminals (Hokfelt & Ljungdahl, 1971).

7.5.3 Functional properties of pre-synaptic plasma membrane uptake

Perfusion of the isolated rat heart with increasing concentrations of noradrenaline demonstrated that the initial rate of uptake of noradrenaline was saturable and could be described by Michaelis-Menten kinetics; as diffusion of this polar molecule into the tissues was minimal, it was suggested that the accumulation of exogenous noradrenaline proceeds initially by a saturable process of membrane transport (Dengler et al, 1961; Iversen, 1963). Accumulation of noradrenaline could be blocked by ouabain, so it was suggested that the uptake process may be mediated by an energy-requiring carrier (Dengler et al, 1961). The source of energy for this carrier is the electrochemical gradient of sodium ions which is generated by the Na^+/K^+-ATPase ("sodium pump"); exclusion of sodium from the extracellular space completely abolishes the active uptake of noradrenaline (Iversen & Kravitz, 1966; Bogdanski & Brodie, 1966). Other alkali metals such as lithium and potassium cannot substitute for sodium, and alkaline earths such as calcium are ineffective (Iversen & Kravitz, 1966; Bogdanski & Brodie, 1966).

The structural properties of phenylethylamines (such as noradrenaline) which are accumulated by the pre-synaptic noradrenaline carrier were studied in greatest detail by Burgen & Iversen (1965). They determined the affinities of phenylethylamines for the carrier in the isolated rat heart, as measured by the ability of these compounds to compete with the uptake of noradrenaline. The conclusions from this and subsequent studies were as follows:

1. Affinity for the uptake process was reduced by the presence of a hydroxyl group on the β carbon, such that dopamine had greater affinity for the carrier than noradrenaline.

2. The steric arrangement of the hydroxyl group on the β carbon is more favourable in the (-) than in the (+) position, such that (-)-noradenaline had greater affinity than (+)-noradrenaline.

3. Presence of a methyl group on the α carbon increased affinity for the carrier, such that amphetamine was more potent than phenylethylamine.

4. A single phenolic hydroxyl group in the *meta* or *para* position enhanced affinity and two phenolic hydroxyl groups in these positions caused a further increase in affinity.
5. Phenolic methoxyl groups caused a striking reduction in affinity, such that normetanephrine was far less potent than noradrenaline. There was a progressive reduction in affinity of compounds which possessed two or three phenolic methoxyl groups, in comparison to phenylethyalmine.
6. Presence of an N-methyl group reduced affinity, such that adrenaline was less potent than noradrenaline. Larger N-substitutions caused even greater reductions in affinity.
7. Substitution of the benzene ring with a reduced ring (such as cyclohexane or cyclopentane) caused little reduction in affinity, and the bicyclic naphthalene substitution was also active. A tryptamine substitution retained some activity, such that serotonin had appreciable affinity.

The above findings will be discussed in greater detail in relation to the structural properties of ligands for Transport-P.

Several important drugs were found to interfere with the uptake of catecholamines into sympathetic nerve terminals, including cocaine, antidepressants, tyramine and amphetamine. Frohlich & Loewi (1910) had reported that cocaine sensitizes organs to the effects of adrenaline. The mechanism of action of cocaine was elucidated in the Department of Pharmacology at Oxford by two of Burn's students, Macmillan and Trendelenburg; the latter then continued the work at Harvard. Burn had concluded that circulating catecholamines are accumulated into tissue stores and that tyramine releases stored catecholamines (see above); his students extended these observations. Trendelenburg (1959) demonstrated that cocaine prolonged the half-life of injected noradrenaline and he concluded that cocaine causes supersensitivity by delaying the inactivation of the hormone. Macmillan (1959) proposed that "cocaine may prevent the uptake of noradrenaline by the tissue stores", thereby increasing the amount of noradrenaline which is available to act on adrenergic receptors. Further evidence for this hypothesis was provided by the finding that cocaine reduces the uptake of [^{3}H]noradrenaline into sympathetically innervated tissues (Whitby et al, 1960). Kinetic analysis indicated that the inhibitory effect of cocaine was of a competitive nature (Iversen & Langer, 1969). Whereas sympathomimetic amines such as tyramine were shown to block uptake and to deplete the tissue content of noradrenaline, cocaine blocked the uptake of circulating noradrenaline but had no effect on the endogenous tissue content of noradrenaline

(Muscholl, 1961). The explanation for these differences is that tyramine, amphetamine and other sympathomimetic amines block the uptake of exogenous noradrenaline and enhance the release of endogenous noradrenaline, whereas cocaine only blocks uptake without enhancing release (Hertting et al, 1961b & c).

Tricyclic antidepressants were found to inhibit the uptake of [^{3}H]noradrenaline in rat brain *in vivo*; as the mechanism of action of these antidepressants was unknown, it was suggested that the "ability of imipramine to prevent the rebinding of noradrenaline by cerebral tissues may be a mechanism for the antidepressant action of this drug. Such an action of the drug would allow more free physiologically active noradrenaline liberated from the central sympathetic neurones to react with the central adrenergic receptors" (Glowinski & Axelrod, 1964). This "pre-synaptic re-uptake hypothesis" remains the prevailing view and will be discussed critically in Section 12.4. Kinetic analysis indicated that the inhibitory effect of the antidepressant desipramine was of a competitive nature (Iversen & Langer, 1969), and the antidepressants generally act in a manner which is similar to cocaine, as will be described in detail in a subsequent section.

7.5.4 Discovery of the uptake of amines in pre-synaptic vesicles

The location of catecholamines within vesicles was discovered in 1953, as a result of simultaneous studies by Hillarp in Sweden and by Blaschko who had by then moved to the Department of Pharmacology at Oxford, which was headed by Burn. Both studies utilised Palade's techniques of differential centrifugation to investigate the location of catecholamines in homogenates of the adrenal medulla. They concluded that "adrenaline is mainly held in particles. We shall call them *pre-secretory granules*" (Blaschko & Welch, 1953). Hillarp et al (1953) reported that the adrenal medullary granules were 0.1-0.6 μm in size. Von Euler & Hillarp (1956) studied the location of catecholamines in neurones and they found noradrenaline to be located in analogous granules which could be sedimented from homogenates of splenic nerves. Dopamine, adrenaline, noradrenaline and serotonin were found to accumulate in the adrenal medullary granules both *in vivo* and *in vitro* (Bertler et al, 1960a & b; Carlsson & Hillarp, 1961).

The distribution of [^{3}H]noradrenaline within sympathetic neurones was investigated in organs which receive a dense noradrenergic innervation, such as the heart, salivary glands

and the vas deferens. [^{3}H]Noradrenaline or [^{3}H]adrenaline were administered to rats *in vivo*, the organs removed and the intracellular localisation of the radiolabel was studied in tissue homogenates; in the heart, vas deferens, submaxillary glands and pineal, the [^{3}H]catecholamines were detected in a light microsomal fraction (presumably representing light vesicles) where endogenous noradrenaline was also located (Potter & Axelrod, 1962 & 1963a). The particles in the microsomal fraction from the heart also contained dopamine-β-hydroxylase, indicating that [^{3}H]noradrenaline was accumulated into the neurosecretory vesicles where endogenous noradrenaline is synthesised and stored (Potter & Axelrod, 1963b). While [^{3}H]noradrenaline and endogenous noradrenaline were located in light particles in sympathetic neurones, in the adrenal medulla, endogenous and radiolabelled catecholamines were located both in the light microsomal fraction and in dense granules (Potter & Axelrod, 1963a).

7.5.5 Functional properties of adrenal medullary and pre-synaptic vesicular uptake

Many of the basic properties of vesicular uptake were discovered by Bertler, Hillarp and Rosengren in Sweden. Rabbits were given the neurotransmitter precursors dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan (5-HTP) and the animals were then killed and adrenal medullary granules were prepared by differential centrifugation. Although the decarboxylase enzyme was known to be located in the cytoplasm, the neurotransmitters dopamine, adrenaline, noradrenaline and serotonin were found to be located predominantly in the granules. "The newly formed dopamine is thus rapidly taken up by the granules". Further, "the mechanism by which catechol amines are taken up and stored does not seem to be specific for catechol amines... Which of the two types of amines a cell containing these granules possesses, seems to be due to its contents of DOPA and 5-HTP synthesising enzymes". In addition, "Pretreatment of the animals with reserpine impaired the uptake of dopamine by adrenal medullary granules...Thus the primary effect of reserpine which is known to deplete the body stores of catechol amines may be its interaction with the uptake of catechol amines by the granules" (Bertler et al, 1960a & b).

The chromaffin granules of the adrenal medulla and the neurosecretory vesicles in pre-synaptic nerve terminals both function as storage vesicles for the neurotransmitter amines, and their functional properties are very similar but not identical. These uptake processes are

not specific for catecholamines, as they are capable of accumulating serotonin and non-catechol phenylethylamines such as tyramine and amphetamine (Carlsson et al, 1963). Uptake of catecholamines in adrenal medullary granules is temperature-dependent, indicating that it is an energy-requiring process (Kirshner, 1962a; Carlsson et al, 1962c &1963). Uptake requires the presence of ATP and is enhanced by the presence of magnesium; ATP alone (but not magnesium alone) increased uptake but a high amine incorporation was obtained only when both had been added (Kirshner, 1962a & b; Carlsson et al, 1963). The maximal effective concentrations of ATP and magnesium were approximately 1mM (Carlsson et al, 1963). Calcium was ineffective and chelating agents such as EDTA reduced uptake in the presence of magnesium (Kirshner, 1962a & b; Carlsson et al, 1963). Alkali metals such as sodium and postassium did not enhance uptake (Kirshner, 1962b). Two hypotheses were considered: first, that the amines diffused freely across the vesicular membrane and were then bound to specific sites within the vesicle, possibly the negatively charged ATP molecules; and second, that the vesicular membrane is impermeable to the amines which react with some component of the membrane, following which they are actively transported across the vesicular membrane by an energy requiring process, and then released to the interior of the vesicles (Kirshner, 1962b). The data were thought to be more consistent with the active transport hypothesis, and subsequent cloning of cDNAs which encode transport proteins which are integral to the vesicular membrane is consistent with this hypothesis (see Section 7.5.14).

The structural properties of phenylethylamines which are accumulated by adrenal medullary vesicles were studied initially by Kirshner (1962b), Carlsson et al (1963) and Slotkin & Kirshner (1971). The following conclusions were made regarding the effects of structural substitutions on the ability of phenylethylamines to inhibit the uptake of [^{3}H]adrenaline in granules from rat adrenal medulla (Slotkin et al, 1975) and the uptake of [^{3}H]noradrenaline in vesicles from rat brain (Slotkin et al, 1979):

1. A single phenolic hydroxyl group in the *para* position enhanced affinity and two phenolic hydroxyl groups caused a further increase in affinity.
2. A phenolic chlorine atom increased affinity for uptake.
3. Phenolic methoxyl groups increased affinity in adrenal medullary vesicles but reduced affinity in brain vesicles.
4. Increasing the length of the side chain enhaned affinity in adrenal medullary granules.
5. Affinity for the uptake process was reduced by the presence of a hydroxyl group on the

β carbon.

6. Affinity for the uptake process in the adrenal granules was enhanced by the presence of a methyl group on the α carbon but this substitution had no effect on affinity for uptake in brain vesicles.
7. Presence of an N-methyl group enhanced affinity for uptake in the adrenal granules but this substitution had no effect on affinity for uptake in brain vesicles.
8. The phenyl group was essential for uptake but aliphatic alkylamines were still active, particulary when the alkyl chain was longer than four carbons.
9. The steric arrangement of the hydroxyl group on the β carbon is more favourable in the (-) than in the (+) position.

The above findings will be discussed in greater detail in relation to the structural properties of ligands for Transport-P.

Several drugs were found to inhibit the vesicular uptake of amines. Brodie and his colleagues in Bethesda had discovered that reserpine depletes animals of serotonin and increases the excretion of the serotonin metabolite 5-hydroxyindoleacetic acid (Shore et al, 1955). Marthe Vogt demonstrated that administration of reserpine and amphetamine *in vivo* depleted the serotonin and noradrenaline content of the hypothalamus and the adrenal medulla (Paasonen & Vogt, 1956; Holzbauer & Vogt, 1956). Bertler's work on the effect of reserpine on granular storage *in vivo* was described above. Subsequently, uptake of catecholamines in adrenal chromaffin granules *in vitro* was found to be inhibited irreversibly by reserpine (Kirshner, 1962a). This compound blocks the uptake of dopamine into the granules; as dopamine is converted to noradrenaline by dopamine-β-hydroxylase within the granules, administration of reserpine depletes the tissue content of noradrenaline, partly by preventing its synthesis from dopamine (Kirshner, 1962a & b).

Sympathomimetic amines such as tyramine and amphetamine are also accumulated by the vesicular uptake process; accumulation of these amines is only partly dependent on ATP and is only inhibited in part by reserpine, presumably because these relatively lipid soluble amines can enter the vesicles by simple diffusion, in addition to their accumulation via the specific uptake process (Carlsson et al, 1963). Sympathomimetic amines displace endogenous neurotransmitter amines from the storage vesicles (Carlsson et al, 1963) and

they increase the rate of metabolism of catecholamines *in vivo* (Axelrod & Tomchick, 1960). This provided evidence that vesicular storage prevents the degradation of catecholamines by monoamine oxidase (which is located in mitochondria). It was proposed that sympathomimetic amines such as amphetamine may neutralise the acidity of the pre-synaptic vesicles and that this may accelarate the release of the neurotransmitters from the vesicles (Sulzer & Rayport, 1990). However, this mechanism is probably insignificant, as mice which lack the pre-synaptic vesicular transporter (and which are therefore incapable of storing catecholamines in neurosecretory vesicles) respond to amphetamines by releasing substantial amounts of catecholamines (Fon et al, 1997). Thus, the primary site of action of amphetamine is likely to be on the pre-synaptic plasma membrane transporters.

7.5.6 The chemiosmotic hypothesis

Peter Mitchell introduced a new concept which unified the fields of metabolism and transport. Mitchell studied in Cambridge, after which he taught in Cambridge and then in Edinburgh. He then carried out his work in a research institute which he established in Cornwall. Mitchell's ideas received much hostility in the 1960s. The following summary of the chemiosmotic hypothesis is based on a review (Mitchell, 1976) which was published two years before he received the Nobel Prize, since when his ideas have been generally accepted.

According to the chemiosmotic hypothesis, the enzymes of the respiratory chain generate an acidic environment in mitochondria. The accumulation of protons in mitochondria generates a chemical and an electrical gradient across the impermeable mitochondrial membrane; this gradient provides the source of power for the ATP synthase, which translocates protons out of the mitochondria and phosphorylates ADP to ATP. The electrochemical proton gradient is also used as a source of energy for various transporters which translocate solutes either in the same direction, or in the opposite direction to the protons.

The chemiosmotic hypothesis was based on four postulates:

1. The ATP synthase is a reversible, proton-translocating ATPase;
2. Respiratory enzyme chains are proton-translocating systems which have the opposite polarity of proton translocation as the ATP synthase;
3. There are proton (or hydroxyl) linked solute transporters which are necessary for osmotic stabilisation and metabolite transport;
4. Systems 1-3 are enclosed in an insulating membrane which forms an osmotic barrier to

the movement of protons, hydroxyl ions and solutes.

Mitchell gathered a large amount of experimental evidence which supported the above postulates, which are now generally accepted. The third postulate is of greatest relevance to the present work. A large amount of evidence was obtained which indicated that there are transporter molecules which could allow the movement of protons out of the acidic environment, in exchange for cations which were translocated in the opposite direction across the impermeable membrane; these were designated "antiporters". Other molecules could transport anions in the same direction as the protons, and were designated "symporters".

The chemiosmotic hypothesis provided an explanation of how chemical reactions which are non-directional could be used to generate directional movement within the cell. It also provided a unitary explanation for the functions of mitochondria, which were no longer regarded as "oxidative phosphorylation", but were better understood as separate oxidative enzymes and phosphorylating enzymes which are coupled to each other via the electrochemical gradient of protons, even though they are quite far apart on a molecular scale. The chemiosmotic hypothesis is applicable to the functions of chloroplasts, the bacteriorhodopsin system of Holobacterium, neurotransmitter storage vesicles, lysosomes and phagosomes, and the principles are relevant to proton translocation across the plasma membranes of specialised cells, such as osteoclasts, gastric parietal cells and renal tubular cells.

7.5.7 Classification of ion motive ATPase molecules

Adenosine triphosphate (ATP) is the source of energy in all cells. It is generated by phosphorylation of ADP by the ATP synthase enzyme in mitochondria. The generation of ATP is a reversible reaction and the enzyme can be made to act (under artificial conditions) as as an ATPase, hydrolysing ATP to ADP and inorganic phosphate. The ATP which is generated by ATP synthase is hydrolysed by a variety of ATPase enzymes which utilise the energy for various cellular functions. A group of these ATPase molecules are described as the "ion motive ATPases", as they translocate ions across cellular membranes. These ion motive ATPases have been classified into three groups (Pederson & Carafoli, 1987):

1. P-ATPases are defined as those which form a covalent phosphorylated (hence the symbol

"P") intermediate as part of their reaction cycle. They include the Na^+/K^+ ATPase (sodium pump) in the plasma membrane, the H^+ ATPase in the plasma membrane, the Ca^{++} ATPase in the endoplasmic and sarcoplasmic reticulum and the K^+/H^+ ATPase in gastric parietal cells. P-ATPases consist of a single peptide which contains the ATP-binding, catalytic and phosphorylation sites. In view of the similarity of the sequences of these ATPases, it seems likely that they may have arisen by duplication of an ancestral gene. All P-ATPases are inhibited by vanadate which acts as an analogue of phosphate. In addition, there are relatively selective inhibitors such as cardiac glycosides (digoxin and ouabain) for the Na^+/K^+ ATPase, omeprazole for the K^+/H^+ ATPase in gastric parietal cells and diethylstilboestrol for the H^+ ATPase in the plasma membrane.

2. V-ATPases are found in vacuoles (hence the symbol "V") of yeasts and plants, and in lysosomes, endosomes, clathrin-coated vesicles, neurotransmitter and hormone storage granules, secretory granules and Golgi vesicles. This class of ATPase translocates protons and the vacuoles in which they are located are therefore acidic. The V-ATPases consist of multiple protein subunits which are described below. Their overall structure is similar to the F-ATPases. This class of ATPases is inhibited selectively by bafilomycinA1 and are unaffected by vanadate or by oligomycin.

3. F-ATPases are the ATP synthase molecules in mitochondria, chloroplasts and bacteria. They consist of a soluble part (designated F_1, hence the symbol "F") which includes the ATP binding and catalytic regions, and a hydrophobic part (designated F_0) which is embedded in the plasma membrane. These ATPases consist of multiple protein subunits which are described below. F-ATPases in mammalian cells are inhibited by oligomycin.

7.5.8 The acidity of the storage vesicles

Mitchell defined "proticity" as the flow of protons, in analogy with electricity which is the flow of electrons. Protons and electrons are the smallest particles involved in chemical reactions; the radius of the proton is 10^{-15} m, whereas the radii of most other ions are approximately 10^{-10} m. The small size of the proton and its large electric field make it exceedingly mobile. "Thus, just as electron-conducting metals are used for the efficient transmission of electric power, so proton-conducting aqueous media are especially suitable

for the efficient transmission of protic power" (Mitchell, 1976). Proticity provides the power for accumulation of neurotransmitters in neurosecretory vesicles and in adrenal medullary chromaffin granules.

The internal pH of chromaffin granules was first measured by quenching of fluorescent amines and was estimated to be 5.5 (Bashford et al, 1975a). In the same seminal study which was carried out at Oxford, it was also noted that chromaffin granule membranes possessed ATPase activity whose functional properties were different from those of the mitochondrial ATPase; this ATPase moved protons to the interior of the chromaffin granules. "Such a movement of protons would lead to the development of both electrical and chemical potential within or across the chromaffin granule membrane; the active uptake of catecholamines may take place by a proton-catecholamine exchange mechanism" (Bashford et al, 1975a). Indeed, inhibition of the ATPase activity was accompanied by inhibition of catecholamine uptake into the granules, confirming the validity of the hypothesis (Bashford et al, 1975b). This novel ATPase was subsequently designated "vacuolar-type ATPase" (V-ATPase; see above).

The chromaffin granule V-ATPase was purified and it was found to be very similar in its properties to the proton-translocating ATPase from mitochondria (Sutton & Apps, 1981). Nonetheless, there were differences which were consistent with Bashford's suggestion that the chromaffin granules possessed a new form of ATPase which had not been identified hitherto (Sutton & Apps, 1981). The V-ATPase is an extremely complex cellular machine which consists of thirteen different proteins. Complementary cDNAs encoding these proteins were obtained both from mammals and from yeast, in which mutations of the proteins result in phenotypes which can only survive in acidic pH, due to disruption of the V-ATPase activity.

The current model of the three-dimensional structure of V-ATPase is based on the knowledge which was gained from the structure of mitochondrial F-ATPase (ATP synthase) which was elucidated by John Walker and his colleagues in Cambridge (Abrahams et al, 1994). Using X-ray diffraction at 2.8 A resolution, they found that the catalytic site of the F-ATPase was a globular structure which consisted of three α and three β subunits which were "arranged alternately like the segments of an orange" around a central shaft. Accordingly, the structure of V-ATPase is also believed to consist of multiple subunits which are organised

into four components: a catalytic unit, a "shaft", a "turbine" and a "hook" (Figure 7.5.3; Nelson & Harvey, 1999). The catalytic unit is believed to consist of alternating A and B subunits which bind and hydrolyse ATP; the A subunit contains a glycine-rich consensus sequence which is a site for binding and hydrolysis of ATP, known as a "Walker motif" (Walker et al, 1982). The B subunit also binds ATP but has no catalytic activity and does not contain a Walker motif. The "turbine" consists of 10-12 copies of a highly lipophilic protein which are arranged in the shape of a barrel which is located in the plasma membrane; this component of the F-ATPase was also crystallised by Walker and his colleagues (Stock et al, 1999). In the F-ATPase, a proton leaving the mitochondrion binds to the carboxyl group in an aspartate residue which is buried in the membrane bilayer, within a subunit of the turbine. As the aspartate becomes protonated, its carboxy terminal helix will rotate. When the aspartate is deprotonated, the carboxy terminal rotates back to its original position; these alternating movements cause the turbine to rotate (Rastogi & Girvin, 1999). The shaft rotates with the turbine (Sambongi et al, 1999). Rotation of the shaft causes a conformational change in the catalytic unit which remains fixed, enabling the phosphorylation of ADP to ATP. The V-ATPase is believed to function in the reverse direction; hydrolysis of ATP causes conformational changes which rotate the "shaft" which in turn rotates the "turbine" which pumps protons across the membrane. The "hook" consists of two dimeric proteins which are anchored in the plasma membrane and prevent the rotation of the catalytic unit. Thus, the ATP-binding site faces the cytoplasm and the V-ATPase moves protons away from the catalytic unit towards the vesicular lumen which becomes acidic (Nelson & Harvey, 1999). Visual evidence for the rotation of F-ATPase was provided in a remarkable experiment in which a fluorescent actin filament was attached to the "shaft" as a marker; in the presence of ATP, the filament rotated in an anticlockwise direction when viewed from the membrane side (Noji et al, 1997). Visual evidence for rotation of the turbine was provided in a complementary experiment in which one of the subunits of the turbine was tagged with a fluorescent actin filament; when ATP was added, the turbine rotated in the same anticlockwise direction and with the same rotational force as had been observed in the case of the shaft (Sambongi et al, 1999). Thus, the rotary torque in the turbine is transmitted by the shaft to the catalytic unit where ATP synthesis or hydrolysis take place (Figure 7.5.3). It is believed that binding of ATP is an energetic step which causes large conformational changes in the molecule; thus, the concentration of ATP determines the rotation rate which in turn determines the rate of catalysis (Yasuda et al, 2001).

The vesicular membranes of neurosecretory vesicles and adrenal chromaffin granules possess transporter molecules which exchange protons from the acidified interior with neurotransmitters from the cytoplasm, thus functioning as antiporters according to the chemiosmotic hypothesis (Mitchell, 1976). These transporter molecules will be described below (Section 7.5.14).

7.5.9 Model of pre-synaptic re-uptake processes

The properties of the uptake system in isolated storage particles were different from, and were not adequate to explain the uptake of catecholamines in intact tissues. In particular, catecholamines are polar molecules which could not be expected to diffuse rapidly through the plasma membrane to the storage particles. In order to reconcile these differences, Iversen suggested that "the initial uptake of catecholamines is mediated by a membrane carrier system in the axonal membrane; once inside the sympathetic nerve, the accumulated amines rapidly enter the intraneuronal storage particles. This second stage in the uptake of exogenous catecholamines may serve to protect the accumulated catecholamines from degradation by intraneuronal enzymes and at the same time provide an amplification stage in the uptake allowing very high tissue/medium ratios to be attained. By removing accumulated catecholamines from the axoplasm the particle uptake may serve to reduce the apparent concentration gradient between the extracellular catecholamines and the concentration of accumulated catecholamine in the axoplasm at the inner surface of the axonal membrane" (Iversen, 1967). This model remains valid; molecular cloning of distinct transporter molecules which are located in the plasma membranes and in the neurosecretory vesicles has been consistent with this hypothesis of the pre-synaptic re-uptake of neurotransmitter amines.

7.5.10 Discovery of the uptake of amines in non-neuronal cells

Accumulation of amines in non-neuronal cells was discovered and studied in greatest detail by Iversen, who suggested that the pre-synaptic re-uptake process in noradrenergic nerve terminals and uptake in non-neuronal cells are designated $Uptake_1$ and $Uptake_2$, respectively (Iversen, 1965). At the same time as Iversen's report on $Uptake_2$ in the rat heart muscle, Fischer et al (1965) reported that denervated salivary glands accumulated [^{3}H]noradrenaline by a reserpine-resistant mechanism and they attributed this to accumulation of the amine in an extraneuronal store.

In the original studies in the rat isolated heart, uptake of adrenaline saturated at approximately $2.7x10^{-6}$ M (Iversen, 1963). However, at adrenaline concentration of $27x10^{-6}$ M, an unexpectedly large uptake of adrenaline was observed. The affinity constants for the uptake of adrenaline and noradrenaline at high perfusion concentrations were $51x10^{-6}$ M and $252x10^{-6}$ M, respectively, and were far greater than the corresponding values for uptake of these amines at low perfusion concentrations; thus, $Uptake_2$ had much lower affinity and greater capacity for these catecholamines than $Uptake_1$ (Iversen, 1965). Further evidence for the presence of this new uptake process was obtained in immunosympathectomised rats, in which uptake of noradrenaline at low perfusion concentrations was abolished, but uptake at high perfusion concentrations was relatively spared; this suggested that accumulation of amines via $Uptake_2$ may be at "an extraneuronal site of uptake" (Iversen, 1965). Subsequent fluorescence microscopy studies demonstrated that after perfusion of the rat heart with high concentrations of noradrenaline, accumulation of the amine via $Uptake_2$ was in the cytoplasm of cardiac muscle cells (Ehinger & Sporrong, 1968; Farnebo & Malmfors, 1969).

$Uptake_2$ could be distinguished from $Uptake_1$ as follows (Iversen, 1965):

1. $Uptake_1$ had much greater affinity and smaller capacity for the catecholamines;
2. $Uptake_1$ favoured the accumulation of noradrenaline whereas $Uptake_2$ favoured adrenaline;
3. $Uptake_2$ did not discriminate between the isomers of adrenaline and noradrenaline;
4. Catecholamines accumulated via $Uptake_2$ were released more easily than via $Uptake_1$;
5. $Uptake_2$ was relatively resistant to cocaine, desipramine and metaraminol.

$Uptake_2$ was first detected in non-neuronal cells in the brain by Snyder and his colleagues in Baltimore. Utilising Iversen's discovery of the accumulation of normetanephrine by $Uptake_2$ but not by $Uptake_1$ (see below), they incubated slices of rat brain with [^{3}H]normetanephrine as a ligand for $Uptake_2$. They found that [^{3}H]normetanephrine accumulated in the brain slices by a mechanism which was independent of sodium ions and resistant to ouabain, desipramine and reserpine, which distinguished it from the accumulation of noradrenaline via $Uptake_1$ and via the vesicular transporter. The affinity of normetanephrine for the uptake process was much less than the affinity of noradrenaline for $Uptake_1$ (Hendley et al, 1970).

Further, using a limited series of compounds, the structural requirements for inhibition of the uptake of [^{3}H]normetanephrine appeared to resemble the requirements for accumulation via $Uptake_2$. The authors concluded that accumulation of normetanephrine in the brain slices was via an extraneuronal uptake process which was likely to be $Uptake_2$ (Hendley et al, 1970). Subsequent investigators reported uptake of adrenaline, noradrenaline or dopamine in purified preparations of glial cells (Henn & Hamberger, 1971) or in primary cultures of glial cells (Kimelberg & Pelton, 1983; Paterson & Hertz, 1989). The neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) is a ligand for $Uptake_2$ and this compound was also reported to accumulate in primary cultures of human glial cells with characteristics which were those of $Uptake_2$ (Russ et al, 1996). Isoprenaline may also accumulate in rat brain slices by a mechanism which resembles $Uptake_2$ (Wilson et al, 1988).

In general, glial cells accumulate far greater quantities of amino acid neurotransmitters (such as GABA, glutamate and aspartate) than amine neurotransmitters (such as catecholamines and serotonin; Henn & Hamberger, 1971; Hosli & Hosli, 1978; Kimelberg & Katz, 1986). Studies on the cellular localization of the uptake of neurotransmitters using autoradiography or fluorescence microscopy demonstrated that monoamines were accumulated mainly by neurones, and the accumulation in glia was minimal; in contrast, amino acid transmitters were accumulated both by neurones and by almost all glial cells (Hosli & Hosli, 1978).

7.5.11 Functional properties of non-neuronal amine uptake

In the initial work, there was no evidence of accumulation of catecholamines via $Uptake_2$ when the catecholamines were perfused at low concentrations; it was therefore suggested that there may be a "trigger mechanism" which brought the process into operation (Iversen, 1965). This was explained in a subsequent study, in which it became clear that $Uptake_2$ was not a threshold phenomenon, but operates in a linear manner at all perfusion concentrations of catecholamines (Lightman & Iversen, 1969). Accumulation of catecholamines via $Uptake_2$ could not be detected at low perfusion concentrations because the catecholamines are rapidly metabolised in non-neuronal cells, where there are no neurosecretory vesicles in which they can be stored. The catecholamines are rapidly degraded in non-neuronal cells by COMT followed by MAO, resulting in highly lipophilic compounds such as MHPG which escape rapidly across cellular membranes (Figure 7.5.2). Thus, when metabolism was inhibited,

$Uptake_2$ was seen to accumulate catecholamines at all concentrations, in a linear, saturable manner (Lightman & Iversen, 1969).

$Uptake_2$ saturated with increasing amine concentrations and could be described by Michaelis-Menten kinetics (Iversen, 1965; Grundemann et al, 1998). The source of energy for this uptake process is unclear. $Uptake_2$ is independent of the electrochemical gradient of sodium ions and is insensitive to drugs which block the Na^+/K^+ ATPase, such as ouabain (Hendley et al, 1970; Paterson & Hertz, 1989). $Uptake_2$ is also independent of the electrochemical gradient of protons, as increasing extracellular pH does not inhibit the accumulation of amines via $Uptake_2$ (Schomig et al, 1992). In the absence of a clear source of energy for $Uptake_2$, it has been suggested that movement of amines via $Uptake_2$ may be by a facilitative diffusion process which does not require cellular energy (Hendley et al, 1970; Paterson & Hertz, 1989). Depolarization of non-neuronal cells inhibits the accumulation of amines via $Uptake_2$, so it was suggested that the amines enter the cells in a protonated form, driven by the negative intracellular potential (Schomig et al, 1992).

The structural properties of phenylethylamines which are accumulated by $Uptake_2$ in the rat isolated heart were studied by Burgen & Iversen (1965). They determined the affinities of phenylethylamines for the carrier, as measured by the ability of these compounds to compete with the uptake of noradrenaline. Their conclusions were confirmed by subsequent investigators (Grohmann & Trendelenburg, 1984) and can be summarised as follows:

1. Presence of an N-methyl group increased affinity, such that adrenaline was more potent than noradrenaline.
2. Affinity for the uptake process was increased by the presence of a hydroxyl group on the β carbon, such that dopamine was less potent than noradrenaline.
3. Phenolic hydroxyl groups reduced affinity, such that phenylethylamine was more potent than dopamine.
4. Presence of a methyl group on the α carbon reduced affinity, such that amphetamine was less potent than phenylethylamine.
5. $Uptake_2$ did not recognise the steric arrangement of the hydroxyl group on the β carbon, such that the (-) and (+) enantiomers of adrenaline and noradenaline were

equipotent (Iversen, 1965).

6. The most striking difference between the specificities of $Uptake_1$ and $Uptake_2$ was in the effect of O-methylation. Phenolic methoxyl groups caused a striking increase in affinity, such that normetanephrine was far more potent than noradrenaline.

These properties were almost all opposite to those which had been observed in ligands for $Uptake_1$. Further, $Uptake_2$ was unaffected by cocaine or by the antidepressant desipramine which are potent inhibitors of $Uptake_1$ (Iversen, 1965; Salt, 1972). $Uptake_2$ was inhibited by high concentrations of the steroid hormones corticosterone, testosterone, 17-β-oestradiol and deoxycorticosterone; the IC_{50} values for these compounds were 2.0 to 4.5×10^{-6} M which is substantially greater than the physiological concentrations of these hormones in plasma (Iversen & Salt, 1970; Salt, 1972). In contrast, of all the steroids tested, only 17-β-oestradiol inhibited $Uptake_1$; its IC_{50} was 36.6×10^{-6} M, indicating that it was a very weak inhibitor of $Uptake_1$ (Salt, 1972). $Uptake_2$ was relatively unaffected by progesterone, hydrocortisone and dexamethasone (Salt, 1972). $Uptake_2$ was also inhibited by clonidine (IC_{50} 16.8×10^{-6} M; Salt, 1972) and by some haloalkylamines which are analogues of phenoxybenzamine and dibenamine (Iversen & Langer, 1969; Iversen et al, 1972). Serotonin and histamine were reported to be substrates for $Uptake_2$, confirming the broad spectrum of compounds which can be accumulated by this uptake process (Grohmann & Trendelenburg, 1984). These findings will be discussed in greater detail in relation to the structural properties of ligands for Transport-P.

7.5.12 Physiological significance of amine uptake in pre-synaptic neurones and in non-neuronal cells

The primary function of the pre-synaptic re-uptake process is to terminate the action of released amine neurotransmitters. In this regard, pre-synaptic re-uptake is far more important than enzymatic degradation as a mechanism for terminating the action of noradrenaline. Thus, the overflow of noradrenaline can be increased by inhibition of the re-uptake process with cocaine, but not by combined inhibition of both MAO and COMT (Brown, 1965). Further, most of the noradrenaline which accumulates in the tissues following peripheral administration remains as unmetabolised noradrenaline (Whitby et al, 1961). These findings

indicated that following release from the nerve terminals, the neurotransmitter amines are inactivated by pre-synaptic re-uptake via the plasma membrane carrier; they are then accumulated via the vesicular carrier in the neurosecretory vesicles where they are protected from enzymatic degradation by monoamine oxidase which is located in the mitochondria. Molecules of noradrenaline which leak from the neurosecretory vesicles to the cytoplasm are metabolised predominantly by MAO; thus, reserpine which blocks vesicular storage causes an increase in the proportion of deaminated metabolites (Kopin & Gordon, 1962). In contrast, circulating noradrenaline is metabolised by COMT in peripheral tissues (such as the liver); thus, tyramine, whose predominant effect is to block the pre-synaptic plasma membrane noradrenaline transporter ($Uptake_1$) causes an increase in the proportion of O-methylated metabolites (Kopin & Gordon, 1962). A significant proportion of deaminated metabolites which are released from noradrenergic neurones are then methylated by COMT in peripheral tissues, resulting in deaminated, O-methylated products. In the brain, methylation takes place in non-neuronal cells such as glia, where COMT is located (Kopin, 1985).

The second function of pre-synaptic re-uptake is to enable replenishment of the store of neurotransmitter via the catecholamine synthesis pathway. This function is carried out by the vesicular transporter which accumulates dopamine; this catecholamine is then converted by dopamine-β-hydroxylase to noradrenaline within the vesicles. Thus, administration of reserpine which blocks the vesicular transporter is followed by depletion of the tissue content of noradrenaline (Kirshner, 1962b). In contrast, the plasma membrane transporters do not contribute significantly to replenishment of the store of noradrenaline, as most of the noradrenaline in tissues is derived from synthesis, and uptake of the catecholamine precursor tyrosine takes place by an independent mechanism (Kopin et al, 1965). Thus, potent inhibitors of the plasma membrane noradrenaline transporter (such as desipramine) do not deplete tissue noradrenaline.

The third function of the pre-synaptic re-uptake processes is to prevent degradation of the neurotransmitters by the intracellular metabolising enzymes. This function is carried out by the vesicular transporters which sequester the neurotramsitters in the neurosecretory vesicles, as described above. Thus, administration of reserpine is followed by a large increase in the tissue content of deaminated and O-methylated metabolites. Sequestration of accumulated

toxins (such as MPP^+) within vesicles may also reduce their toxic effects on intracellular enzymes (Reinhard et al, 1987; Daniels & Reinhard, 1988).

A fourth function of the vesicular transporters is to maintain the favourable concentration gradient of neurotransmitter across the plasma membrane, by preventing the accumulation of neurotransmitters on the cytoplasmic surface of the plasma membrane transporters (Iversen, 1967).

The function of $Uptake_2$ in non-neuronal cells is to remove circulating catecholamines, as well as neurotransmitter molecules which diffuse away from pre-synaptic nerve terminals, where $Uptake_1$ is located; the catecholamines are then metabolised initially via COMT which is abundant in non-neuronal cells. $Uptake_2$ may also be important in removal of certain drugs such as isoprenaline which are poor substrates for $Uptake_1$ (Lightman & Iversen, 1969).

7.5.13 Isolation of cDNAs which encode plasma membrane neurotransmitter transporters

Pre-synaptic nerve terminals accumulate GABA by a process which is dependent on sodium and chloride (Iversen & Neal, 1968). A cDNA encoding the plasma membrane GABA transporter was isolated By Kanner and his colleagues in 1990. In prior work (Radian et al, 1986), the GABA transporter was purified from rat brain membranes. The purification procedure was based on the assumption that the GABA transporter was a glycoprotein; this assumption was subsequently corroborated by Zaleska & Ericinska (1987). Rat brain membranes were extracted with cholate as detergent and proteins were precipitated with ammonium sulphate. Cholate was then removed on a Sephadex G-50 column and replaced with the non-ionic detergents Triton X100 and β-octyl-glucoside (Radian & Kanner, 1985). The proteins were then fractionated by diethylaminoethyl (DEAE) chromatography in the presence of the detergents. The column fractions were further purified by affinity chromatography, using Wheat Germ Agglutinin-Sepharose, which is a lectin which binds to sugar residues. At each chromatographic step, the column fractions were assayed for the presence of GABA transport activity by reconstitution of the peptide in liposomes (Radian & Kanner, 1985). The chromatographic steps resulted in 100 to 400 fold enrichment of GABA transport activity. This was accompanied by 60% loss of activity of the purified protein, possibly due to presence of the detergents. SDS-polyacrylamide gel electrophoresis revealed

the presence of a band of 80 kDa, presumably representing the GABA transporter. There were additional bands of 160 and 240 kDa which presumably represented dimer and trimer forms of the 80 kDa band. Antibodies against the 80 kDa polypeptide were raised by injecting the peptide into rabbits. This antiserum precipitated GABA transport activity from a crude preparation of rat brain membranes. This provided evidence that the 80 kDa protein represented the GABA transporter. Liposomes in which the 80 kDa peptide had been reconstituted exhibited uptake of GABA which was dependent on both sodium and chloride, with a Km of 3 μM; these features were similar to the properties of the transporter which are native to the rat brain (Radian et al, 1986).

The 80 kDa protein was subjected to cyanogen bromide degradation and several of the resulting fragments were sequenced (Guastella et al, 1990). The sequence of the longest peptide was used to design oligonucleotide probes which were used to screen a rat brain cDNA library by plaque hybridisation. Two positive plaques were found. Synthetic RNA was transcribed *in vitro* from these clones and was tested for its ability to reproduce the uptake of GABA by injection into Xenopus oocytes. One of the clones exhibited uptake of GABA and was therefore designated GAT-1 (GABA Transporter-1; Guastella et al, 1990). In oocytes injected with GAT-1 RNA, uptake of GABA was saturable and followed Michaelis-Menten kinetics with a Km of 7 μM. Uptake was dependent on extracellular sodium and chloride and could be blocked with compounds which are known to inhibit uptake of GABA in rat brain. When GAT-1 RNA was translated *in vitro*, a 67 kDa protein was obtained and was shown to react with the antiserum which had been raised against the 80 kDa protein from rat brain membranes. The protein encoded by the GAT-1 clone therefore shared antigenic determinants with the GABA transporter peptide which is native in rat brain. The difference in molecular weights was presumably due to the fact that the native protein is glycosylated (Radian et al, 1986). A fragment of the GAT-1 cDNA was labelled with ^{32}P and used as a probe to hybridise to RNA extracted from different regions of rat brain. A single hybridisation band of 4.2 kb was visualised in RNA extracted from cerebrum, cerebellum and the brain stem.

The GAT-1 clone contained an insert of 4054 nucleotides, consisting of 149 nucleotides at the 5' untranslated end, an open reading frame of 1797 bases, and a 3' untranslated end of 2108 nucleotides (Guastella et a, 1990). The open reading frame predicted a protein of 599

amino acids with a molecular weight of 67 kDa, which agrees with the molecular weight of the translation product. Both the amino- and carboxy-termini were predicted to be intracellular. A hydropathy plot of the deduced amino acid sequence using the Kyte and Doolittle analysis suggested that there may be 12 hydrophobic regions which may represent α helical membrane-spanning domains, each 21 residues in length. The second extracellular loop is large and includes three potential asparagine-linked glycosylation sites. There are three intracellular serine and threonine residues which may represent regulatory sites for potential phosphorylation by protein kinase A and protein kinase C.

The overall structure of the GAT-1 protein was similar to other membrane transport proteins, but the amino acid sequence was different from any other known protein. This suggested that GAT-1 may represent the first member of a new family of transport proteins.

In 1991, a cDNA encoding the second member of this family was isolated. The pre-synaptic plasma membrane noradrenaline transporter ($Uptake_1$) cDNA was isolated by an expression cloning strategy (Pacholczyk et al, 1991). A cDNA library was constructed from a human noradrenergic neuronal cell line (SK-N-SH cells; Section 10.3.2) and pools of clones were transfected into COS-1 cells. Cells expressing the transporter were identified by uptake of the noradrenaline analogue *meta* -iodobenzylguanidine ([^{125}I]MIBG); the high specific activity of the ligand enabled detection of uptake by autoradiography following exposure to X-ray film for 48-72 hours. Single positive cells were then picked by scraping an area of approximately 7 mm^2 around the area corresponding to the autoradiographic spot, and the DNA recovered and electroporated into bacteria. Positive pools were subdivided into sub-pools containing diminishing numbers of clones, until a single clone was identified.

The cDNA insert consisted of 1,983 bp, including a 1,851 bp open reading frame (Pacholczyk et al, 1991). The sequence encodes a 617 amino acid protein. Hydropathy analysis indicates that the molecule includes 12 membrane-spanning domains, each consisting of 18 to 25 amino acids. As in the case of the GABA transporter, there is a large second extracellular loop containg three potential glycosylation sites. The amino acid sequence of the noradrenaline transporter was 46% identical to the human GABA transporter. Hybridisation with the noradrenaline transporter probe revealed that there are two sizes of RNA in SK-N-SH cells and in the brain stem, representing 3.6 kb and 5.8 kb;

the larger RNA probably represents the cloned cDNA and the nature of the smaller RNA was unknown. The noradrenaline transporter cDNA clone was transfected into HeLa cells and uptake of [^{3}H]noradrenaline was monitored. Uptake was sodium dependent and saturable, with a Km of 457 nM. Uptake was blocked by tricyclic antidepressants, amphetamine and cocaine, with affinities which were similar to the expected affinities of these drugs for the native noradrenaline transporter (Pacholczyk et al, 1991).

Other members of this group of transport proteins were identified by screening cDNA libraries with degenerate oligonucleotides whose sequences were based on conserved regions of the two cloned transporters. In this way, cDNAs encoding the plasma membrane transporters for dopamine and serotonin were obtained (Shimada et al, 1991; Kilty et al, 1991; Hoffman et al, 1991; Blakely et al, 1991). These four transporters have the same overall structure and approximately 30% of the amino acid sequences are identical, most particularly in the membrane-spanning regions. Homologous cDNAs were also isolated encoding a sodium- and chloride-dependent transporter for the amino acid neurotransmitter glycine (Liu et al, 1992a; Smith et al, 1992; Guastella et al, 1992).

Acetylcholine is hydrolysed in the synaptic cleft to its constituents, choline and acetic acid, as described above. Choline is then transported across the pre-synaptic plasma membrane and used in the synthesis of new molecules of acetylcholine. Degenerate primers were designed, based on the sequence of the plasma membrane GABA transporter (GAT-1), and used in PCRs which employed as template cDNA synthesised from rat spinal cord (Mayser et al, 1992). A PCR product of 440 bp was obtained, the sequence of which was homologous to GAT-1. The PCR product was labelled with ^{32}P and used as a probe to screen a rat spinal cord cDNA library by plaque hybridisation. Clones were obtained encoding a protein of 635 amino acids which were approximately 40% identical with other plasma membrane neurotransmitter transporters, particularly in the membrane-spanning regions. The overall structure was identical to other plasma membrane neurotransmitter transporters, in which both the amino- and carboxy-termini are located in the cytoplasm, presence of 12 hydrophobic regions which are presumably membrane-spanning domains, a large second extracellular loop containing extracellular sites for asparagine-linked glycosylation, and intracellular phosphorylation sites. RNA could not be detected in tissues by northern analysis, presumably because RNA concentrations were low, but reverse transcription PCR demonstrated the presence of RNA in brain, spinal cord and the heart. Synthetic RNA was

transcribed from the clone and injected into Xenopus oocytes which accumulated choline by a saturable, sodium-dependent mechanism, with a Km of $9x10^{-6}$ M. Surprisingly, uptake of choline was not blocked by the choline transport blocker hemicholinium, suggesting that there may be additional choline transporters (Mayser et al, 1992). Subsequently, the cDNA was expressed in mammalian cells and was found to be much more effective in inducing the uptake of creatine than choline; the cDNA was therefore re-designated a creatine transporter (Schloss et al, 1994). To isolate other choline transporters, Okuda et al (2000) designed PCR primers which were based on the sequences of cDNAs which are predicted to be sodium-dependent transporters in *C elegans*. They systematically expressed putative transporters one by one in Xenopus oocytes, and they identified a clone which encoded a protein which functioned as a sodium- and chloride-dependent, hemicholinium-sensitive choline transporter. They then isolated a rat homologue which encoded a protein consisting of 580 amino acids and was homologous to the sodium-dependent glucose transporters. The protein encoded by the rat clone also functioned as a sodium- and chloride-dependent, hemicholinium-sensitive choline transporter when injected in oocytes, with a Km of $2x10^{-6}$ M. RNA corresponding to this clone was detected in the major cholinergic cell groups in rat striatum, spinal cord and the basal forebrain (Okuda et al, 2000).

Glutamate transporters represent a different class of proteins. Uptake of glutamate is accompanied by the co-transport of sodium and the counter-transport of potassium (Amara, 1992). Three homologous clones of approximately 50% identity were isolated in 1992, two of which are probably glial cell glutamate transporters (Storck et al, 1992; Pines et al, 1992) and the third probably a pre-synaptic re-uptake transporter which is located in the plasma membranes of glutamatergic neurones (Kanai & Hediger, 1992). The mammalian glutamate transporters are homologous to bacterial glutamate transporters. These transporters accumulate aspartate with approximately the same affinity as glutamate, but they do not accumulate adrenaline, noradrenaline, dopamine or serotonin. A fourth homologous clone was isolated with distinct functional properties; it acted both as a glutamate and aspartate transporter and as a ligand-gated chloride channel (Fairman et al, 1995). In addition to terminating the actions of glutamate and aspartate as neurotransmitters, these transporters may help to prevent the extracellular concentrations of glutamate from rising to neurotoxic levels.

Disruption of the dopamine transporter gene in mice was associated with an increase in

locomotor activity and this was consistent with the known effects of dopamine in the regulation of motor function (Giros et al, 1996). Further, cocaine and amphetamine increased locomotor activity in normal mice but not in mice which lack the dopamine transporter; this was consistent with previous work indicating that cocaine and amphetamine exert their major effects on the pre-synaptic plasma membrane re-uptake transporters (Section 7.5.3). Studies on the kinetics of dopamine release in slices of caudate demonstrated that absence of the dopamine transporter causes dopamine to remain in the extracellular space far longer than in normal circumstances; this presumably accounts for the increased locomotor activity. These findings in transgenic animals confirmed that pre-synaptic re-uptake is the main mechanism of inactivation of dopamine, as had already been demonstrated using pharmacological blockers of pre-synaptic re-uptake (Section 7.5.12). Amphetamine increased dopamine release in the wild-type animals but not when the dopamine transporter was absent. Cocaine had no effect on dopamine release in any of these animals. These findings explain the lack of effects of these drugs on locomotor activity in the transgenic animals, and they are consistent with the view that amphetamine blocks re-uptake and increases amine release, whereas cocaine only blocks uptake without increasing release (Section 7.5.3). The knockout mice had lower concentrations of RNA for dopaminergic receptors, which is presumably a manifestation of down-regulation of these receptors in the presence of high concentrations of the neurotransmitter (Giros et al, 1996).

Disruption of the pre-synaptic $Uptake_1$ plasma membrane noradrenaline transporter gene was associated with reduction of brain concentrations of noradrenaline and increased activity of tyrosine hydroxylase, presumably as a result of removal of the inhibitory effect of noradrenaline on the activity of this enzyme (Section 7.3.7). Noradrenaline release was reduced and noradrenaline clearance from the extracellular space was slowed (Xu et al, 2000). Extracellular noradrenaline concentrations were increased and this was accompanied by a decrease in the density of α_1 adrenergic receptors, presumably as a result of down-regulation. There was an increase in the sensitivity of striatal dopaminergic receptors, the mechanism of which was unclear (Xu et al, 2000). In general, these findings were consistent with the known effects of the pre-synaptic $Uptake_1$ plasma membrane noradrenaline transporter, based on the effects of blocking the transporter by pharmacological tools (see Sections 7.5.2 and 7.5.3).

7.5.14 Isolation of cDNAs which encode vesicular neurotransmitter transporters

The model which was proposed by Bashford et al (1975a & b) remains valid and applies to all neurotransmitter storage vesicles, both in the adrenal medulla and in nerve endings: the interior of the neurosecretory vesicles is acidified by V-ATPase which generates an electrochemical gradient of protons across the vesicular membrane; the neurotransmitters are then accumulated by exchange with protons (antiport) via specific transporter proteins which are located in the vesicular membranes (Johnson, 1988; Schuldiner et al, 1995). Pre-synaptic vesicular uptake processes have been observed for all the neurotransmitters, including acetylcholine, the amines (adrenaline, noradrenaline, dopamine, serotonin and histamine) and amino acids (GABA, glutamate, aspartate and glycine). The first of these vesicular neurotransmitter transporters to be cloned was the chromaffin granule transporter from the adrenal medulla.

The neurotoxin precursor MPTP accumulates in glial cells by a non-specific process of diffusion. It is then oxidised by MAO B in the mitochondria to the quaternary amine MPP^+. Dopaminergic neurones in the substantia nigra then accumulate the MPP^+ by uptake through the plasma membrane dopamine transporter, and the MPP^+ then exerts its neurotoxic effect on the respiratory chain in the mitochondria of the dopaminergic neurones (Marini et al, 1992). The chromaffin cells of the adrenal medulla also accumulate MPP^+ via the plasma membrane noradrenaline transporter, but they are resistant to the neurotoxic effects of this compound; these cells sequester the neurotoxin in the chromaffin storage granules, thus protecting the mitochondria (Reinhard et al, 1987; Daniels & Reinhard, 1988). Surprisingly, Chinese hamster ovary (CHO) cells, which do not possess a plasma membrane noradrenaline transporter, are more sensitive to the neurotoxic effects of MPP^+ than PC12 rat adrenomedullary phaeochromocytoma cells (Liu et al, 1992b). In order to understand the factors which confer resistance to MPP^+ toxicity in the adrenal medulla, a cDNA library was constructed from PC12 cells and the plasmid DNA was transfected into CHO cells by the calcium phosphate method. This resulted in approximately 500,000 stably transformed CHO cells. These cells were then grown in medium containing 1 mM MPP^+. Almost all the cells died and gradually detached from the culture plate over the next three weeks. However, at four weeks, a single colony of cells grew and rapidly covered the plate. These cells were

resistant to the toxic effects of MPP^+, and the resistance was reversed by exposing the cells to reserpine. As reserpine is known to block the vesicular transporter in chromaffin cells, the acquired resistance to MPP^+ in the transfected cells was attributed to transfer of a vesicular transporter from the PC12 cells to the CHO cells; the vesicular transporter would sequester the MPP^+ away from the site of its toxic effect in the mitochondria (Liu et al, 1992b). In view of the absence of a plasma membrane catecholamine transporter in CHO cells, transfected and wild type cells were grown in the presence of a high concentration of dopamine (1 mM) for 24 hours and the intracellular distribution of dopamine was then studied with glyoxylic acid-inducd fluorescence. In the wild type cells, fluorescence was diffuse, presumably representing cytoplasmic accumulation; in contrast, in the resistant cells, the fluorescence was in a perinuclear and punctate cytoplasmic location, presumably representing accumulation in the Golgi complex and in lysosomes; this pattern was abolished by reserpine (Liu et al, 1992b).

The generation of stably transformed mammalian cells implies that the transfected plasmid had been incorporated into the genome of the cells. In order to retrieve the plasmid which had conferred resistance, the genomic DNA from the stably transformed, resistant cells was digested with an enzyme, *Not* I, which cuts the plasmid in a single site. The digestion reaction was then re-ligated and introduced into competent bacteria by electroporation (Liu et al, 1992c). Plasmid pools were then introduced into wild type CHO cells and resistance was again observed. An individual clone was obtained by preparing pools containing diminishing numbers of plasmids (Liu et al, 1992c). The clone was expressed in CHO cells and pharmacological experiments performed on homogenates of transfected cells. The transfected cells accumulated [^{3}H]dopamine by a proton-dependent mechanism which was sensitive to reserpine and resistant to cocaine. Competition studies indicated that adrenaline, noradrenaline and serotonin were ligands for uptake (Liu et al, 1992c). These pharmacological properties were consistent with those of the chromaffin granule amine transporter (see above), so this clone was designated CGAT.

The clone contained an insert of 2.5 kb which encoded a protein of 521 amino acids (Liu et al, 1992c). Hydropathy analysis suggested that the protein contained 12 membrane-spanning domains. The amino- and carboxy-termini were predicted to be in the cytoplasm and there was a large loop projecting into the vesicular lumen, between the first and second membrane-

spanning domains. This loop contains three sites for asparagine-linked glycosylation. There are serine and threonine residues which may be consensus sites for phosphorylation by protein kinase A and protein kinase C on the cytoplasmic surface of the protein. The sequence of the protein was different from other known mammalian transport proteins, but there was some homology with bacterial transporters which are responsible for antibiotic resistance (Liu et al, 1992c).

The CGAT insert was radiolabelled with ^{32}P and used as a probe to screen a rat brain cDNA library by colony hybridisation. Several incomplete clones were obtained and assembled, resulting in a homologous sequence which was designated SVAT (for synaptic vesicle amine transporter). In situ hybridisation demonstrated the presence of RNA for this transporter in noradrenergic, dopaminergic and serotonergic neurones in the brain.

Disruption of the VMAT gene in transgenic mice causes depletion of the intracellular stores of catecholamines and serotonin in the brain, confirming that the neurosecretory vesicles are the primary site of amine storage (Fon et al, 1997). In these transgenic animals, amphetamine induces the release of substantial amounts of catecholamines, indicating that the vesicular store of catecholamines is not a primary site of action of amphetamine.

Acetylcholine is synthesised by choline acetyltransferase in the cytoplasm of cholinergic neurones, from choline which has been accumulated by the plasma membrane choline transporter and acetyl coenzyme A which is synthesised in the mitochondria. Acetylcholine is then stored in neurosecretory vesicles by a vesicular transporter which accumulates acetylcholine in exchange for protons from the acidified interior of the vesicles. The vesicular acetylcholine transporter can be blocked by vesamicol (Parsons et al, 1993; Nguyen & Parsons, 1995).

Identification of the vesicular transporter for acetylcholine was the result of work on the nematode worm *Caenorhabditis elegans* (*C elegans*). The genetic analysis of *C elegans* was initiated by Brenner in Cambridge. He chose to study this organism because it is a small animal (1 mm in length) which can be grown on agar, feeding on E coli. Most of the animals are hermaphrodites which have two X chromosomes; they reproduce prodigiously and rapidly, the entire cycle from adult to adult being 3.5 days. A few male worms are produced which have only one X chromosome; they can be maintained by mating with the

hermaphrodites. A large number of mutants was isolated after treatment with the chemical mutagen ethyl methanesulphonate, and a large number of genes was characterized from these mutants (Brenner, 1973). The genetic studies were carried out on the hermaphrodites which become homozygous. The males were used as tools to transmit genetic material, thus allowing the construction of hermaphrodites with appropriate mutations (Brenner, 1974). Most of the mutants are defective in movement, so the genes which are associated with the uncoordinated movements were designated *"unc"* genes (Brenner, 1974). In tandem with these genetic studies, Brenner studied the nervous system of *C elegans,* by reconstruction of serial section electron microscope photomicrographs. There are only 600 somatic cells in the hermaphrodite *C elegans,* of which 302 are neurones. The complete structure of the nervous system was obtained and the neurological appearances of the uncoordinated mutants were documented. It was therefore possible to determine the effects of gene mutations on the neuroanatomy and the motor function of these simple animals (White et al, 1986).

Unc-17 mutants have deficits in neuromuscular function and these mutant worms are resistant to acetylcholinesterase inhibitors, suggesting that the mutation may be in a gene which encodes a protein which is important for cholinergic function (Brenner, 1974). The *unc-17* gene was cloned and used as a probe to isolate clones from *C elegans* cDNA libraries; the sequence of the encoded protein was approximately 40% identical to the SVAT and CGAT proteins which accumulate amines in chromaffin granules and neurosecretory vesicles; the regions of greatest sequence identity were the membrane-spanning regions (Alfonso et al, 1993). Antibodies were raised against peptides derived from the predicted protein sequence and used to localise the expression of the *Unc-17* gene product in *C elegans.* The protein was present only in cholinergic neurones and was expressed in a punctate distribution near synaptic regions, confirming that it may function as a vesicular acetylcholine transporter (Alfonso et al, 1993). Using probes which were derived from the coding region of the *Unc-17* sequence, homologous cDNAs were obtained from the marine ray *Torpedo* brain electric lobe which has a rich cholinergic innervation, and from rat adrenal and human neuroblastoma cell cDNA libraries. These homologous clones were expressed in mammalian cells and were shown to accumulate acetylcholine and to bind the antagonist vesamicol; uptake of acetylcholine was blocked by vesamicol but not by reserpine or tetrabenazine (Varoqui et al, 1994; Erickson et al, 1994). Uptake is sensitive to temperature and is dependent on the presence of ATP (Varoqui & Erickson, 1996). In the rat, the RNA was expressed exclusively in cholinergic neurones in the brain and in the peripheral

autonomic nervous system (Erickson et al, 1994). The entire sequence of the human vesicular acetylcholine transporter gene is contained within the first intron of the choline acetyl transferase gene; transcription of both genes seems to be regulated by the same promoter, possibly by nerve growth factor as the transcription factor (Erickson et al, 1994).

Identification of the vesicular transporter for GABA was also a result of work on *C elegans.* McIntire et al (1993a) studied the GABAergic nervous system in *C elegans.* They found that of the 302 neurones, 26 immunostained with GABA. Individual GABAergic neurones were then killed by laser microsurgery; the movement abnormalities were documented and compared to the coordination disorders in the *C elegans* mutants which had been identified by Brenner (1974). Five mutations were identified in which the phenotype resembled the effects of ablation of GABAergic neurones (McIntire et al, 1993b). The mutant *Unc-47* accumulates large amounts of GABA in the GABAergic neurones but it responds normally to the GABA agonist muscamol; this suggested that the defect in these animals was pre-synaptic, possibly in the storage of GABA (McIntire et al, 1993b). Cosmids generated from the region of the *unc-47* gene were injected into the gonads of mutant worms and two clones reversed the uncoordinated abnormalities in the progeny of the injected worms (McIntire et al, 1997). An open reading frame was identified in this genomic region and the cDNA encoded a protein of 486 amino acids which were arranged in ten membrane-spanning domains, with both amino- and carboxy-termini in the cytoplasm. This sequence was tagged to green fluorescent protein and was shown to be expressed in all GABAergic neurones in *C elegans,* and only in GABAergic neurones. A homologous cDNA clone was obtained from a rat brain library. The amino acid sequence differed from the sequences of the amine and acetylcholine vesicular transporters and there were no obvious sites for asparagine-linked glycosylation. In the rat, the RNA was colocalised with the RNA for glutamic acid decarboxylase, which is the synthetic enzyme for GABA. Immunocytochemistry demonstrated that the peptide is distributed in a punctate manner, suggesting a vesicular location. Vesicles from mammalian cells expressing the peptide accumulated GABA in a saturable manner with a Km of 5 mM (McIntire et al, 1997).

Glutamate accumulates in synaptic vesicles; uptake is dependent on the presence of ATP and is driven by a proton gradient (Naito & Ueda, 1983). The vesicular glutamate transporter does not accumulate aspartate (Naito & Ueda, 1983), in contrast to the sodium-dependent plasma membrane glutamate transporters (Section 7.5.13). The vesicular glutamate

transporter does not accumulate the glutamate analogue glutamine, or other amino acid neurotransmitters such as GABA or glycine (Naito & Ueda, 1983). The vesicular uptake of glutamate is not entirely stereospecific, as D-glutamate competes with L-glutamate (Naito & Ueda, 1983). The vesicular uptake of glutamate appears to be mediated by a transporter which was originally identified as a sodium-dependent inorganic phosphate transporter (Ni et al, 1994). The cDNA clone encoding this transporter was isolated from primary cultures of rat cerebellum using a subtractive hybridization procedure. The sequence, which probably has six to eight membrane-spanning domains, was homologous to a renal sodium-dependent phosphate transporter, so the new clone was designated BNPI (for brain Na^+-phosphate inorganic). Indeed, when BNPI was expressed in *Xenopus* oocytes, it mediated sodium-dependent accumulation of inorganic phosphate. However, RNA encoding this protein was detected only in the brain (Ni et al, 1994). *In situ* hybridisation histochemistry and immunocytochemistry indicated that the RNA and protein for BNPI is restricted to neurones which are known to release glutamate (Ni et al, 1995; Bellocchio et al, 1998). Electron microscopy and subcellular fractionation suggested that the BNPI protein may be located in synaptic vesicles (Bellocchio et al, 1998). Synaptic vesicles from rat brain were immunopurified using an antiserum against BNPI; such synaptic vesicles accumulated glutamate (Takamori et al, 2000). Expression of the BNPI cDNA in mammalian cells resulted in uptake of glutamate which was dependent on protons and ATP and could be blocked by the V-ATPase inhibitor bafilomycinA1; these functional properties are typical of a vesicular neurotransmitter transporter (Takamori et al, 2000; Bellocchio et al, 2000). As in the native brain vesicles, accumulation of glutamate in transfected cells was facilitated by chloride (Bellocchio et al, 2000). Uptake conforms to the Michaelis-Menten model with a Km of 1 mM (Bellocchio et al, 2000). The other amino acid neurotransmitters (aspartate, glycine and GABA) were poor substrates for the transporter (Takamori et al, 2000; Bellocchio et al, 2000). Glutamate is released from mammalian cells which had been transfected with the BNPI clone, confirming that this cDNA is likely to encode a vesicular glutamate transporter (Takamori et al, 2000). In the brain, inorganic phosphate accelerates the synthesis of glutamate from glutamine, so it is possible that transport of phosphate by BNPI may serve a modulatory role in the rate of synthesis of this neurotransmitter (Bellocchio et al, 1998). Not all glutamatergic neurones express BNPI (Bellocchio et al, 1998), so there may be other proteins which function as vesicular glutamate transporters. A human homologue of BNPI has been identified (Aihara et al, 2000) and it is possible that this protein may be a member of a family of transport molecules.

7.5.15 Isolation of a cDNA which encodes Uptake$_2$ in non-neuronal cells

A cDNA encoding the Uptake$_2$ carrier was isolated from a library constructed from a human kidney cell line, using probes which were derived from the sequences of renal amphiphilic solute transporters. Cationic drugs are transported across cell membranes in the kidney, liver and intestines. A cDNA encoding uptake of the quaternary amine tetraethylammonium was isolated from a rat kidney cDNA library by expression in Xenopus oocytes (Grundemann et al, 1994). The clone, designated OCT1 (for organic cation transporter) encoded a protein of 556 amino acids with eleven membrane-spanning domains; the sequence had no significant homologies to other transporters. RNA was expressed in proximal renal tubules, hepatocytes and in the intestine. Uptake of [^{14}C]tetraethylammonium in the oocytes was saturable and followed Michaelis-Menten kinetics with a Km of 9.5×10^{-5} M, which was similar to the affinity of this drug for the transport process in the kidney. Uptake was inhibited by other quaternary amines and desipramine (Ki 2.8×10^{-6} M) but reserpine was a poor ligand (Grundemann et al, 1994). Homologous clones which encode other transporters were isolated from kidney cDNA libraries (Okuda et al, 1996; Grundemann et al, 1997; Schomig et al, 1998).

The substrate specificity of one such renal transporter, OCT2, was similar to that of the Uptake$_2$ carrier, in that OCT2 was sensitive to corticosterone and O-methylisoprenaline (Grundemann et al, 1997). Accordingly, PCR primers were designed, based on the sequence of the OCT2 carrier, and used for amplification of cDNA from the kidney cell line (Grundemann et al, 1998). A PCR product was obtained, whose sequence was similar to the sequences of amphiphilic solute transporters. The PCR product was used to screen the cDNA library by colony hybridisation. Incomplete, overlapping clones were obtained and then assembled to yield a cDNA which encodes a protein of 556 amino acids. The sequence is homologous to the sequences of renal transporters for organic anions and cations. The gene for this transporter is located on chromosome 6 and RNA encoding the transporter was detected by RT-PCR in the brain, liver and heart. Mammalian cells expressing the cDNA accumulated [^{3}H]noradrenaline and the neurotoxin [^{3}H]MPP$^+$, and ligands for the uptake process included adrenaline and serotonin. Uptake of noradrenaline was saturable and followed Michaelis-Menten kinetics, with low affinity (Km 5.1×10^{-4} M) and high capacity.

Uptake was inhibited by corticosterone and was resistant to reserpine and desipramine (Grundemann et al, 1998). These features are similar to the functional properties of $Uptake_2$, described above.

7.5.16 The synaptic vesicle 2 (SV2) "orphan" transporters

Regis Kelly and his colleagues in California prepared rabbit antisera and monoclonal antibodies against cholinergic synaptic vesicles which had been purified from the electric organ of the marine ray *Narcine brasiliensis* and the electric fish *Discopyge ommata* (Sanes et al, 1979). The biochemical properties of the antigens in the synaptic vesicles suggested that they may be glycoproteins (Carlson & Kelly, 1983). The monoclonal antibodies detected four antigens which were designated SV1-SV4 (Caroni et al, 1985). The SV2 antigen in the intact vesicles was fully accessible to the monoclonal antibody whereas SV1, SV3 and SV4 only became accessible after the vesicles had been sonicated or treated with detergent; this suggested that the SV2 antigen was located on the surface of the vesicles whereas the other antigens were exposed only to the inside of the vesicles (Caroni et al, 1985). SV2 was found to occur in all nerve terminals of the electric fish (Caroni et al, 1985). The SV2 antibody cross-reacted with components of the mammalian and amphibian central nervous system; in the rat and in the frog, the antibody was seen to bind in a punctate manner in all regions of the nervous system. Immunogold studies demonstrated that the binding was predominantly to vesicles in pre-synaptic nerve terminals (Buckley & Kelly, 1985). SV2 antibody-coated beads precipitated most of the vesicles which had been prepared from rat brain, suggesting that most synaptic vesicles in rat brain possess the SV2 glycoprotein (Floor & Feist, 1989). The SV2 antibody also bound to cells of the peripheral neuroendocrine system, including the adrenal medulla, the pituitary and the islet cells of the pancreas; in contrast, there was no binding to secretory cells which are not of neuroendocrine origin (such as the thyroid) or to exocrine cells (such as the salivary glands; Buckley & Kelly, 1985). The antibody was seen to bind to neuroendocrine secretory cells both in tissues obtained from the whole animal and to cells in culture (Buckley & Kelly, 1985; Lowe et al, 1988). Electron microscopic studies demonstrated that the SV2 antigen was present both in the small (40 nm diameter) neurosecretory vesicles of nerve terminals and in large (100 nm diameter) dense core vesicles which are typical of neuroendocrine cells, such as adrenal medullary cells (Lowe et al, 1988). The SV2 antigen which was detected in cultured cells had the properties of a membrane-spanning glycoprotein (Buckley & Kelly, 1985) and subsequent, more detailed studies suggested that it is likely to be a keratan sulphate proteoglycan with oligosaccharide

side chains N-linked to a core protein of 80 kDa (Scranton et al, 1993; Carlson, 1996).

The cDNA clones which encode SV2 were isolated simultaneously by three groups of investigators (Bajjalieh et al, 1992; Feany et al, 1992; Gingrich et al, 1992). Bajjalieh et al (1992) purified an immunoreactive peptide fragment from rat brain synaptic vesicles and determined its amino acid sequence; this was then used to design PCR primers which were used to amplify the rat brain library. The resulting PCR product was then used as a probe to obtain a full-sized clone. Feany et al (1992) used an expression cloning strategy: as the SV2 antigen had been detected in the adrenal medulla (Buckley & Kelly, 1985), a cDNA encoding SV2 seemed likely to exist in a cDNA library which had been constructed from PC12 rat phaeochromocytoma cells (Liu et al, 1992b & c). Plasmid DNA from the library was transfected into CHO cells and stable transformants were selected (Feany et al, 1992). The transfected cells were screened with the monoclonal antibody for SV2. Genomic DNA was prepared from one of the SV2-expressing cell lines and the integrated plasmids were recovered by digestion with *Not* I which cleaves the plasmid once, followed by re-ligation of the plasmid (Feany et al, 1992). Transfection into COS cells of one of the retrieved plasmids resulted in expression of SV2. Gingrich et al (1992) detected the SV2 antigen as an 87,000 dalton protein which co-purified with the dopamine receptor during chromatographic isolation procedures. Fragments of the protein were sequenced and oligonucleotide probes were designed and used to screen a rat brain cDNA library and a bovine brain cDNA library. The rat and bovine clones were highly homologous (Gingrich et al, 1992).

The translated sequence of SV2 consists of 742 amino acids which are likely to be arranged in 12 membrane-spanning domains (Figure 11.9.4; Bajjalieh et al, 1992; Feany et al, 1992; Gingrich et al, 1992). The amino-terminal half of the molecule was homologous to mammalian and bacterial sugar and antibiotic transporters. Feany et al (1992) suggested that the carboxy-terminal half may be homologous to the plasma membrane transporters for the neurotransmitter amines (noradrenaline, dopamine and serotonin) but this suggestion was not supported by subsequent investigations (Bajjalieh et al, 1993). The best fits of SV2 were with the human and rat glucose transporters and with the proton-dependent bacterial transporters for xylose, arabinose, citrate and tetracycline (Gingrich et al, 1992).

There was no obvious signal sequence in SV2, indicating that the amino terminal is likely to be cytoplasmic. The amino terminal contains many aspartate and glutamate residues which

make it highly negatively charged (Figure 11.9.4). There were three sites for asparagine-linked glycosylation on the luminal side and other sites for phosphorylation by protein kinase A and protein kinase C on the cytoplasmic side (Bajjalieh et al, 1992; Feany et al, 1992; Gingrich et al, 1992). There were two consensus sites for ATP binding Walker motifs (GXGXXG) which were located at amino acids 129-143 and 266-288 (Gingrich et al, 1992). There is a large luminal loop between the seventh and eighth membrane-spanning domains; this loop contains a region which is rich in phenylalanine and is homologous to the mcbg protein which is involved in antibiotic resistance in bacteria (Bajjalieh et al, 1993). The amino terminal half of SV2 contains regions which had been shown to be conserved in transporters for sugars, citrate and tetracycline (Henderson & Maiden, 1990; Bajjalieh et al, 1992). These include the sequences RXGRR (where X is any amino acid) between the second and third membrane-spanning domains, PESPR at the end of the sixth membrane-spanning domain and $RX_3GX_3GX_6PXYX_2EX_6RGX_6QX_5G$ from the fourth to the fifth membrane-spanning domains; in the last motif, the proline (P) has been substituted for phenylalanine (F) and the glutamine (Q) has been substituted for cysteine (C) in SV2. There is also a basic amino acid, arginine, before the 6th membrane-spanning region (Bindra et al, 1993); this had been shown to be conserved in all proton symporters (Griffith et al, 1992).

In situ hybridisation and Northern analysis revealed that the RNA encoding SV2 is present throughout the central and peripheral nervous system but was undetectable in peripheral tissues such as the liver, spleen, kidneys, muscle, heart and lung (Feany et al, 1992; Bajjalieh et al, 1992; Gingrich et al, 1992). *In situ* hybridisation studies in embryonic mice revealed that the SV2 RNA first appears in the ventral neural tube on embryonic day 10.5, following which it becomes detectable in all regions of the central and peripheral nervous system, including the noradrenergic neurones of the sympathetic nervous system (Marazzi & Buckley, 1993).

Further work indicated that SV2 is likely to be a member of a small family of mammalian molecules. Bajjalieh et al (1993) used a cDNA which encodes the amino-terminal half of SV2 as a probe to screen a rat brain cDNA library for homologous molecules. They isolated a clone which encodes a protein consisting of 682 amino acids with an acidic amino-terminal, twelve membrane-spanning regions and three asparagine-linked glycosylation sites. The amino acid sequence was 65% identical to the sequence of SV2. The new clone and the protein which it encodes were therefore designated SV2B and the original molecule SV2A

(Bajjalieh et al, 1993). The most obvious difference between the two molecules was that SV2B had a shorter amino-terminal than SV2A. The anti-SV2A antibody recognised the SV2B peptide, suggesting that the widespread immunoreactivity to the antibody in the central nervous system represents both SV2A and SV2B antigens (Bajjalieh et al, 1993). As in the case of SV2A, RNA encoding SV2B was detected in all parts of the brain but not in non-neuronal tissues (Bajjalieh et al, 1993). The third member of the family, SV2C, was isolated by screening a rat brain cDNA library with an expressed sequence tag (EST; Janz & Sudhof, 1999). The protein consists of 727 amino acids which are arranged in twelve membrane-spanning regions, and the overall sequence is 62% identical to SV2A and 57% identical to SV2B. The phosphorylation and N-linked glycosylation sites were conserved in this molecule. Northern blot analysis demonstrated that the RNA is most abundant in the brain, but there was probably some hybridisation in non-neuronal tissues as well; this is in contrast to the distribution of RNA for other members of this family, which is restricted to neural tissues (Janz & Sudhof, 1999). Immunoblot studies on subcellular fractions indicated that as in the case of other members of this family, SV2C is likely to be located in synaptic vesicles. In contrast to SV2A, SV2B and SV2C were absent from chromaffin granules but were present in a microsomal fraction of the adrenal medulla (Janz & Sudhof, 1999).

The fourth member of this family, designated SVOP, was also isolated by screening a rat brain cDNA library with an EST (Janz et al, 1998). The translated sequence also suggests twelve membrane-spanning domains but in contrast to the SV2 isoforms, SVOP is not glycosylated. There are homologues of SVOP in *C elegans* and *Drosophila* whereas the other SV2 isoforms have no known homologues in invertebrates; this suggests that SVOP may be the older protein in evolutionary terms, and that the SV2 isoforms may be specialised vertebrate versions of SVOP (Janz et al, 1998). Further evidence for this hypothesis is that the amino acid sequence of SVOP is more closely related to the invertebrate molecules than to the SV2 isoforms (48% vs 22%). It was therefore suggested that SVOP is not an isoform but is likely to be a separate protein which is likely to have been independently conserved during evolution. The sequence of SVOP was also related to the sequence of the organic cation transporter OCT1 (see Section 7.5.15). Immunoblotting studies on subcellular fractions suggested that in the rat brain, SVOP is present in the synaptic vesicle fraction; however, in the adrenal medulla, SVOP was present in a microsomal fraction but was relatively sparse in the chromaffin granules, in contrast to SV2A.

The anatomical distribution of the SV2 isoforms was studied by *in situ* hybridisation histochemistry and by immunohistochemistry, using cRNA probes and antisera which are specific for the isoforms (Bajjalieh et al, 1994; Janz & Sudhof, 1999). Both techniques demonstrated that SV2A is ubiquitous in the rat brain, whereas the SV2B isoform was more restricted in its distribution. Both isoforms were present in the hypothalamus. In contrast to the widespread distribution of SV2A and SV2B, the SV2C isoform was restricted to phylogenetically old parts of the rat brain, such as the striatum, substantia nigra, pons and the medulla, but was absent from cortical regions (including the cerebral and cerebellar cortices), the hippocampus and the thalamus (Janz & Sudhof, 1999). The distribution of the SV2 isoforms was not consistent with the distribution of any known neurotransmitter, which makes it unlikely that SV2 functions as a transporter for a specific neurotramsnitter (Bajjalieh et al, 1994; Janz & Sudhof, 1999). Some neurones appeared to possess both SV2A and SV2B, and it is possible that some vesicles may contain both isoforms of SV2 (Bajjalieh et al, 1994). In the rat, the RNA for SVOP was detectable only in the brain, and immunohistochemical studies demonstrated that the distribution of SVOP is very similar to the distribution of SV2 in several regions of the rat brain (Janz et al, 1998).

Mice which are homozygous for disruption of the SV2A gene are viable but they only develop normally up to the seventh post-natal day (Crowder et al, 1999; Janz et al, 1999). Following that, they fail to thrive and they suffer from generalised seizures, culminating in death by the 23rd post-natal day. The heterozygotes are also prone to seizures but they are of normal weight and are fertile, although their mortality rate is slightly greater than the control animals. In contrast, mice which are homozygous for disruption of the SV2B gene are apparently normal and fertile, and SV2A/SV2B double knockout mice were not more severely affected than mice in which only the SV2A gene had been disrupted (Janz et al, 1999). Thus, loss of the SV2B gene does not aggravate the lethality of the SV2A deficiency. Crowder et al (1999) reported that the predisposition to seizures was associated with reduction in inhibitory GABAergic neurotransmission in the hippocampus. However, Janz et al (1999) found that GABAergic transmission was normal but that glutamatergic neurones from the SV2A knockout mice were more responsive to electrical stimulation; they suggested that the function of SV2A may be to sequester cytoplasmic calcium which would otherwise accumulate during repetitive stimulation of glutamatergic neurones. Electron microscopy revealed no obvious defects in the morphology of synapses, the expression of other synaptic proteins was unaffected and the number and size of the synaptic vesicles appeared normal

(Crowder et al, 1999; Janz et al, 1999).

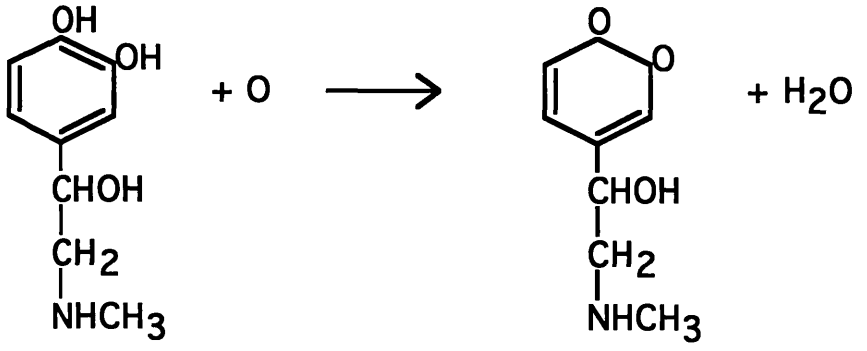
These findings in the transgenic mice indicated that the SV2 proteins are unlikely to be important for the formation of an anatomically normal synapse, but that SV2A is required for normal neurotransmission. However, the functions of the SV2 proteins are unknown. The following possibilities have been discussed:

1. The location of SV2 in acidic synaptic vesicles, its homology to bacterial proton-dependent transporters and the conservation of the arginine at the beginning of the 6th membrane-spanning region all suggested that this molecule may function as a proton-dependent transporter. However, pharmacological studies on the SV2 isoforms and SVOP failed to demonstrate uptake of radiolabelled neurotransmitters, sugars or amino acids (Gingrich et al, 1992; Janz et al, 1998). Further, the anatomical distribution of the SV2s in the brain is not consistent with the distribution of any known neurotransmitter (Bajjalieh et al, 1994; Janz & Sudhof, 1999).
2. It was therefore suggested that the SV2s may function as chloride channels, by analogy with the cystic fibrosis trans-membrane conductance regulator (CFTR) and the multiple drug resistance transporter (MDR; Bajjalieh et al, 1992; Feany et al, 1992). It was suggested that SV2 could function as an efflux transporter, allowing the release from the vesicles of sodium and chloride ions which may enter the vesicles at the cell membrane during exocytosis and the vesicle re-cycling process (Buckley, 1994). Alternatively, SV2 may allow entry of chloride into the vesicles; this would neutralise the electrical component of the electrochemical gradient of protons which is required for uptake of neurotransmitters into the vesicles (Bajjalieh et al, 1994).
3. The amino terminal of SV2 is highly charged and contains several stretches of acidic residues which were postulated to bind calcium (Bajjalieh et al, 1992). Janz et al (1999) suggested that SV2 may pump cytoplasmic calcium into the vesicles and that this would prevent the accumulation of calcium in the cytoplasm during repetitive stimulation of the nerve terminal (see above).
4. It has been suggested that the SV2 family may have served a transport function early in evolution, but that these proteins may now act as matrix receptors by interacting with matrix proteins such as laminin (Carlson, 1996; Son et al, 2000). The SV2A isoform also binds to the synaptic vesicle docking protein synaptotagmin (Schivell et al, 1996) and the interaction is enhanced when SV2A is phosphorylated on serine and threonine residues by casein kinase I (Pyle et al, 2000); the significance of this interaction is unknown.

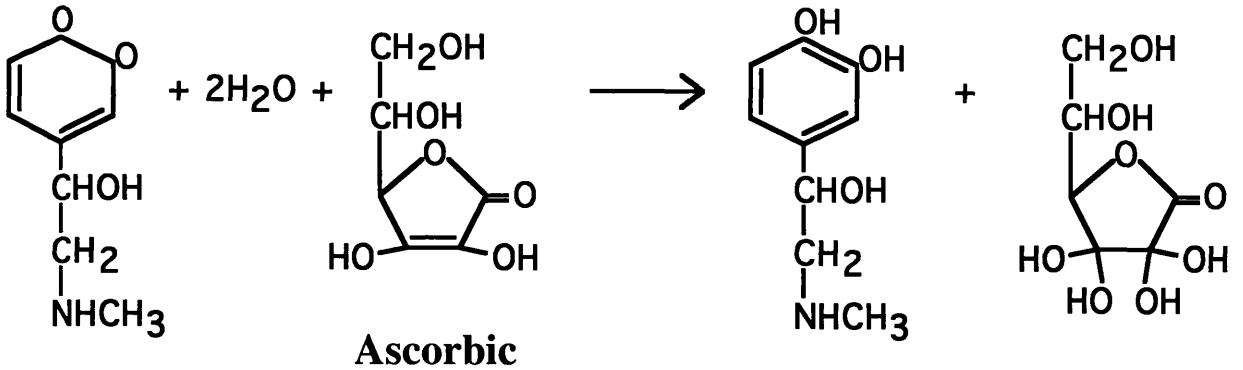
In summary, the SV2 proteins are ubiquitous in the neurosecretory vesicles of neurones and in the secretory granules of neuroendocrine cells. The functions of these proteins are unknown and they are regarded as "orphan" transporters.

Figure 7.5.1:

Auto-oxidation of adrenaline to a quinone and its reduction by ascorbic acid



Adrenaline



Ascorbic acid

Adrenaline

Figure 7.5.2:

Metabolism of adrenaline and noradrenaline by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). In neurones, metabolism proceeds along the MAO path whereas the COMT path is followed in non-neuronal cells.

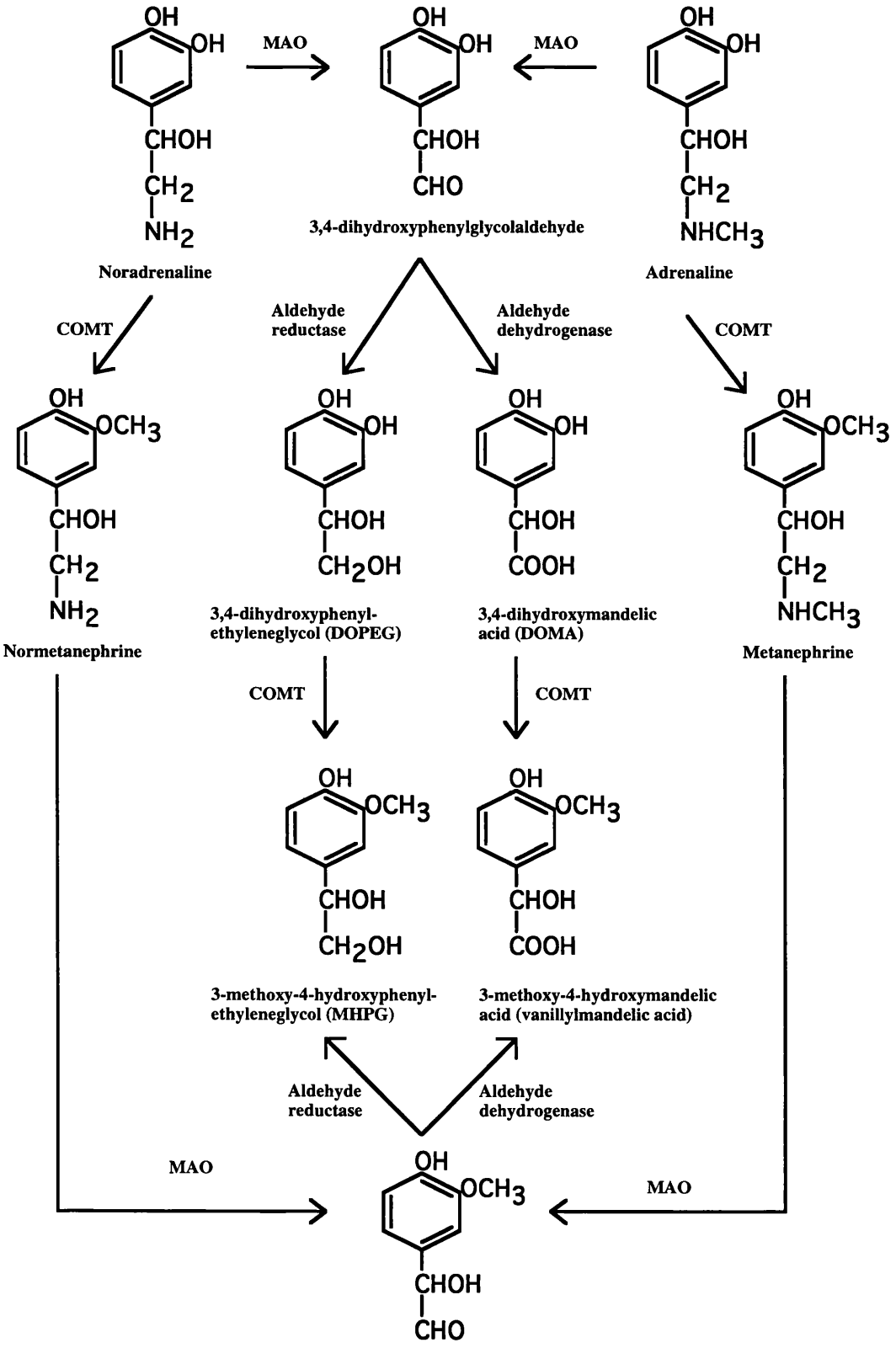
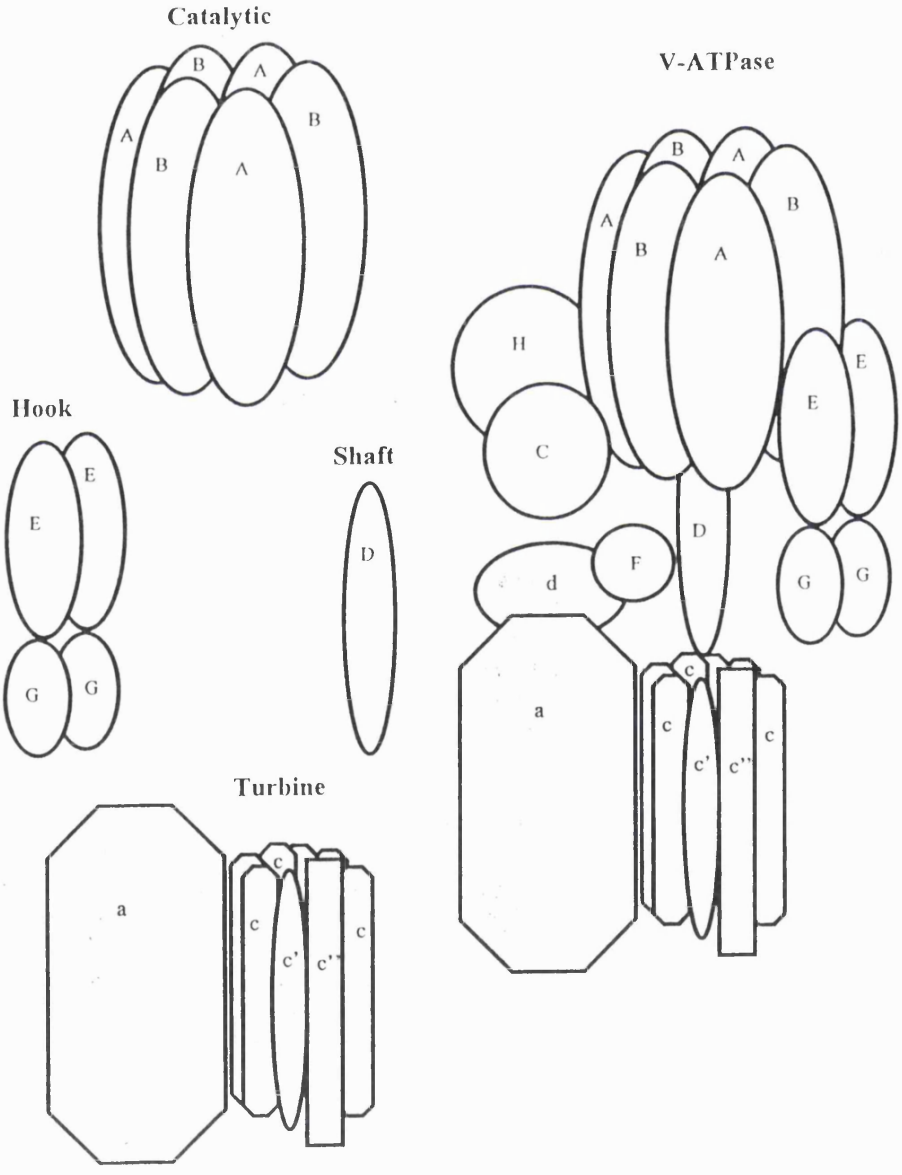


Figure 7.5.3:
Current model of the three-dimensional structure of V-ATPase, based on the structure of mitochondrial F-ATPase. The structure of V-ATPase is also believed to consist of multiple subunits which are organised into four components: a catalytic unit, a "shaft", a "turbine" and a "hook". The catalytic unit is believed to consist of alternating A and B subunits which bind and hydrolyse ATP. The "turbine" is located in the plasma membrane. Hydrolysis of ATP causes conformational changes which rotate the "shaft" which in turn rotates the "turbine" which pumps protons across the membrane. The "hook" is anchored in the plasma membrane and prevents rotation of the catalytic unit. Thus, the ATP-binding site faces the cytoplasm and the enzyme moves protons away from the catalytic unit towards the vesicular lumen which becomes acidic. Reproduced from Nelson & Harvey (1999).



8. HYPOTHESIS

The α_1 adrenergic ligand [^{3}H]prazosin binds to peptidergic neurones and is displaced by unlabelled prazosin in concentrations up to 10^{-7} M. However, at greater concentrations of unlabelled prazosin, there is a paradoxical increase in accumulation of [^{3}H]prazosin. The following hypothesis was formulated to explain these findings:

A. Displacement of [^{3}H]prazosin is from α_1 adrenoceptors in the peptidergic neurones;

B. The increase in radioactivity (the prazosin paradox) is due to the presence in peptidergic neurones of an unusual uptake process which is activated by its ligand (prazosin). This uptake process was designated Transport-P.

9. AIMS OF THE RESEARCH

The aims of the research were to test the hypothesis as follows:

A. Displacement of [^{3}H]prazosin is from α_1 adrenoceptors:

1. To look for RNA which encodes α_1 adrenoceptors in the peptidergic neurones.

2. To identify the sub-type of α_1 adrenoceptors in the peptidergic neurones.

3. To isolate an individual clone encoding the peptidergic neurone α_1 adrenoceptor.

4. To compare the affinity of prazosin for the cloned peptidergic neurone α_1 adrenoceptor with its affinity for the binding sites in the peptidergic neurones.

B. The prazosin paradox is due to an unusual uptake process which is activated by its ligand:

1. To examine the possibility that the prazosin paradox could be due to internalisation of ligand-receptor complexes.
2. To determine the source of energy for the prazosin paradox.
3. To determine whether the prazosin paradox is a general feature of all cells or a specialised function of peptidergic neurones, by examining the effects of prazosin in pre-synaptic neurones and in non-neuronal cells.
4. To examine the peptidergic neurones for the presence of specific binding sites for antidepressants which had been found to inhibit the prazosin paradox.
5. To provide visual evidence for the uptake of amines in peptidergic neurones.
6. To determine whether the proposed uptake process requires a defined chemical structure in its ligands.
7. To determine whether the amines can be released by an energy-dependent process.
8. To isolate a cDNA which encodes the uptake proess.

10. METHODS

10.1 CULTURES OF MAMMALIAN CELLS

10.1.1 Primary cultures of hypothalamic cells from fetal and neonatal rats

The earliest reference which I could find to neuronal cultures was the report by Harrison (1907) in which he described the growth of pieces of frog embryos which he had placed on cover slips covered with frog lymph. The coverslip was then inverted over a hollow slide and the rim sealed with paraffin. "When reasonable aseptic precautions are taken, tissues will live under these conditions for a week and in some cases specimens have been kept alive for nearly four weeks". He then observed the growth of nerve fibres "which left the mass of nerve tissue and extended out into the surrounding lymph clot." Since then, primary cultures of various regions of animal brains have become established procedures. In the present work, dissociated primary cell cultures were prepared from the rat hypothalamus using a method which was based on published procedures (Wilkinson et al, 1974; Loudes et al, 1983; Shoemaker et al, 1983; Clarke et al, 1987).

Pregnant rats (15 days) were caged individually and given food and water *ad libitum.* On day 18 of pregnancy, the pregnant rat was killed and the abdomen and the uterus opened to expose the fetuses which were decapitated with sharp scissors. The dorsum of the skull was removed and the olfactory nerves were cut with sharp scissors. The brain was then inverted and the optic chiasma was cut to expose the hypothalamus which was dissected under a microscope at the following borders: junction of the anterior cerebral arteries anteriorly; caudal border of the mamillary bodies posteriorly and lateral sulci and perforating vessels dorsolaterally. The hypothalami were placed in ice-cold dissociation buffer consisting of NaCl 137 mM, KCl 5 mM, Na_2HPO_4 0.7 mM, HEPES 25 mM, gentamicin 0.1 g/l, pH 7.4. The hypothalami were then washed three times with 10 ml of the dissociation buffer, cut in half and incubated for 60 minutes at 37^0C in dispersion solution consisting of 0.2% collagenase II (activity 149 U/mg), 0.4% bovine serum albumin, 0.2% glucose and 0.05% DNAse I (activity 510 Kunitz units/mg) in dissociation buffer. The fragments were then dispersed by aspiration about 20 times into a sterile plastic Pasteur pipette and incubated for a further 30 minutes at 37^0C. The tissues were again dispersed and the cells transferred to a sterile 50 ml tube via a sterile mesh with 200 um pores. The cells were washed with dissociation buffer followed by culture medium, and were finally dispersed in fresh culture medium. The cell suspension was diluted with culture medium to the desired cell density and incubated in sterile culture dishes or chamber slides in a humidified atmosphere containing

5% CO_2 in air. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (ratio 1:1) containing 10% heat-inactivated fetal bovine serum (FBS), sodium bicarbonate 3.7 g/l and gentamicin 100 mg/l. Culture media were changed at 48-hour intervals and the experiments were carried out two to eight days following culture.

10.1.2 Cultures of immortalised cell lines

The cell lines which were used in these studies are described below. The same methods were used for the dispersion, growth, propagation and storage of all the cell lines.

Immortalised cells were thawed from liquid nitrogen by placing them on ice for 10 minutes, then at 37^0C for 5 minutes. They were then transferred to a Corning 25 cm^2 flask containing 7 ml of culture medium which consisted of DMEM and Ham's F-12 (ratio 1:1) containing 10% FBS and sodium bicarbonate 3.7 g/l. The cells were then grown in a humidified atmosphere containing 5% CO_2 in air. Culture media were changed at 48-hour intervals. When the cells reached confluence, they were dispersed by incubation for 5 minutes at 37^0C with phosphate buffered saline (PBS) containing 0.05% trypsin, 0.05% EDTA, 0.05% $NaHCO_3$, 0.04% DNAse I and 0.1% glucose. The cell suspension was centrifuged for 5 minutes at 400 g and the supernatant discarded. The cells were washed once in culture medium and were then re-suspended in fresh medium at the required density and incubated in flasks, dishes, culture wells or chamber slides as required. Culture media were changed at 48-hour intervals. To store cells in liquid nitrogen, a flask of cells was dispersed as described above and the cell suspension was centrifuged. The supernatant was discarded and the cells were re-suspended in the above culture medium. An aliquot of the cells was diluted in trypan blue and counted in a haemacytometer. The remaining cells were centrifuged and re-suspended in 10% DMSO in FBS at a density of 10^7 cells/ml. The cells were placed on ice for an hour, transferred to -70^0C for four hours and then to liquid nitrogen.

10.1.2.1 GT1-1 gonadotrophin-releasing hormone peptidergic neurones

The GT1-1 gonadotrophin-releasing hormone (GnRH) neuronal cell line was produced by Mellon et al (1990) in California, by targetting an oncogene, the simian virus 40 (SV40) tumour antigen (T antigen), to GnRH neurones, using the GnRH promoter.

SV40 is a DNA tumour virus which was discovered as a contaminant in the rhesus monkey

cell cultures which were used to prepare the Salk and Sabin polio vaccines (Watson et al, 1987). Although SV40 causes tumours in rodents, there has been no evidence of increased incidence of cancer in humans who received the polio vaccines. The genome of SV40 consists of double-stranded, circular DNA of 5243 base pairs which integrates into the host cell genome following infection. The genome consists of three regions (Figure 10.1.1):

1. The "early region" encodes the T antigens which are responsible for viral replication.
2. The "late region" encodes three viral coat proteins and the agnogene which encodes a protein thought to be involved in viral assembly or release. These late genes are expressed only after DNA synthesis begins, about 12 to 14 hours after infection.
3. The region of DNA which lies between the early region and the late region contains the regulatory sequences for replication, including the origin of DNA replication.

The early region of SV40 encodes two proteins (T antigens) which are derived by alternative splicing of a single RNA; the "large T antigen" is 94 kilodaltons and the "small T antigen" is 17 kilodaltons; these two proteins have the same amino termini but they differ at the carboxyl ends because of differential splicing (Figure 10.1.1). The function of these proteins is to induce the synthesis of enzymes which are involved in DNA replication during the cell cycle (Watson et al, 1987). It appears that the large T antigen is sufficient for inducing tumours in rapidly dividing cells such as lymphocytes, but that the combination of large T and small T antigens is necessary for the induction of tumours in slowly dividing cells (Choi et al, 1988).

Hanahan (1985) linked these SV40 oncogenes with the rat insulin gene to produce tumours of the β cells of the islets of Langerhans in transgenic mice. In that study, two hybrid genes were constructed, combining the promoter region and a transcriptional enhancer from a rat insulin gene with the SV40 T antigens; in one of the genes, the insulin promoter/enhancer was aligned to promote transcription of the T antigens, whereas in the second gene the insulin promoter/enhancer was inverted in relation to the coding region of the T antigens. The two fusion genes were injected into fertilised one-cell mouse embryos, which were then inserted into the oviducts of pseudopregnant female mice and allowed to develop. The transgenic mice which were produced were found to have hyperplasia of the islets of Langerhans and died early, presumably because of hypoglycaemia; when they were given a high sugar diet, they lived longer and they developed tumours of the β cells of the islets of Langerhans in which the large T antigen was detectable by immunocytochemistry.

Expression of the large T antigen was confined to the β cells of the islets of Langerhans, indicating that expression was tissue specific. Interestingly, the fusion gene in which the promoter/enhancer region had been inverted also caused hyperplasia and tumour formation, and the large T antigen was expressed in a tissue-specific manner, indicating that the insulin gene component could function in a bidirectional manner. Serial studies on mice of different ages suggested that the hybrid insulin/T antigen gene was expressed in all β cells, which gradually all became hyperplastic, but only a few cells eventually became malignant within the time course of the study (Hanahan, 1985).

Mellon et al (1990) used the same technique to generate mice bearing tumours of GnRH neurones, from which the GT1-1 cells were obtained. The transgene consisted of the GnRH promoter which was placed upstream to the T antigen. The construct was injected into fertilised one-cell mouse embryos and nine transgenic mice were obtained. Two of these mice had tumours of the anterior hypothalamus and northern blot analysis indicated that the T antigen was expressed selectively in the tumour tissue. The tumour cells were dispersed mechanically in the presence of collagenase and DNAse and grown in culture. Following six months of repeated passage, three cell lines were obtained from the heterogeneous cultures and they were designated GT1-1, GT1-3 and GT1-7. When grown in serum-free medium, the GT cells have a neuronal appearance and they extend neurites which contact other cells or end in growth cones. Northern blot analysis and immunocytochemistry demonstrated that the GT cells possessed specific neuronal proteins, such as GnRH, neurone-specific enolase and neurofilament protein. The cultured cells were capable of neurosecretory function as they secreted GnRH into the culture medium. They also expressed RNA for synaptic vesicle proteins, including Vamp-2, SNAP-25 and chromogranin. Indeed, electron microscopic examination indicated that the GT cells possessed neurosecretory granules, clathrin coated pits and fine structural specialisations which are typical of synaptic contacts. Secretion of GnRH could be stimulated by exposure of the cells to high concentrations of potassium which depolarises neurones via calcium channels. GnRH secretion could also be stimulated by exposure to veratradine which depolarises neurones by opening sodium channels. The basal secretion of GnRH was inhibited by the sodium channel blocker tetrodotoxin. These findings indicated that the GT cells possess many specialised features of neurones, including ultrastructural components, channels and peptides which are important for the process of regulated neurosecretion. While the GT cells possessed RNA for GnRH, they did not

possess RNA for other hypothalamic hormones such as somatostatin, pro-opiomelanocortin, corticotrophin-releasing hormone or growth hormone-releasing hormone. This indicated that the GT cells possessed the specific characteristics of differentiated GnRH neurones. In contrast, the GT cells did not possess RNA for specific glial proteins such as glial fibrillary acid protein, myelin basic protein or myelin proteolipid protein, which confirms the neuronal nature of these cells (Mellon et al, 1990).

A large amount of information has been obtained on the GT cells, confirming that they are indeed immortalised GnRH peptidergic neurones and that they possess many of the properties of GnRH neurones in intact animals. The secretion of GnRH *in vivo* occurs in a pulsatile manner; similarly, secretion of GnRH by GT cells is pulsatile and dependent on the presence of calcium in the extracellular space (Martinez de la Escalara et al, 1992a; Wetsel et al, 1992; Krsmanovic et al, 1992). Synchronization of secretory activity may be mediated by synaptic contacts or by intercellular cytoplasmic "bridges", both of which have been demonstrated between GT cells (Mellon et al, 1990; Wetsel et al, 1992). These intercellular "bridges" or "passageways" have also been observed in GnRH neurones *in vivo* (Witkin et al, 1995). Synchronisation of neuronal firing via gap junctions (also described as "electrical synapses") has been observed in other regions of the brain which have defined networks whose components must discharge in a coordinated manner (Galarreta & Hestrin, 1999; Gibson et al, 1999; Bennett, 2000). Synchronization of GnRH secretion may also be the result of a feedback effect of GnRH on its own release, possibly mediated via GnRH autoreceptors which are expressed in the GT cells (Krsmanovic et al, 1993 & 1999; Li et al, 1996). Electron microscopic studies demonstrated that GnRH is present in the neurosecretory vesicles in GT cells (Liposits et al, 1991); both large dense core vesicles and small synaptic vesicles appear to be present in the GT cells (Ahnert-Hilger et al, 1998).

Under physiological conditions, GnRH stimulates the secretion of the pituitary gonadotrophins which in turn stimulate the secretion of gonadal steroids; the steroid hormones in turn exert a negative feedback action on the hypothalamo-pituitary gonadal axis. Northern and western blot analysis demonstrated that GT cells possess RNA and peptide for the androgen receptor, and androgens bind with high affinity to a cytosolic receptor in these cells (Poletti et al, 1994; Belsham et al, 1998). Further, dihydrotestosterone inhibits the synthesis of GnRH mRNA and the effect is blocked by an androgen antagonist, confirming that the androgen receptors are functional (Belsham et al, 1998). These findings are

consistent with the inhibition of GnRH secretion by androgens under physiological conditions *in vivo*. The GT cells also possess mRNA and high-affinity binding sites for oestradiol (Poletti et al, 1994; Shen et al, 1998). Both the α and β oestrogen receptors are detectable by northern and western blotting analysis and oestradiol reduces GnRH mRNA in these cells (Roy et al, 1999). Oestradiol stimulates the transcription of galanin mRNA in GT cells and the effect is blocked by oestrogen antagonists; this demonstrates that the oestrogen binding sites are functional and the effect is consistent with the effect of oestradiol on the synthesis of galanin in GnRH neurones *in vivo* (Shen et al, 1998). The 5α-reductase enzyme which converts testosterone to dihydrotestosterone and the 3α-hydroxysteroid dehydrogenase enzyme which converts dihydrotestosterone to 5α-androstan-3α,17β-diol are present in GT cells (Poletti et al, 1994). These cells do not possess cytochrome P-450 aromatase which converts androgens to oestrogens (Poletti et al, 1994), and this is consistent with the absence of this enzyme from the GnRH neurones of adult rodents (Sanghera et al, 1991). Thus, GT cells possess the biochemical mechanisms which are required for the actions of gonadal steroids, and which are characteristic of mature GnRH neurones.

Noradrenaline influences the secretion of GnRH in rats in *vivo* (Barraclough & Wise, 1982; Kalra & Kalra, 1983; Herbison, 1997). The GT cells possess RNA and high affinity binding sites for β_1 adrenergic receptors and noradrenaline and isoprenaline stimulate the secretion of GnRH from these cells; the effect of noradrenaline is accompanied by an increase in the intracellular content of cAMP and β_1 adrenergic antagonists inhibit these actions of noradrenaline (Martinez de la Escalara et al, 1992b; Findell et al, 1993). These findings indicate that noradrenaline stimulates the secretion of GnRH by activating β_1 adrenergic receptors in the GT cells. GT cells also possess RNA and binding sites for D_1 dopaminergic receptors and dopamine stimulates the secretion of GnRH from GT cells via D_1 dopaminergic receptors which are linked to adenylate cyclase (Martinez de la Escalara et al, 1992c; Findell et al, 1993). The secretion of GnRH *in vivo* is also influenced by GABA, glutamate, neuropeptide Y, histamine, opioid peptides, endothelin, acetylcholine and prostaglandins; these neurotransmitters influence the secretion of GnRH in the GT cells and receptors for these substances have been detected in the GT cells (Favit et al, 1993; Mahachoklertwattana et al, 1994; Besecke et al, 1994; Noris et al, 1995; Maggi et al, 1995a

& b; Krsmanovic et al, 1991 & 1998; Pimpinelli et al, 1999). GnRH neurones express glucocorticoid receptors *in vivo* and gluococorticoids inhibit the synthesis and secretion of GnRH, presumably accounting in part for the inhibition of reproductive activity during conditions of stress; similarly, GT cells possess glucocorticoid receptors and dexamethasone inhibits the synthesis of GnRH in these cells (Chandran et al, 1994). Peptides are colocalised with amine or amino acid neurotransmitters in many neurones (Section 7.1.2). GABA immunoreactivity is detectable in some GnRH neurones *in vivo* (Tobet et al, 1996), and GABA immunoreactivity has also been detected in GT cells (Ahnert-Hilger et al, 1998). Thus, a large amount of information has been obtained on these cells, confirming that they have many of the properties of differentiated GnRH peptidergic neurones.

We demonstrated the presence of α_1 adrenoceptor binding sites in GT1-1 cells, as evidenced by binding of the selective radioligands [^{3}H]prazosin and [^{125}I]HEAT and their displacement by unlabelled α_1 adrenergic ligands (Al-Damluji et al, 1993). However, in GT1-7 cells, the stimulant effect of noradrenaline on the secretion of GnRH was unaffected by the α adrenergic antagonists phentolamine, prazosin and yohimbine (Martinez de la Escalara et al, 1992b). Noradrenaline increases intracellular calcium concentrations in GT1-7 cells and depolarises the membrane potential; these effects are blocked by the β adrenergic antagonist propranolol but not by the α antagonist phentolamine (Uemura et al, 1997). Section 11.1 of this Thesis presents definitive evidence for the presence of α_1 adrenoceptors in GT1-1 cells.

10.1.2.2 SK-N-SH noradrenergic neurones

In May 1970, a four-year old girl presented with a lung tumour at the Memorial Sloan-Kettering Cancer Center in New York. At operation, she was found to have a neuroblastoma, the major part of which was excised. Urinary catecholamine and VMA excretion was increased. She was given radiotherapy to the chest followed by chemotherapy (vincristine, cyclophosphamide and daunomycin) but she developed metastases in the femur, bone marrow, liver and epidural space. Additional radiotherapy was given to the metastases followed by chemotherapy (trifluoromethyl-2'-deoxyuridine and adriamycin) with little response. A bone marrow aspiration was obtained in December 1970, from which the SK-N-SH cell line was established. The patient died in January, 1971 (Biedler et al, 1973).

The cells were plated many times on glass and plastic and individual colonies were selected for propagation. The doubling time of SK-N-SH cells was 44 hours. When these cells were implanted in the cheek pouches of hamsters, histologically proven tumours were obtained (Biedler et al, 1973). When grown at low density, the cells projected long, thin processes resembling axons. They contained large amounts of dopamine β-hydroxylase and they converted [^{3}H]dopamine to [^{3}H]noradrenaline, confirming their origin from noradrenergic neurones (Biedler et al, 1973; Richards & Sadee, 1986).

SK-N-SH cells accumulate the noradrenaline analogue *meta* -iodobenzylguanidine ([^{125}I]MIBG) by a saturable process which conforms to Michaelis-Menten kinetics (Buck et al, 1985). Noradrenaline competes with MIBG with a Ki of $3x10^{-7}$ M which is similar to the affinity of noradrenaline for the pre-synaptic plasma membrane noradrenaline transporter ($Uptake_1$; Section 7.5.3). Uptake of MIBG was temperature-sensitive and could be blocked by ouabain, confirming that it is an energy-dependent process which relies on the electrochemical gradient of sodium ions which is generated by the Na^+/K^+ ATPase (Buck et al, 1985). These are characteristics of the $Uptake_1$ noradrenaline transporter (Section 7.5.3). SK-N-SH cells also accumulate [^{3}H]noradrenaline and [^{3}H]dopamine by a saturable, active process and uptake was blocked by tricyclic antidepressants with relative potencies which were appropriate for $Uptake_1$ (Richards & Sadee, 1986). A cDNA encoding the pre-synaptic $Uptake_1$ plasma membrane noradrenaline transporter was isolated from SK-N-SH cells by expression cloning (Pacholczyk et al, 1991; Section 7.5.13). After removal of the uptake buffer containing MIBG, a substantial amount of MIBG was retained in the cells, suggesting that it may be accumulated in intracellular vesicles (Buck et al, 1985). However, other investigators concluded that most of the MIBG and [^{3}H]noradrenaline were accumulated in the cytoplasm of SK-N-SH cells (Smets et al, 1989).

10.1.2.3 DDT_1 MF-2 smooth muscle cells

The DDT_1 cell line was isolated from a leiomyosarcoma in the ductus deferens of a Syrian hamster which had been exposed to chronic treatment with diethylstilboestrol and testosterone (Norris et al, 1974). The cultured cells which were derived from the tumour

possessed receptors for androgens and glucocorticoids and exposure to androgens stimulated DNA synthesis (Norris et al, 1974; Norris & Kohler, 1977). It was subsequently noted that a sub-clone of these cultured cells contracted upon exposure to noradrenaline (Cornett & Norris, 1982). The sub-clone was designated DDT_1 MF-2. These cells were found to bind the α_1 adrenergic ligand [^{3}H]dihydroergocryptine in a saturable manner and Scatchard analysis of the data resulted in a linear plot, indicating a single class of binding sites. The radioligand was displaced stereospecifically by noradrenaline, the (-) isomer having the greater affinity. [^{3}H]Dihydroergocryptine was also displaced by adrenergic agonists and antagonists with affinities which were typical of α_1 adrenergic receptors (Cornett & Norris, 1982). A cDNA encoding the α_{1B} sub-type of α_1 adrenergic receptors was isolated from these cells (Cotecchia et al, 1988; Section 7.2.4). Further, pharmacological analysis of the α_1 adrenergic receptors in DDT_1 MF-2 cells suggests that they contain exclusively the α_{1B} sub-type, as the receptors in these cells are inactivated by chlorethylclonidine and are relatively resistant to the α_{1A} antagonists WB4101, phentolamine, 5-methylurapidil and niguldipine (Han et al, 1992).

The α_{1B} adrenergic receptors in the DDT_1 MF-2 cells rapidly become desensitised following exposure of the intact cells to α_1 agonists. Noradrenaline binds to the α_1 adrenergic receptors in these cells and this is followed by breakdown of membrane polyphosphoinositides to diacylglycerol and phosphatidylinositol; this process can be followed in the DDT_1 MF-2 cells by monitoring the incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol (Leeb-Lundberg et al, 1987). Pre-incubation of the cells with noradrenaline followed by extensive washing of the cells at low temperature results in a dramatic decrease in the ability of the α_1 adrenergic receptors to mediate subsequent stimulation of phosphatidic acid and phosphatidylinositol phosphorylation. This desensitisation process is accompanied by phosphorylation of the receptor protein and reduction of the density of [^{3}H]prazosin binding sites in the intact cells. These effects of noradrenaline are maximal after 10 minutes. The following mechanism was suggested for the

desensitisation of the α_1 adrenergic receptors in the DDT_1 MF-2 cells: binding of noradrenaline to the α_1 adrenoceptors activates protein kinase C via diacyl glycerol which is generated during hydrolysis of membrane phospholipids. Protein kinase C then phosphorylates the α_1 adrenoceptors and this leads to loss of function of the receptors and sequestration of the receptors away from the plasma membrane. The receptors are then dephosphorylated by intracellular phosphatases (Leeb-Lundberg et al, 1987). Indeed, this work in intact cells was supported by the finding that the purified α_{1B} adrenergic receptor from DDT_1 MF-2 cells could be phosphorylated by the purified catalytic units of protein kinase C or protein kinase A, and that occupancy of the purified α_1 adrenergic receptor by noradrenaline increased the rate of phosphorylation by protein kinase C (Bouvier et al, 1987). Further support for this hypothesis was provided by the finding that some of the effects of noradrenaline on α_1 adrenergic receptors in DDT_1 MF-2 cells can be reproduced by phorbol esters which mimic the action of diacyl glycerol (Leeb-Lundberg et al, 1985). More recent work has demonstrated that the α_{1B} adrenoceptor can be phosphorylated by the β adrenergic receptor kinases (βARKs) which specifically phosphorylate the agonist-occupied conformation of G protein coupled receptors (Diviani et al, 1996). Indeed, it may be that the β adrenergic receptor kinases mediate most of the homologous agonist-induced phosphorylation of the α_{1B} adrenoceptor. The phosphorylated receptor then binds to the protein β arrestin which dissociates the receptor from the G protein (Diviani et al, 1996).

Sequestration of α_1 adrenoceptors was demonstrated in the DDT_1 MF-2 cells (Fratelli & DeBlasi, 1987). The cells were incubated with adrenaline or noradrenaline at 37^0C for 20 minutes, placed on ice, then washed extensively at 4^0C. Binding assays with [^{3}H]prazosin were performed at 4^0C on the intact cells. Exposure of the cells to the agonists for a few minutes at 37^0C caused loss of approximately 40% of the [^{3}H]prazosin binding sites. The loss of binding sites was a reversible process. This agonist-induced loss of binding sites was not seen when the binding assays were performed on broken cells, presumably indicating

that the lost receptors were sequestered in some intracellular compartment which was inaccesible to [^{3}H]prazosin in the intact cells. Further, loss of binding sites was not seen if the incubation with agonists was at 4^{0}C, suggesting that it is an active process requiring cellular energy. Loss of binding sites was not seen if the binding assay was performed at 37^{0}C, presumably due to dephosphorylation and recycling of the receptors at the physiological temperature (Fratelli & DeBlasi, 1987; Cowlen & Toews, 1987). Subsequent studies demonstrated that the reduction in the density of α_1 adrenoceptors in the intact DDT$_1$ MF-2 cells was accompanied by a shift in the binding sites from the plasma membrane fraction to a vesicular fraction in sucrose density gradients (Cowlen & Toews, 1988).

In the above studies, the cells were exposed to agonists for 20 minutes; the density of surface receptors declined but there was no change in receptor density in membrane preparations, indicating that receptors had simply been redistributed to an intracellular compartment (Fratelli & DeBlasi, 1987). However, when the intact DDT$_1$ MF-2 cells were exposed to noradrenaline for 24 hours, there was a significant loss of receptors from the membrane preparations, presumably due to gradual hydrolysis of the receptors in the intracellular compartment where they are sequestered (Colucci et al, 1988). The molecular mechanisms of this long term process are not well understood. Removal of the arginine-rich part of the carboxyl terminal tail of the α_{1B} adrenoceptor resulted in mutant receptors which were defective in long term downregulation, suggesting that this region of the receptor may be required for this function (Wang et al, 2000).

Visual evidence has been obtained for agonist-induced internalisation of α_{1B} adrenoceptors expressed in mammalian kidney cells. Immunocytochemistry combined with confocal microscopy demonstrated that exposure to noradrenaline or to drugs which activate protein kinase C causes a rapid translocation of the α_{1B} adrenoceptors from the cell surface to internal vesicles. Following removal of the agonist, the internalised α_{1B} adrenoceptors are rapidly reactivated and translocated back to the cell surface (Fonseca et al, 1995). Thus, following exposure to a pulse of agonist, the α_{1B} adrenoceptors undergo a cycle of phosphorylation, uncoupling from the G protein, internalisation, dephosphorylation and

translocation back to the cell membrane.

In the original studies in the DDT_1 MF-2 cells and on the purified α_{1B} adrenoceptor, it was concluded that exposure to noradrenaline and phosphorylation of the receptor reduced the density of α_1 adrenoceptors without affecting the affinity of agonists for the receptors (Leeb-Lundberg et al, 1987; Bouvier et al, 1987). However, in subsequent studies, it became clear that exposure to agonists may result in the appearance of a form of the receptor which has low affinity for agonists (Fratelli et al, 1987; Toews, 1987; Cowlen & Toews, 1987). Thus, when the binding assays were performed on intact cells at 37^0C, adrenaline, noradrenaline and phenylephrine displayed biphasic competition curves in the DDT_1 MF-2 cells, whereas antagonists such as prazosin and phentolamine displayed monophasic curves. The low affinity form of the receptor was not evident when the binding assays were performed on intact cells at 4^0C or on membrane preparations. These findings suggested that under basal conditions, most of the receptors exist in a high affinity form for the agonists, while during the interaction of agonists with intact cells at 37^0C, a significant proportion of receptors was converted to a low affinity form for agonists (Fratelli et al, 1987). The changes in affinity are likely to involve some conformational change in the receptor, as mutation of a single amino acid (alanine 293) increases the binding affinity of noradrenaline and its potency for activating phosphatidyl inositol hydrolysis, but has no effect on the affinities of antagonists for the receptor (Cotecchia et al, 1990 & 1995).

The third intracellular loop of the α_{1B} adrenoceptor plays a major role in binding and activating G proteins (Cotecchia et al, 1990; Luttrell et al, 1993; Hawes et al, 1994). Protein kinase A appears to phosphorylate a serine residue in position 278, which is in the third intracellular loop of this receptor (Alonso-Llamazares et al, 1997). Phosphorylation of the receptor at this site followed by uncoupling from the G protein would be a plausible mechanism for desensitisation of the receptor upon exposure to agonists. In addition, G protein coupled receptors have a common sequence $NP(X)_nY$ which is located near the end of the seventh membrane spanning domain; in the α_{1B} adrenoceptor, this is represented by the sequence NPIIY. Mutation of this tyrosine in position 348 to alanine uncoupled the receptor from the G protein, without affecting agonist binding (Wang et al, 1997). The

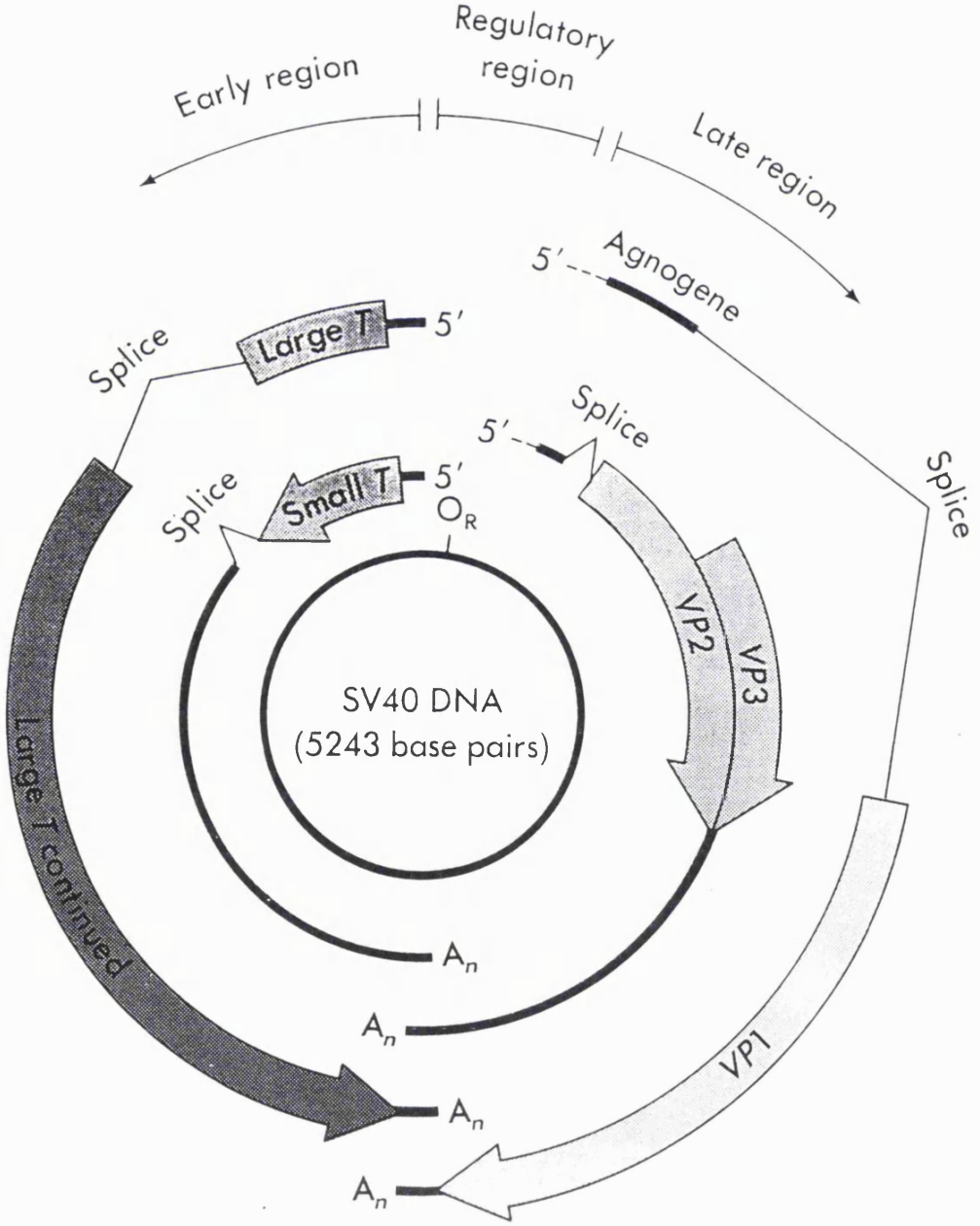
carboxyl terminus tail of the α_{1B} adrenoceptor is also important for desensitisation and internalisation of this receptor; a truncated mutant which lacks most of the carboxyl terminus tail binds adrenaline with normal affinity and activates protein kinase C, confirming that the carboxyl terminus is not required for agonist binding or activation of G proteins; however, following exposure to adrenaline, there was evident impairment of the phosphorylation and desensitisation of the truncated mutant, and the internalisation was slower than the natural receptor (Lattion et al, 1994). Exposure to adrenaline results in phosphorylation of serine, but not of threonine or tyrosine residues in the α_{1B} adrenoceptor (Diviani et al, 1997). The serine residues are located in positions 404, 408 and 410, in the carboxyl terminus tail of the α_{1B} adrenoceptor; replacement of these residues by alanines abolishes the ability of adrenaline to induce phsophorylation and desensitisation of the receptor, whereas re-introduction of these serines restores the ability of adrenaline to induce these functions (Diviani et al, 1997). Protein kinase C phosphorylates serines which are in positions 394 and 400 in the carboxyl terminus tail of the α_{1B} adrenoceptor (Diviani et al, 1997). It is possible that protein kinase C may mediate heterologous desensitisation of the α_{1B} adrenoceptor, ie, desensitisation which is caused by activation of other protein kinase C-linked receptors.

10.1.2.4 COS-7 kidney cells

CV-1 is a simian kidney cell line in which the SV40 virus can propagate. These cells were transfected with a plasmid which contained the sequence of a mutant SV40 virus in which there was a 6-base deletion in the origin of replication (Gluzman, 1981). No infectious virus was detected in cells transfected with the mutant DNA. Three transformed cell lines were obtained and were designated COS lines (for CV-1 origin defective SV40). These cells were found to have integrated the SV40 DNA, as determined by Southern transfer followed by hybridisation. Circular DNA was not detected, indicating that the SV40 DNA was present only in integrated form in the cellular genome. The COS cells permitted the replication of SV40 mutants in which there were deletions in the Early Region, indicating that the COS cells were producing T antigens which are required for the replication of the mutant viral DNA (Section 10.1.2.1). The development of these cells allowed the use of plasmid vectors which contain the SV40 origin of replication; such plasmids replicate in the COS cells under the influence of the T antigens which these cells express. This allows the expression of large amounts of cloned protein in these mammalian cells (Gluzman, 1981).

Figure 10.1.1

The genome of the SV40 virus. The genome consists of double-stranded, circular DNA of 5243 base pairs which consists of three regions: The "early region" encodes the T antigens which are responsible for viral replication. The large T antigen and the small T antigen are two proteins which are derived by alternative splicing of a single RNA. The "late region" encodes the three viral coat proteins (VP1, VP2 and VP3) and the agnogene which encodes a protein thought to be involved in viral assembly or release. The "regulatory region" which lies between the 5' ends of the early region and the late region contains the regulatory sequences for replication. O_R is the origin of DNA replication. The translated portion of each RNA is shown by an arrow. Reproduced from Watson et al (1987).



10.2 BINDING ASSAY

Binding studies were performed on intact cells which were grown as described above. Briefly, the cells were grown in Corning 75 cm^2 or 150 cm^2 flasks in culture medium consisting of DMEM and Ham's F-12 (ratio 1:1) containing 10% FBS and sodium bicarbonate 3.7 g/l, in a humidified atmosphere containing 5% CO_2 in air. Culture media were changed at 48-hour intervals. When the cells reached confluence, they were dispersed and incubated in Corning or Nunc 12-well plates which had been coated with poly-D-lysine (2.5 $\mu g/cm^2$; MW 70,000-150,000) and laminin (0.25 $\mu g/cm^2$). Cell density at seeding was $2X10^6$ cells/well in 2 ml culture medium. Culture media were changed at 48-hour intervals. Drugs were dissolved in buffer consisting of DMEM with 25 mM HEPES and 0.5 mM sodium ascorbate, pH 7.4. After four days in culture, the cells were washed twice with buffer at 25^0C then incubated at 37^0C or at 0^0C for 60 min in the presence of the radioligand (eg, [^{3}H]prazosin $2x10^{-10}$ M) and unlabelled compounds in the indicated concentrations. At the end of the incubation period, the buffer was removed and the culture plates were placed on ice. The cells were then washed twice with buffer at 0^0C; the washing procedure occupied less than 30 seconds. The buffer was then removed and the cells were solubilised with two ml of a warm solution of 0.1% sodium dodecyl sulphate and 0.1 M sodium hydroxide. Fifty microlitre aliquots were removed for protein assay and 10 ml of scintillation liquid was then added to the cell extract, mixed and radioactivity was measured in a scintillation spectrometer with an efficiency of 50%. Protein content was measured by the bicinchoninic acid modification of the biuret reaction (Smith et al, 1985) using albumin standards and reagents supplied by Pierce & Warriner.

The data are presented both as dpm radioligand/well, and as moles ligand (labelled and unlabelled)/mg protein, by accounting for the fall in specific activity of the radioligands consequent upon isotope dilution. Non-specific binding was defined as the amount of ligand which was bound in the presence of a specific antagonist. Specific binding was obtained by subtracting non-specific binding from total binding. Each experimental point was carried out in triplicate and each experiment was replicated at least once. The minimum number of estimations for each experimental point was therefore six. The data are expressed as the means ± S.E.M. Standard error bars are not shown where they are smaller than the sizes of the symbols.

Half-maximal inhibitory concentrations (IC_{50}) values were calculated from concentration-response curves. The affinity constant (K_{Dpraz}) of prazosin for α_1 adrenoceptor binding sites was calculated from plots of bound *vs* bound/free ligand, in which the gradient = $-1/K_D$ (Scatchard, 1949). The affinities of other ligands were calculated using the equation: $K_D = IC_{50}/(1 + [Praz]/K_{Dpraz})$, where [Praz] is the concentration of [^{3}H]prazosin (Cheng & Prusoff, 1973).

10.3 UPTAKE ASSAY

Uptake studies were performed on intact cells which were grown as described above in 12-well plates which had been coated with poly-D-lysine and laminin. Cell density at seeding was $2x10^6$ cells/well in 2 ml culture medium. Culture media were changed at 48-hour intervals. Drugs were dissolved in buffer consisting of DMEM with 25 mM HEPES and 0.5 mM sodium ascorbate, pH 7.4. After four days in culture, the cells were washed twice with buffer at 25^0C then incubated at 37^0C for the indicated times in the presence of a radioligand and unlabelled compounds in the indicated concentrations. At the end of the incubation period, the culture plates were placed on ice and the buffer was removed. The cells were then washed twice with buffer at 0^0C; the washing procedure occupied less than 30 seconds. The buffer was then removed and the cells were solubilised with two ml of a warm solution of 0.1% sodium dodecyl sulphate and 0.1 M sodium hydroxide. Fifty microlitre aliquots were removed for protein assay and 10 ml of scintillation liquid was then added to the cell extract, mixed and radioactivity was measured in a scintillation spectrometer.

Compounds were tested for the ability to compete with prazosin (inhibition of Transport-P) and for the ability to activate Transport-P, as follows:

1. Competitive inhibition of the uptake of prazosin:

Compounds were tested for the ability to inhibit the uptake of prazosin 10^{-6} M in GT1-1 GnRH peptidergic neurones. The cells were incubated for 60 minutes at 37^0C in the presence of a tracer dose of [^{3}H]prazosin (eg, $2x10^{-10}$ M) and unlabelled prazosin 10^{-6} M; this concentration of prazosin activates Transport-P. Each experiment included a positive control (the antidepressant desipramine 10^{-4} M). Non-specific uptake was defined as uptake

in the presence of desipramine 10^{-4} M and specific (desipramine-sensitive) uptake was obtained by subtracting non-specific from total uptake. Efficacy was defined as % inhibition of the uptake of prazosin at 10^{-6} M when the test compound was used in a concentration of 10^{-4} M. Efficacy was expressed as % of the effect of a maximal inhibitory concentration of desipramine (10^{-4} M). Half-maximal inhibitory concentrations (IC_{50} values) were calculated from the concentration-response curves. IC_{50} values were calculated only for compounds which achieved a maximal inhibitory response, defined as 90% of the inhibitory effect of desipramine 10^{-4} M. When a compound did not achieve the maximal inhibitory response, IC_{50} values were not calculated and the data were expressed only as efficacy (% inhibition relative to desipramine 10^{-4} M). Relative potencies were calculated as follows:
Relative potency = (IC_{50} drug A/IC_{50} drug B) - 1.
It is necessary to subtract '1' from the quotient to take account of the fact that equipotent compounds have equal IC_{50} values.

Each experimental point was carried out in triplicate and each experiment was carried out at least twice; the minimum number of estimations for each experimental point was therefore six. The data are expressed as the means ± S.E.M. Standard error bars are not shown where they are smaller than the sizes of the symbols.

A representative member of each group of compounds was tested for its ability to inhibit the uptake of prazosin competitively. This was done by examining the effects of different concentrations of the test compound in the presence of different concentrations of unlabelled prazosin, while the concentration of [^{3}H]prazosin was kept constant.

In the initial studies, the concentration of [^{3}H]prazosin was approximately $2x10^{-9}$ M. In subsequent experiments, the concentration of the radioligand was reduced to approximately $2x10^{-10}$ M. This change reduced non-specific uptake of [^{3}H]prazosin and drug affinities were therefore generally higher than in the earlier studies.

The test compounds were dissolved in water, uptake/binding buffer or dimethylsulphoxide (DMSO). When DMSO was used as solvent, it was also present in all solutions to 1:1000.

In six consecutive experiments which compared DMSO to water as solvent, DMSO had no qualitative or quantitative effect on the results; specific uptake with DMSO was 192 ± 12 pmoles/mg protein, representing 79 ± 1% of total uptake; in the absence of DMSO, specific uptake was 186 ± 7 pmoles/mg protein, representing 79 ± 1% of total uptake.

The conclusions of these experiments are based on the potencies of compounds at Transport-P, as indicated by inhibition of the uptake or prazosin. Inhibition of uptake does not necessarily indicate that these compounds are themselves internalised by the uptake process; that would require direct measurement of the accumulation of labelled compounds in the cells. Further studies therefore examined the accumulation of labelled compounds which possess the structural properties which enable interaction with Transport-P, as identified in these studies. Such labelled compounds included [^{3}H]verapamil and BODIPY FL prazosin.

2. Activation of Transport-P:

The compounds were tested for their ability to increase the uptake of a tracer dose of [^{3}H]prazosin in the peptidergic neurones, as can be seen in the lower panel of Figure 11.1.1. The cells were incubated in the presence of [^{3}H]prazosin $2x10^{-10}$ M and concentrations of the test compounds, ranging from 10^{-9} M to 10^{-4} M. Each experiment included a positive control (unlabelled prazosin 10^{-6} M). Basal uptake was defined as the amount of [^{3}H]prazosin accumulated by the cells in the presence of [^{3}H]prazosin $2x10^{-10}$ M. For the purpose of these experiments, activation of Transport-P was defined as the increase in the uptake of [^{3}H]prazosin which is caused by unlabelled prazosin 10^{-6} M; the value for activation of Transport-P was therefore obtained by subtracting basal uptake from uptake in the presence of unlabelled prazosin 10^{-6} M. The increase in uptake of [^{3}H]prazosin was expressed as % of the basal value. Each experimental point was carried out in triplicate and each experiment was carried out twice.

The compounds were dissolved in water, uptake/binding buffer or DMSO. When DMSO was used as solvent, it was present in all solutions to 1:1000. In six consecutive experiments, DMSO had no effect on the accumulation of [^{3}H]prazosin when the cells were exposed to this radioligand at a concentration of $2x10^{-10}$ M (DMSO: 26.9 ± 1.8 fmoles/mg

protein; water: 26.5 ± 1.9 fmoles/mg protein). However, DMSO diminished the activation of Transport-P which was caused by unlabelled prazosin 10^{-6} M. In six consecutive experiments, the increase in uptake in the presence of DMSO was 19.8 ± 2.7 fmoles/mg protein, representing 76 ± 12% increase above basal; in the absence of DMSO, the increase in uptake was 34.3 ± 4.0 fmoles/mg protein, representing 132 ± 16% increase above basal. Despite this effect of DMSO, there were no qualitative differences in the experiments, and the conclusions were the same, regardless of whether DMSO was included or not. In view of the diminution of the effect of prazosin by DMSO, the data from experiments in which DMSO was used are not included in the analysis.

10.4 RELEASE ASSAY

GT1-1 GnRH neuronal cells were cultured as described above and incubated in Corning or Nunc 12-well plates ($2x10^6$ cells/well) which had been coated with poly-D-lysine and laminin. Release studies were performed on intact cells. Drugs were dissolved in the same uptake/binding buffer (see above). After four days in culture, the cells were washed twice with buffer at 25^0C then incubated at 37^0C for 60 minutes in the presence of [^{3}H]prazosin $2x10^{-9}$ M and unlabelled prazosin 10^{-6} M. Accumulation of prazosin reaches equilibrium within 60 minutes. At the end of the incubation period, the buffer was removed and the '0 minute' wells were washed twice with buffer at 25^0C. Washing the '0 minute' wells occupied less than 30 seconds. The remaining wells were washed once with buffer at 25^0C and incubated in uptake buffer with or without the indicated compounds. After various time intervals, the buffer was removed and the cells were solubilised with two ml of a warm solution of 0.1% sodium dodecyl sulphate and 0.1 M sodium hydroxide. Fifty microlitre aliquots were removed for protein assay and 10 ml of scintillation liquid was then added to the cell extract, mixed and radioactivity was measured in a scintillation spectrometer.

Initial studies indicated that desipramine does not inhibit amine release from GnRH cells; subsequent release studies were therefore carried out in the presence of desipramine 10^{-5} M to prevent re-uptake of released prazosin from the incubation medium.

Release of prazosin from GnRH cells followed an exponential curve which fitted the equation $y=k10^{ct}$ where y is the specific cellular content of prazosin at time t, k is the

specific cellular content of prazosin at time 0 minutes, c is the release constant ($seconds^{-1}$) and t is time. The release constant c is also equal to the gradient of the regression trendline in the log-linear plots. The half-life of retained cellular prazosin was calculated from these log-linear plots which were obtained using an exponential curve-fitting function in Microsoft Excel on a Macintsoh computer. The reliability of a curve fit was assessed using the R^2 value.

The effect of temperature on the release of prazosin was analysed using both linear and Arrhenius plots. In the linear plots, the temperature quotient (Q_{10}) was defined according to Price & Stevens (1989) as the ratio of the release constant at (T+10K)/TK where T is 300K (27^0C). Q_{10} is also given by the expression $e^{Ea/75000}$ where "e" is the natural logarithm (2.71828) and "Ea" is the activation energy in $kJmol^{-1}$ (Price & Stevens, 1989). The activation energy was calculated from the Arrhenius plot in which the inverse of absolute temperature is plotted against the natural logarithm of the release constant (Cornish-Bowden, 1995). In such a plot, the gradient is given in the expression -Ea/R where "R" is the gas constant (8.31451 $Jmol^{-1}K^{-1}$).

The data are presented as cellular content of prazosin (labelled and unlabelled) by accounting for the fall in specific activity of [^{3}H]prazosin consequent upon mixing with unlabelled prazosin, and expressed as pmoles prazosin/mg protein. These units were omitted when data were log-transformed in the exponential plots. Each experimental point was carried out in triplicate and each experiment was replicated at least once. The minimum number of estimations for each experimental point was therefore six. The data are expressed as the means ± S.E.M. Standard error bars are not shown where they are smaller than the sizes of the symbols. Statistical comparisons were by analysis of variance (ANOVA; single factor) which was performed using a function on Microsoft Excel. P values less than 0.05 were considered significant.

10.5 SPECTROPHOTOFLUORIMETRY

Compounds were dissolved in Krebs-Ringer-HEPES buffer (KRH buffer) consisting of NaCl 125 mM, KCl 4.8 mM, $MgCl_2.6H_2O$ 0.5 mM, Na_2HPO_4 0.7 mM, NaH_2PO_4 1.5 mM, $CaCl_2.2H_2O$ 2.5 mM, glucose 10 mM and HEPES 23 mM (pH 7.4). Fluorescence was examined in acrylic cuvettes in an SLM Aminco 8000 spectrophotofluorimeter. Excitation and emission polarisers were both set horizontal to suppress scattered light and slits were 8 nm wide. The excitation and emission spectra of test compounds were examined at wavelengths which are indicated in the relevant Figures. Fluorescence of test compounds was measured relative to a rhodamine standard.

Cells were cultured as described above, dispersed in culture medium to a density of 10^6 cells/ml and grown at a density of $4x10^5$ cells/cm^2 on glass coverslips which had been coated with poly-D-lysine (5 μg/cm^2; MW 70,000-150,000) and laminin (0.5 μg/cm^2). Studies were carried out after two days in culture, when the cells had become confluent. The cell-covered coverslips were washed in KRH buffer, then placed in a cuvette containing 3 ml KRH buffer, at an angle of 45^0 to the incident light, such that specular reflection would be diverted away from the detector. Autofluorescence was then measured, with an excitation wavelength of 450 nm. This non-peak wavelength was chosen to reduce further interference from light scattered over the cell-covered coverslips. Excitation and emission polarisers were horizontal and slits were 8 nm wide. The coverslips were then placed in a cuvette containing 3 ml of the fluorescent compound, (usually BODIPY FL prazosin), with or without the relevant unlabelled compounds and incubated at 37^0C for 60 minutes. The coverslips were then rinsed in 3 ml KRH buffer, placed in another cuvette containing 3 ml fresh KRH buffer and fluorescence was measured as described above.

10.6 FLUORESCENCE MICROSCOPY

Hypothalamic cells from fetal rats were dispersed in culture medium containing 10% FBS as described above, at a density of 250,000 cells/ml. The cells were then incubated at a density of 125,000 cells/cm^2 in glass chamber slides coated with poly-D-lysine (5 μg/cm^2) and laminin (0.5 μg/cm^2). GT1-1 cells were grown in culture medium containing 10% FBS or in serum-free medium consisting of DMEM and F12 (ratio 1:1), insulin 5 mg/l, progesterone

6.2 μg/l, selenium 5.1 μg/l, putrescine 16.1 mg/l, arachidonic acid 1 mg/l, docosahexanoic acid 0.5 mg/l, bovine transferrin 100 mg/l, sodium bicarbonate 3.7 g/l and gentamicin 100 mg/l. The cells were dispersed at a density of 100,000 cells/ml then incubated at a density of 25,000 cells/cm^2 in glass chamber slides coated with poly-D-lysine and laminin.

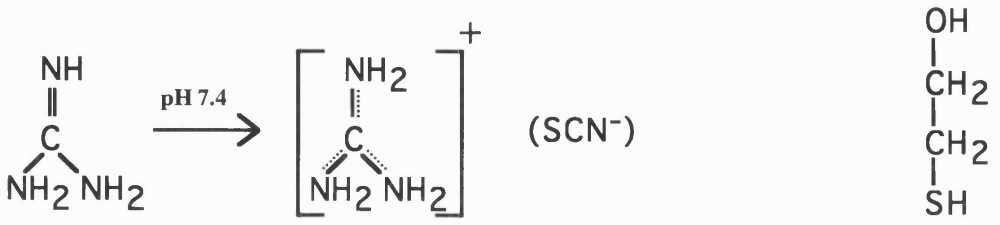
Uptake of fluorescent compounds was studied after 2-3 days in culture. The cells were washed twice with 2 ml of KRH buffer at room temperature, then incubated at 37^0C for 60 minutes in the presence of the fluorescent compound BODIPY FL prazosin $1.77x10^{-7}$ M, with or without unlabelled compounds in the indicated concentrations in KRH buffer. The cell-covered chamber slides were then placed on ice and the incubation medium was aspirated. The chambers and gaskets were removed and the slides were washed in ice-cold KRH buffer. The cells were then fixed in ice cold 3.7% formalin (pH 7.0) for 15 minutes. The cell-covered slides were then dried in a stream of warm air and mounting medium (light, white, mineral oil) was added followed by a glass cover slip. Fluorescence was examined with a Nikon Microphot-FX or a Nikon Eclipse E800 fluorescence microscope with a 470-490 nm excitation filter, 520-560 nm emission filter and a 510 nm dichroic mirror.

To study the release of BODIPY FL prazosin from immortalised GnRH neurones, the cells were washed twice with KRH buffer then incubated at 37^0C for 60 minutes in the presence of BODIPY FL prazosin $1.77x10^{-7}$ M in KRH buffer. The cells were then washed twice with KRH buffer and incubated in the presence of desipramine 10^{-5} M, with or without the indicated compounds in KRH buffer for 15 minutes. The purpose of the desipramine was to inhibit the re-uptake of BODIPY FL prazosin which had been released into the medium. The cell-covered chamber slides were then placed on ice and the incubation medium was aspirated. The chambers and gaskets were removed and the slides were washed in ice-cold KRH buffer. The cells were then fixed as described above and examined with the fluorescence microscope.

10.7 EXTRACTION OF TOTAL CELLULAR RNA FROM GnRH NEURONS

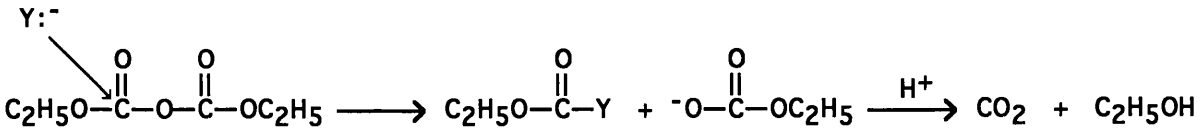
Preparation of total cellular RNA was based on previously described procedures with minor modifications (Glisin, 1974; Chirgwin et al, 1979; Okayama et al, 1987). The preparation of RNA is hindered by the widespread presence of ribonucleases (RNAse). RNAse is a protein consisting of a single chain of 124 amino acids whose tertiary structure is determined by four disulphide bonds and the hydrogen-bonded hydroxyl groups of tyrosine residues (Sela et al, 1957). Within cells, ribonucleases appear to be sequestered in compartments such as secretory granules; their hydrolytic activity is therefore not evident in intact cells. However, when cells are disrupted, the hydrolytic RNAse activity results in rapid degradation of cellular RNA molecules. RNA degradation can be inhibited by using 'chaotropic' agents which denature all cellular proteins, including RNAse, by disrupting hydrogen bonds and disulphide bridges. The unfolding and loss of three-dimensional structure results in loss of the enzymatic activity of these proteins. Anfinsen and his colleagues at the NIH discovered that this process is reversible, such that when the chaotropic agents are removed, enzymatic activity is recoverable (Sela et al, 1957). In the present method, the chaotropic agents were guanidinium thiocyanate and 2-mercaptoethanol. The guanidinium ion is the most effective of a large series of chaotropic agents; in guanidinium thiocyanate, both the anion and the cation are strongly chaotropic (Jencks, 1969). The purpose of 2-mercaptoethanol is to reduce the disulphide bonds which are essential for the function of RNAse (Sela et al, 1957). The method which was used in the present studies was comprised of three steps:

1. Cell membranes were disrupted with the detergent lauryl sarcosine and protiens were inactivated simultaneously with the chaotropic agents.
2. The RNA was separated from cellular protein by centrifugation through a caesium trifluoroacetate cushion, utilising the greater density of RNA in comparison to other cellular macromolecules.
3. Salts were removed by washing the RNA pellet repeatedly with 70% ethanol.



Guanidine **Guanidinium (thiocyanate)** **2-mercaptoethanol**

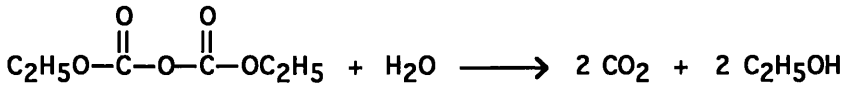
Water was treated with diethyl pyrocarbonate (DEPC; 0.2%) then boiled thoroughly to remove traces of the reagent. DEPC was synthesised originally at Bayer and was intended as a cold steriliser for beverages. The compound has nuclease inhibiting properties and in 1966, Fedorcsak & Ehrenberg suggested that it could be used for extraction of undegraded nucleic acids (see Ehrenberg et al, 1974). This compound takes part in reactions which are based on attack by a nucleophile, Y^-, on the carbonyl group in DEPC, as follows:



DEPC **Carbethoxylated product**

There are three important types of reactions in which DEPC takes part:

1. DEPC reacts with water to form ethanol and this is the basis of its use as a steriliser for beverages.



2. DEPC inactivates enzymes by reacting with the nucleophilic residues in the proteins. In the case of RNAse, inactivation occurs by reaction of DEPC with the histidine residues of this enzyme (Ehrenberg et al, 1974).

3. In high concentrations, DEPC may react slowly with nucleic acids. DEPC reacts only with single-stranded nucleic acids; its reaction with double-stranded nucleic acids is prevented by the fact that the reactive groups of nucleic acids are protected within the double helix. The reaction of DEPC with RNA requires much greater concentrations of DEPC and proceeds much more slowly than the reaction with RNAse. It is therefore possible to find conditions in which RNAse is inactivated without denaturing the RNA (Ehrenberg et al, 1974).

In the experiments described below, DEPC-treated water was used in all procedures which involved the preparation of RNA. The advantage of this method is that complete inactivation of RNAse in the water is accompanied by rapid hydrolysis of DEPC; the small amounts of ethanol and CO_2 which are generated are boiled off and have no deleterious effects in any case (Ehrenberg et al, 1974).

Glassware was heated to 180^{0}C for 16 hours to denature RNAse irreversibly. Sterile gloves were used throughout and care was taken not to contaminate the materials and apparatus with RNAse from the skin. Sterile pipette barrier tips and sterile plastic consumables were used whenever possible.

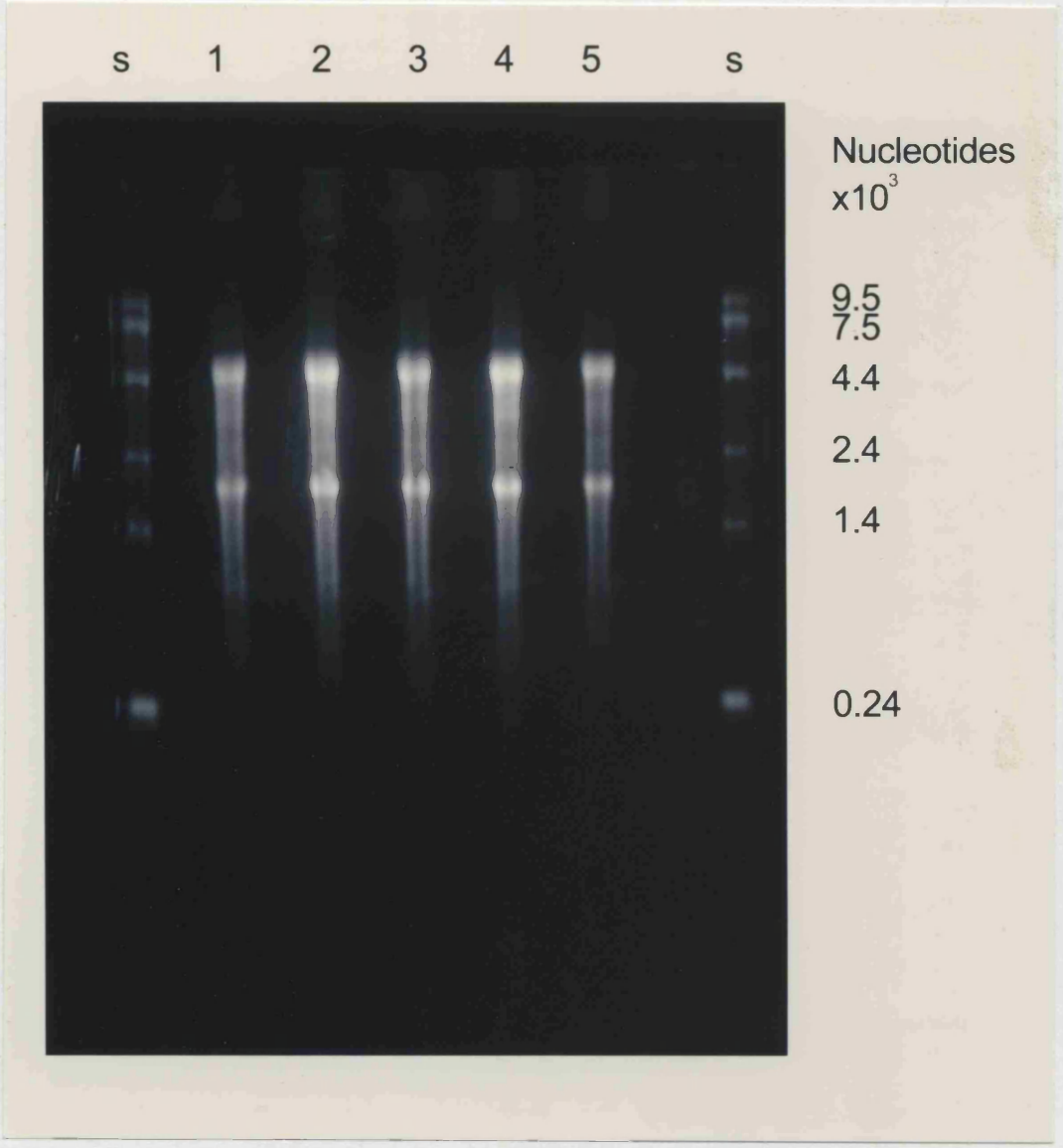
GT1-1 GnRH cells were grown in Falcon 150 mm dishes ($3x10^7$ cells/dish) and media were changed at 48 hour intervals. After ten days, the cells were washed with PBS then disrupted by adding to each dish 5 ml of 'GTC solution' (5.5 M guanidinium thiocyanate, 25 mM sodium citrate and 0.5% lauryl sarcosine, pH 7.0) with 0.2 M 2-mercaptoethanol in DEPC-treated water. The viscous cell lysate was scraped with a cell lifter and transferred to a sterile 50 ml tube using a 10 ml pipette. The cell lysate was sheared first with a Polytron homogeniser, then further sheared by passing twice through an 18-guage needle attached to a 50 ml syringe. The procedure was repeated by passing the cell lysate through needles of 20-guage then 23-guage. The cell lysate was then overlayed on 17 ml of a caesium trifluoroacetate cushion (Pharmacia; density 1.51 g/ml) in a Beckman centrifuge tube. The tubes were centrifuged at 25,000 rpm for 26 hr at 15^{0}C. The supernatant and the interface containing the cellular DNA and protein were aspirated and discarded. DEPC-treated water was added above the cushion to wash any remaining protein or DNA. The caesium cushion was removed with a sterile Pasteur pipette, leaving the RNA pellet in the tube. The bottom of the tube was cut off with a hot, sterile blade and the top of the tube was discarded. The pellet was redissolved in diluted 'GTC solution' (4 M) and transferred to a sterile 2 ml tube. The tube was centrifuged and the supernatant was transferred to a fresh tube; the pellet was discarded. The RNA was precipitated by adding 20 µl of 1 M acetic acid and 600 µl of ethanol. The tube was kept overnight at -20^{0}C and the RNA precipitate was then pelleted by centrifugation and washed with 70% ethanol. Two more precipitation cycles with NaCl (final 0.2 M) and ethanol were carried out to remove any remaining salts and the RNA was dissolved in TE buffer. The concentration of RNA was measured by absorption spectrometry at 260 nm. The RNA was stored at -80^{0}C as a salt and ethanol precipitate.

The quality of the total cellular RNA was tested in denaturing agarose gels followed by visualisation of ribosomal RNA bands in UV light. Formaldehyde and formamide were used

to disrupt the secondary structure of RNA, in order to enable assessment of the size of RNA molecules from the distance migrated by the linear molecules in a gel. Precautions regarding contamination with RNAse were as described above. The gel box, tray and comb were washed with detergent, dried with ethanol then soaked in 3% hydrogen peroxide for 10 minutes. They were then rinsed thoroughly in DEPC-treated water. Agarose was melted to a concentration of 0.8% or 1.2% in electrophoresis buffer consisting of 0.02 M MOPS (3-[N-morpholino]-propanesulphonic acid), 0.005 M sodium acetate, 0.001 M EDTA and 2.2 M formaldehyde (pH 7.0). RNA was diluted 1:3 in denaturing buffer consisting of 50% formamide and 2.2 M formaldehyde in electrophoresis buffer. The diluted RNA sample was heated to 70^0C for 10 minutes to denature then cooled on ice to remove kinetic energy which could enable reconstitution of the secondary structure. The fluorescent dye ethidium bromide (final 0.5 μg/μl) was added to the samples which were then placed in wells of appropriate sizes in the gel. RNA markers of known sizes were purchased commercially and were treated in the same manner as the RNA samples. The gel was electrophoresed at 40 V overnight, then viewed and photographed under ultraviolet light (Figure 10.7.1). Ribosomal RNA constitutes approximately 75% of total cellular RNA and consists mostly of the 28s rRNA (approximately 5000 nucleotides) and the 18s rRNA (approximately 2000 nucleotides) which constitute the final ribosome (Alberts et al, 1989). Presence of these ribosomal bands indicates that the RNA in this preparation is not degraded.

Figure 10.7.1:

The quality of the total cellular RNA was tested in denaturing agarose gels followed by visualisation of ribosomal RNA (rRNA) bands in UV light. Formaldehyde and formamide were used to disrupt the secondary structure of RNA, in order to enable assessment of the size of RNA molecules from the distance migrated by the linear molecules in a gel. Ribosomal RNA constitutes approximately 75% of total cellular RNA and consists mostly of the 28s rRNA (approximately 5000 nucleotides) and the 18s rRNA (approximately 2000 nucleotides) which constitute the final ribosome (Alberts et al, 1989). Presence of these ribosomal bands indicates that the RNA in this preparation is not degraded.



10.8 PREPARATION OF POLY(A)RNA

Messenger RNA (mRNA) constitutes approximately 3% of total cellular RNA, the majority of which is comprised of ribosomal RNA and transfer RNA (Alberts et al, 1989). The aim of this procedure was to isolate mRNA, utilising the fact that mature mammalian mRNA molecules possess a terminal chain of adenosine residues. In the presence of high concentrations of sodium, poly(A)RNA anneals to oligo(dT) which is bound to cellulose. This hybridisation procedure cannot take place in the absence of the cation, because of the strong repulsion between the negatively charged phosphate residues in the backbones of the hybridising molecules; sodium ions neutralise the negative charges and enable hybridisation to take place. Thus, total cellular RNA is added to the oligo(dT)-cellulose in the presence of a high concentration of sodium and the poly(A)RNA is then recovered from the column in a low sodium wash. This method was based on published work (Aviv & Leder, 1972).

Pre-packed oligo(dT)-cellulose columns were purchased commercially and rehydrated with TE buffer. The RNA was heated to 70^0C for 5 minutes to denature then cooled rapidly on ice. Sodium chloride was added to a final concentration of 0.5 M and the sample was applied immediately to the column. The effluent was collected by gravity flow and re-applied to the column three times. The column was then washed with buffer consisting of TE solution with 0.5 M NaCl, in order to remove molecules which had not hybridised to the oligo(dT)-cellulose. The RNA was eluted from the column with TE solution in the absence of sodium. The RNA was stored at -80^0C as a salt and ethanol precipitate.

10.9 TESTING THE RNA IN *XENOPUS* OOCYTES

The quality of the poly(A)RNA was tested by injecting it into *Xenopus laevis* oocytes. Immortalised GT1-1 GnRH neurones express autoreceptors for GnRH (Krsmanovic et al, 1993). Poly(A)RNA from the GT1-1 cells was injected into the oocytes which were later tested for expression of the GnRH autoreceptors, as indicated by mobilisation of intracellular calcium upon exposure to GnRH or one of its analogues. Mobilisation of intracellular calcium was detected using the calcium-binding photoprotein aequorin. Expression of GnRH autoreceptors was taken to indicate the presence of full-sized, undegraded mRNA molecules.

The use of frog oocytes for the study of mRNA and its protein products was introduced by Gurdon et al (1971) in Oxford. In the South African frog *Xenopus laevis,* oogenesis is a continuous, asynchronous process and oocytes in all stages of development are present in

the ovary at all times during adult life (Dumont, 1972). As the oocyte matures, it develops a dark brown "animal" hemisphere and a light "vegetal" hemisphere in which yolk is stored. The nucleus is eccentrically situated near the animal pole (Dumont, 1972). A mature *Xenopus* oocyte is a giant single cell of approximately 1 mm in diameter. It is surrounded by several thousand small follicle cells. Before an oocyte is injected, it is important to remove these follicular cells, which may otherwise affect the composition and synthesis of ovarian material (Gurdon & Wickens, 1983). The oocyte synthesises RNA and translates it to protein but is totally inactive in DNA synthesis (Gurdon & Wickens, 1983). The oocytes are meiotioc cells which accumulate a vast store of enzymes, organelles and other precursors for use during the early development of the fertilised egg (Melton, 1987). In the original work, oocytes were injected with rabbit RNA for haemoglobin and it was found that the oocytes synthesised haemoglobin at the same rate as they synthesised their endogenous proteins for at least 24 hours. Haemoglobin RNA was translated far more efficiently in oocytes than in cell free systems (Gurdon et al, 1971). Subsequent studies indicated that injected mRNAs compete with endogenous oocyte mRNA for the translation mechanisms; when 100 ng of globin mRNA is injected into an oocyte, approximately 50% of the newly synthesised oocyte protein is globin (Melton, 1987). The proteins which are translated from injected mRNA are correctly modified by post-translational processes, including cleavage of precursor proteins, phosphorylation and glycosylation, and the proteins are then either directed into the appropriate cellular membranes or secreted appropriately from the cells. Further, oocytes injected with mixtures of mRNAs assembled multi-subunit proteins into functional complexes (Melton, 1987).

In the original work, it was predicted that oocytes could be used to identify the unknown mRNA for a known protein, by separating cellular RNA into different fractions, injecting these fractions into oocytes and assaying for the synthesis of the protein (Gurdon et al, 1971). Indeed, this principle was subsequently applied to identify the RNAs encoding many proteins in mammalian cells.

Bioluminescence is the phenomenon in which visible light is emitted by an organism. Several marine creatures exhibit bioluminescence which is caused by calcium-binding photoproteins. Aequorin was obtained from the coelenterate jellyfish *Aequorea* which is native to the Pacific in British Columbia and Washington. Aequorin is a self-contained bioluminescent system in which all the organic components which are required for

luminescence are contained within a single protein. It emits blue light at a rate that is highly sensitive to the concentration of free calcium ions, independently of cofactors such as ATP, oxygen or NADH which influence other bioluminescent reactions. Although aequorin emits blue light, it occurs in many coelenterates together with green fluorescent protein, which gives these creatures a green colour. The green fluorescent protein is readily separated from aequorin and should not be detectable in the same preparation (Blinks et al, 1982). Aequorin cDNAs have been isolated and the crystal structure of the molecule was reported recently (Head et al, 2000). The aequorin molecule contains four helix-loop-helix domains, three of which can bind calcium. The molecule also contains coelenterazine as its chromophoric ligand; binding of calcium results in decomposition of the protein into apoaequorin, coelenteramide and CO_2, accompanied by emission of light. The reaction is reversible, as aequorin can be regenerated in the absence of calcium, by incubation with coelenterazine, oxygen and a thiol agent (Head et al, 2000). The jelly fish regulates its bioluminescence by neural control of calcium release and sequestration in aequorin-containing cells (Prendergast, 2000).

The procedure which was used in these studies was based on published methods which had used the emission of light by aequorin to detect mobilisation of intracellular calcium upon exposure of oocytes to agonists (Sandberg et al, 1988). Briefly, female *Xenopus* frogs were washed upon arrival with potassium permanganate 80 mg/litre of dechlorinated, de-ionised water for 20 minutes. After that, four frogs were kept in each water tank, and they were washed with potassium permanganate for an hour once a month thereafter. Partial oophorectomy can be performed at intervals of three weeks, approximately three times on each ovary. On the day of the procedure, a frog was placed in a bucket of anaesthetic (3-aminobenzoic acid ethyl ester, 0.1%) and then removed after approximately 10 minutes. The frog was then placed supine on ice and a horizontal paramedian incision was made in the skin and in the abdominal musculature. The ovary then became evident and was pulled out with forceps. A piece of ovary was cut with scissors and placed in a Petri dish containing ice-cold OR-2 medium (NaCl 82.5 mM, KCl 2.5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, HEPES 5 mM, Na_2HPO_4 1 mM, supplemented with penicillin, streptomycin and gentamicin 100 mg/l). The cut end of the ovary was ligated to prevent bleeding and three sutures were placed in each of the muscle and skin layers. After recovery from the anaesthetic, the frog was returned to the water tank. The cut ovary was rinsed in OR-2 buffer and transferred to fresh buffer. It was then cut into large clumps which were placed in collagenase solution

(Collagenase A, Boehringer, 2 mg/ml in calcium-free OR-2 medium), in which it was incubated at room temperature for 30 minutes. The partially digested ovary was then rinsed five times with calcium-free OR-2 medium, using a Pasteur pipette. The oocyte clumps were then transferred to OR-2 medium in Petri dishes and the connective tissue was torn with forceps to expose the oocytes to the buffer. The oocytes were left in the buffer overnight at 16^0C. The following morning, the follicular cells were stripped from the oocytes using fine forceps under a dissecting microscope. The oocytes were then incubated at 16^0C overnight in fresh OR-2 medium.

Injection needles were R-6 glass tubing (outer diameter 0.63 mm, inner diameter 0.2 mm, length 11 cm; Drummond). The needles were prepared in a Flaming Brown micropipette puller (Sutter Instruments) and were then heated to 180^0C for 16 hours to denature RNAse irreversibly.

Poly(A)RNA (1 μg/μl) was heated to 65^0C to denature then cooled on ice. A small amount of RNA was placed on a piece of Parafilm or a baked glass slide and aspirated into an injection needle using an automated system. Oocytes were placed on a plastic mesh to prevent movement, and were nearly covered in OR-2 medium to prevent dehydration. The oocytes were injected into the vegetal (white) side of the equator, to avoid the nucleus. Injection volumes were 31 nl under pressure of approximately 30 psi, and were delivered within 1-2 seconds. Control oocytes were injected with water. The injected oocytes were placed in a glass Petri dish containing 45 ml of fresh OR-2 buffer and incubated at 16^0C. The buffer was changed at the end of the day and degraded oocytes were discarded. After that, the OR-2 buffer was changed daily. Pharmacological experiments were carried out three days after injection.

Expression of GnRH autoreceptors was examined by studying the mobilisation of intracellular calcium, using the calcium-binding photoprotein aequorin. On the day of the experiment, an injection needle was washed with 1 mM EDTA to remove traces of calcium which may consume the aequorin. The oocytes (which had been pre-injected with RNA or water) were then injected with 33 nl of aequorin (1 ng/μl in calcium-free water), transferred to Petri dishes containing fresh OR-2 medium and incubated at 16^0C for an hour. An oocyte

was then placed in a scintillation vial containing 0.5 ml OR-2 medium and the vial was transferred to a Beckman LS250 scintillation spectrometer in which the coincidence function was switched off, to detect the emission of light using a single channel. After a stabilisation period, the relevant compounds (GnRH or its analogues, with or without selective antagonists) were added and the time course of the effect of the drug on the emission of light was followed.

Figure 10.9.1 shows that in oocytes which had been injected with GT1-1 GnRH neurone poly(A)RNA, exposure to the GnRH analogue [D-Ala^6, des-Gly^{10}]-GnRH-ethylamide $2.5x10^{-7}$ M causes a rapid emission of light, indicating an increase in the intracellular concentration of calcium. In these RNA-injected oocytes, exposure to the vehicle ("medium") alone has no significant effect. As a further control, the GnRH analogue has no effect on the emission of light in water-injected oocytes. The increase in light emission which is caused by $2.5x10^{-7}$ M GnRH can be blocked by the GnRH antagonist [D-Phe^2, Pro^3, D-Phe^6]-GnRH ($2.5x10^{-7}$ M), confirming the specificity of the effect of GnRH (Figure 10.9.2). These findings indicated that the RNA-injected oocytes expressed functional GnRH receptors. The RNA preparation therefore contains full-sized RNA molecules which can be translated by the oocytes into functional protein.

Figure 10.9.1:

Xenopus oocytes were injected with GT1-1 GnRH neurone poly(A)RNA or water ("controls"). Three days later, they were injected with the calcium-binding photoprotein, aequorin. The oocytes were then exposed to the GnRH analogue [D-Ala^6, des-Gly^{10}]-GnRH-ethylamide or to the vehicle in which it had been dissolved ("medium"). The GnRH analogue causes a rapid emission of light, indicating an increase in the intracellular concentration of calcium. In these RNA-injected oocytes, exposure to the vehicle alone has no significant effect. As a further control, the GnRH analogue has no effect on the emission of light in water-injected oocytes. The findings indicate that the RNA-injected oocytes expressed functional GnRH receptors. The RNA preparation therefore contains full-sized RNA molecules which can be translated by the oocytes into functional protein.

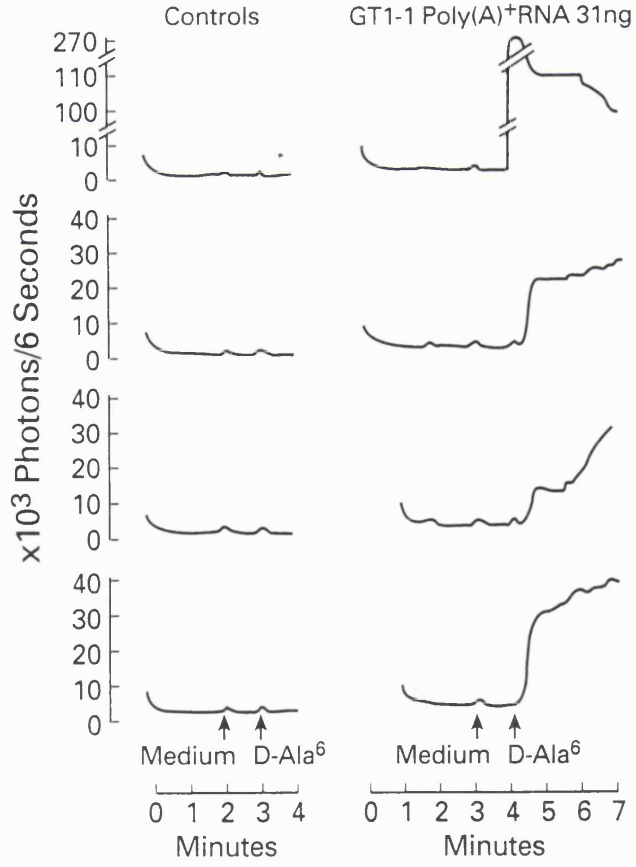
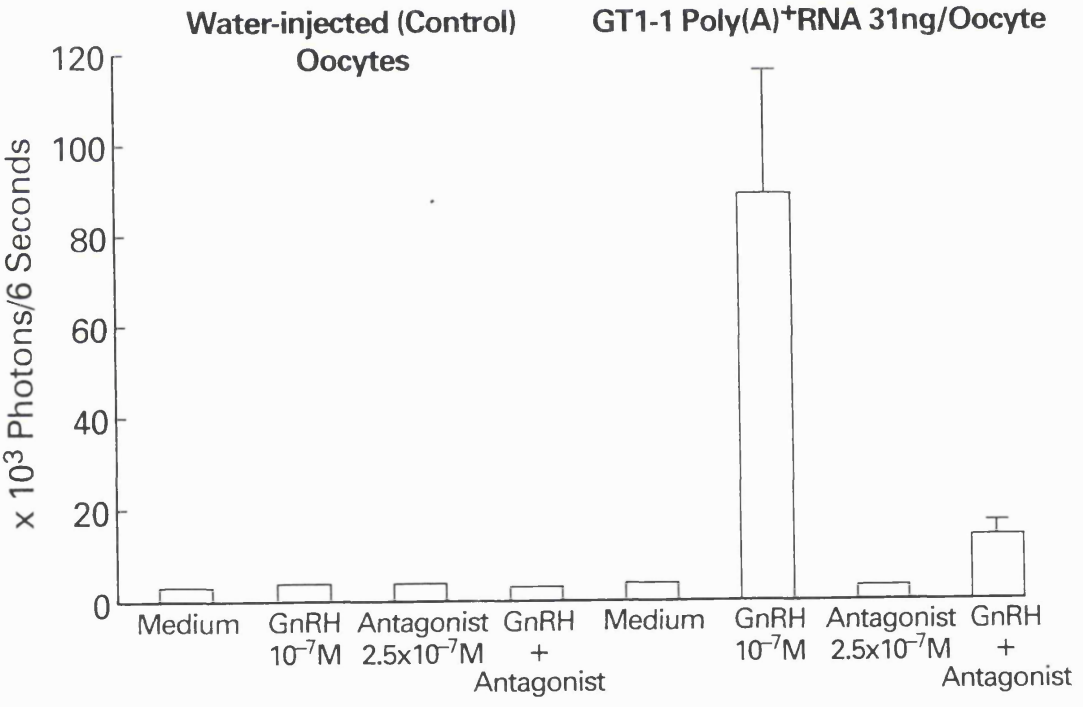


Figure 10.9.2:

Xenopus oocytes were injected with GT1-1 GnRH neurone poly(A)RNA or water (control). Three days later, they were injected with the calcium-binding photoprotein, aequorin. The oocytes were then exposed to GnRH, with or without the selective GnRH antagonist [D-Phe^2, Pro^3, D-Phe^6]-GnRH. The increase in light emission which is caused by GnRH can be blocked by the GnRH antagonist, confirming the specificity of the effect of GnRH. These findings confirm that the RNA-injected oocytes expressed functional GnRH receptors by translating full-sized RNA molecules which are present in the RNA preparation.



10.10 CONSTRUCTION AND ANALYSIS OF A cDNA LIBRARY FROM GnRH NEURONES

Construction of the cDNA library was based on the method of Gubler & Hoffman (1983) in which poly(A)RNA is transcribed into the first strand of cDNA using an oligo(dT) primer which anneals to the poly(A) tail; the RNA is then partly degraded with RNAse H and the resulting RNA fragments act as primers for synthesis of the second cDNA strand in the presence of DNA polymerase and DNA ligase which ensures continuity of the second cDNA strand. Small cDNA molecules are removed in order to increase the statistical chances of finding the cDNA of interest. An adapter is ligated to each end of the cDNA molecules and these are digested to reveal cohesive ends which are ligated to the plasmid vector. In the method used in this study, the sequence of the oligo(dT) primer contained a *Not* I restriction site which, following digestion with *Not* I, forms the 3' end of the cDNA molecules. The primer which is ligated to the 5' end of the cDNA contains a *Sal* I restriction site as part of a four-base extension. These asymmetrical restriction sites enable the construction of a "directional library" in which all the cDNA molecules are in the correct orientation in relation to the plasmid. This is particularly important in a mammalian expression plasmid, in order to ensure that the regulatory elements in the plasmid result in transcription of the cDNA in the correct orientation. The cDNA with cohesive ends is ligated to a plasmid which has been digested with *Not* I and *Sal* I to expose complementary sites; the recombinant DNA is then introduced into E coli.

The pSVSPORT1 plasmid is included in the GIBCO BRL Superscript kit (Figure 10.10.1). The plasmid contains the bacterial origin of replication from pUC vectors, the β-lactamase gene which confers ampicillin resistance and an extensive multiple cloning site. For expression in COS cells, it contains the SV40 origin of replication, early promoter and small t intron (Sections 10.1.2.1 & 10.1.2.4 and Figure 10.1.1). The plasmid also contains a poly(A) addition site which is believed to increase the stability of the transcripts. The multiple cloning site is flanked by SP6 and T7 promoters which allow *in vitro* transcription of either strand of the cDNA insert, depending on the choice of RNA polymerase. When used as part of the GIBCO BRL Superscript kit, there is a restriction site for *Mlu* I in the *Sal* I adapter (see above) at the 5' end of the cDNA and another *Mlu* I site in the plasmid at the other end of the multiple cloning site (position 450-455). Digestion with *Mlu* I therefore releases cDNA from plasmid DNA, enabling assessment of the size of the cDNA insert.

Using poly(A)RNA from the GnRH cells, a size-selected, directional cDNA library was constructed in the pSVSPORT1 vector, using the GIBCO BRL Superscript kit with modifications. Briefly, the first cDNA strand was synthesised using 5 μg poly(A)RNA and oligo(dT)-*Not* I primer-adapter (1 μg). The mixture was heated to 70^0C for 10 minutes to denature the RNA then cooled on ice to enable annealing of the primer to the poly(A) tail. Superscript II reverse transcriptase (1000 units) and its buffer were then added and the reaction was incubated at 37^0C for 60 minutes. The RNA was degraded and the second cDNA strand was synthesised by simultaneous addition of *E coli* RNAse H, *E coli* DNA polymerase and *E coli* DNA ligase, together with the appropriate buffer and nucleotide triphosphates. This reaction was incubated at 16^0C for two hours. The double-stranded cDNA was then extracted with phenol:chloroform:isoamylalcohol, precipitated with ammonium acetate and residual salts were removed with 70% ethanol. The cDNA was dried at 37^0C to remove remaining ethanol and the DNA was re-dissolved in DEPC-treated water. The *Sal* I/*Xho* I cohesive adapter was ligated to the 5' end of the cDNA and the oligo(dT)-*Not* I primer-adapter at the 3' end was digested with *Not* I to reveal the cohesive site.

In order to enrich the cDNA with molecules of larger size, the cDNA was electrophoresed in 0.8% agarose and DNA greater than 1000 bp was cut from the gel. This procedure also removes the excess amounts of adapters which would otherwise interfere in the ligation reaction. The DNA was extracted from the cut piece of gel using a Qiaex kit (Qiagen). This procedure is based on the method of Vogelstein & Gillespie (1979) in which agarose is solubilised with sodium iodide and the DNA is extracted with glass powder; in the Qiaex procedure, a proprietory solution is used to solubilise the agarose and the DNA is extracted with glass beads. The beads are then washed with 70% ethanol to remove salts and the DNA is recovered from the glass beads by heating the suspension to 50^0C in the presence of TE buffer.

The cDNA was then ligated to pSVSPORT1 which had been digested with *Not* I and *Sal* I. The ligation reaction consisted of 50 ng plasmid, cDNA (see below), DNA ligase buffer and T4 DNA ligase 1 Unit, in a reaction volume of 20 μl. The reaction was incubated at room temperature for three hours, according to the manufacturer's recommendations. The DNA was then precipitated with salt and ethanol, washed with 70% ethanol and re-dissolved in 5

µl TE buffer. Half the ligate (2.5 µl) was introduced into *E coli* strain MC 1061 (Bio-Rad) by electroporation using a Bio-Rad GenePulser set at the following parameters: 2.5 kV; 25 uF; 400 ohms. The electroporated bacteria were placed in SOC medium in a shaking incubator at 37^0C for one hour, then grown overnight on LB/ampicillin plates at 37^0C. The number of transformed bacteria was obtained by counting the number of colonies on the plates. The ratio of cDNA:plasmid in the ligation reaction was optimised to achieve the maximum number of transformants. The results were as follows:

cDNA	Plasmid	Transformants/ml	Recombinants/reaction
0.80 µl	5 ng	4800	9600
0.40 µl	5 ng	2400	4800
0.20 µl	5 ng	1100	2200
0.10 µl	5 ng	400	800
0.05 µl	5 ng	200	400
None	5 ng	None	
None	None	None	

From the above, it can be seen that:

1. Electroporation of bacteria in the absence of DNA results in no growth, indicating that antibiotic selection is adequate.
2. Transfection of bacteria by the cut plasmid without cDNA does not result in transformation, indicating that self-ligation of the plasmid does not take place.
3. Increasing the amount of cDNA in the ligation reaction results in increased recombinant molecules, as indicated by the number of transformed bacteria.

A mammalian cell contains about 300,000 RNA molecules (Alberts et al, 1989), so the library was designed to contain $2x10^6$ recombinants, in order to increase the statistical chances of finding the molecule of interest. A full-scale ligation reaction was carried out, using 50 ng plasmid and 8 µl cDNA, as described above in detail. The ligation was electroporated into bacteria as described above and this resulted in $2.1x10^6$ transformed bacteria, indicating that the library contained $2.1x10^6$ recombinants. The electroporated

bacteria were placed in SOC medium at 37^{0}C in a shaking incubator for one hour then transferred to a semi-solid medium (FMC Sea-Prep agarose 3 g in one litre of 2x LB medium with ampicillin 50 μg/ml) and amplified by overnight growth in a static incubator. The semi-solid medium ensures representative amplification of the clones; if amplification is carried out in liquid medium, the bacteria which contain recombinants with small cDNA inserts would grow at a faster rate, resulting in skewing of the library towards small cDNAs (Kriegler, 1991). The amplified bacteria were recovered from the semi-solid medium by centrifugation (Beckman J2-21 centrifuge) at 8000 rpm at 37^{0}C for 20 minutes, then re-suspended in 40 ml LB/ampicillin (50 μg/ml) medium. DMSO was added to 7% and the suspended bacteria were divided into aliquots which were stored in liquid nitrogen. Ten μl were removed from one of the aliquots, diluted serially in LB medium and grown overnight on LB/ampicillin plates. Colony counts revealed that the original 2.1x10^6 transformed bacteria had been amplified to 7.28x10^{10} bacteria (1.82x10^9 bacteria/ml).

Twenty three colonies were picked at random and grown overnight in LB/ampicillin in a shaking incubator at 37^{0}C. Plasmid DNA was extracted using Qiagen kits (see below), digested with *Mlu* I to separate the plasmid from the insert, then run in 0.8% agarose and cDNA insert sizes calculated. Twenty one of the twenty three colonies contained inserts (91%; Figure 10.10.2). Analysis of the 21 randomly selected clones indicated that average insert size was 1493 $\pm$ 213 bp (Figure 10.10.2). This is similar to the average size of cellular mRNA which is quoted as 1500 nucleotides (Alberts et al, 1989).

Extraction of DNA and further analysis of the library was carried out as follows: a frozen one ml aliquot of bacteria was thawed and diluted in one litre of 2x LB medium/agarose (semi-solid medium; see above). The resulting suspension was divided into 25 ml aliquots which were placed on ice to allow the agarose to harden, then transferred to a 37^{0}C static incubator. Following overnight growth, the aliquots were transferred to four 250 ml bottles which were centrifuged (Beckman J2-21 centrifuge) at 8000 rpm at 37^{0}C for 20 minutes. The supernatant was discarded and the bacteria were washed twice with 10 ml PBS. Plasmid DNA was extracted from the bacteria using a modified Qiagen Maxiprep kit which is based on the procedure of Birnboim & Doley (1979) and Birnboim (1983). The principle of the

method is that bacterial cell walls and plasma membranes are destroyed with the combination of sodium hydroxide and the detergent sodium dodecyl sulphate (SDS). The alkaline conditions denature the high molecular weight chromosomal DNA while the covalently closed circular (plasmid) DNA remains double-stranded. Upon neutralisation, chromosomal DNA and protein form an insoluble clot, leaving plasmid DNA in the supernatant. In the Qiagen procedure, the bacteria are suspended in buffer (50 mM Tris/10 mM EDTA) containing RNAse A which is intended to hydrolyse bacterial RNA. The bacteria are then lysed at room temperature by adding 0.2 M NaOH/1% SDS. Chromosomal DNA, polysaccharides and protein are precipitated with 3 M potassium acetate (pH 5.5) at 0^0C and the supernatant is recovered by centrifugation at 4^0C at 16,000 rpm for 45 minutes. Plasmid DNA is then purified by applying the supernatant to a proprietory anion exchange column. The column is washed with high salt buffer (1 M NaCl/50 mM MOPS/15% ethanol; pH 7) and plasmid DNA is then eluted into a centrifuge tube. An equal volume of isopropanol is added to precipitate the DNA in the presence of salt and the tube is centrifuged at 4^0C for 30 minutes at 16,000 rpm. The supernatant is discarded and the pellet is re-dissolved in TE solution. In a modification of the Qiagen procedure, the plasmid DNA was further purified with phenol/chloroform/isoamyl alcohol. The DNA was then recovered by precipitation with NaCl (0.2 M) and 50% ethanol at -80^0C for 10 minutes. The pellet was washed twice with 70% ethanol to remove residual salts. The ethanol was then evaporated at room temperature and the DNA was re-suspended in 100 µl TE solution (1 µg/µl) and left to dissolve at 4^0C overnight. DNA concentration was measured using the optical density at 260 nm and the samples were stored at -20^0C.

The plasmid DNA was extracted from a one ml aliquot of the library containing 1.82×10^9 amplified bacteria (see above). Assuming that recovery of plasmid DNA was nearly complete, the DNA (100 µl) which was extracted from these bacteria can be expected to contain approximately 1.82×10^9 amplified recombinants (1.82×10^7 amplified recombinants/µl). As the library originally contained 2.1×10^6 recombinants, 1 µg (1 µl) of plasmid DNA represents 8.7-fold the original number of recombinants in the library.

In order to assess the approximate size of the cDNA inserts in the plasmid preparation, 1 µg

of DNA was digested with *Mlu* I and fractionated in a 0.8% agarose gel (Figure 10.10.3). In lane 1, the digested plasmid is seen to be 3000 bp in size and the cDNA as a smear of 1000 to 5000 bp. The bulk of the cDNA is approximately 1600 bp (Figure 10.10.3). This is consistent with the result which was obtained by analysis of the randomly selected clones (average insert size 1493 ± 213 bp; Figure 10.10.2). This is also similar to the average size of cellular mRNA which is quoted as 1500 nucleotides (Alberts et al, 1989).

In summary, a size-selected, directional cDNA library was constructed from GT1-1 GnRH neurone poly(A)RNA in the pSVSPORT1 mammalian expression plasmid and placed in *E coli* strain MC 1061. The characteristics of the library are as follows:

1. The library consists of 2.1×10^6 transformed bacteria which have been amplified to 7.28×10^{10} bacteria (1.82×10^9 bacteria/ml).
2. The cDNA inserts are 1000 to 5000 bp.
3. Analysis of 23 randomly selected clones revealed that 91% contained inserts; average insert size is 1493 ± 213 bp.
4. Plasmid DNA prepared from the library contains 1.82×10^7 recombinants/µg.

Figure 10.10.1:

The pSVSPORT1 vector is included in the GIBCO BRL Superscript kit. The plasmid contains the bacterial origin of replication from pUC vectors, the β-lactamase gene which confers ampicillin resistance ("Ap") and an extensive multiple cloning site. For expression in COS mammalian cells, it contains the SV40 origin of replication, early promoter and small t intron (Sections 10.1.2.1 and 10.1.2.4). The plasmid also contains a poly(A) addition site which is believed to increase the stability of the transcripts. The multiple cloning site is flanked by SP6 and T7 promoters (not shown) which allow in vitro transcription of either strand of the cDNA insert, depending on the choice of RNA polymerase.

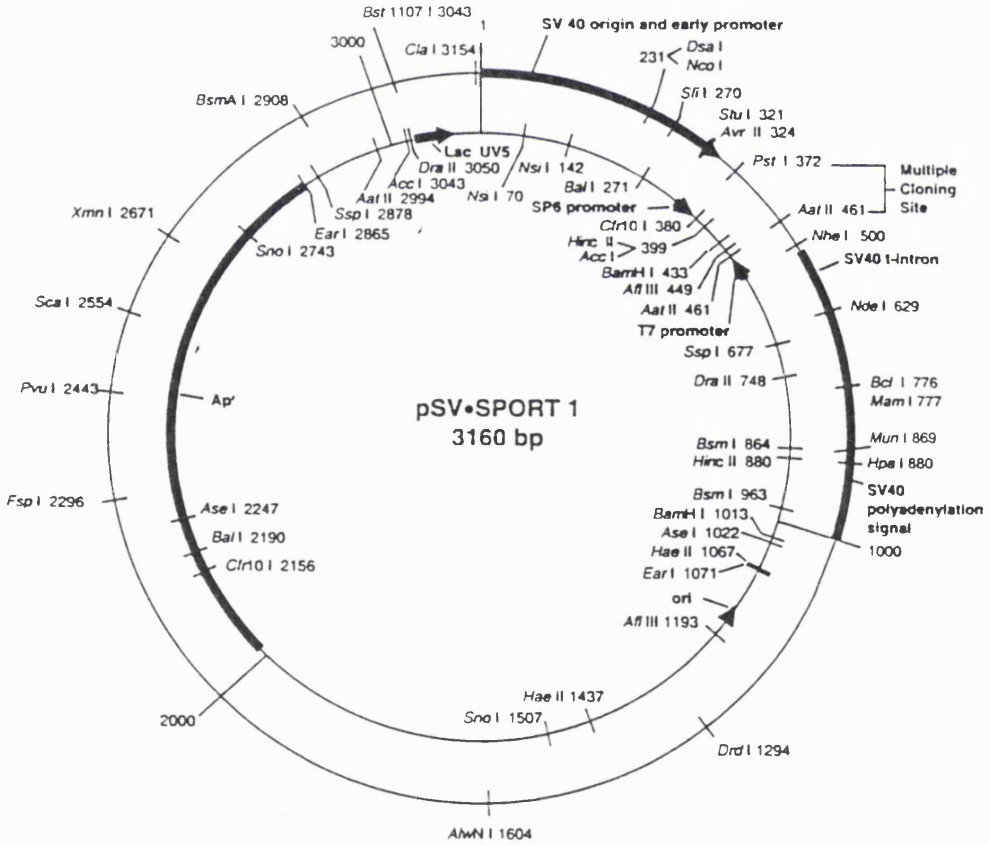


Figure 10.10.2:

Analysis of the cDNA library: Twenty three colonies were picked at random and grown overnight. Plasmid DNA was extracted, digested with *Mlu* I to separate the plasmid from the insert, then run in 0.8% agarose and cDNA insert sizes calculated. This revealed that 21 clones (91%) contained inserts; average insert size is 1493 ± 213 bp. This is similar to the average size of cellular mRNA which is quoted as 1500 nucleotides (Alberts et al, 1989). Lane 24 contains digested library DNA as a control.

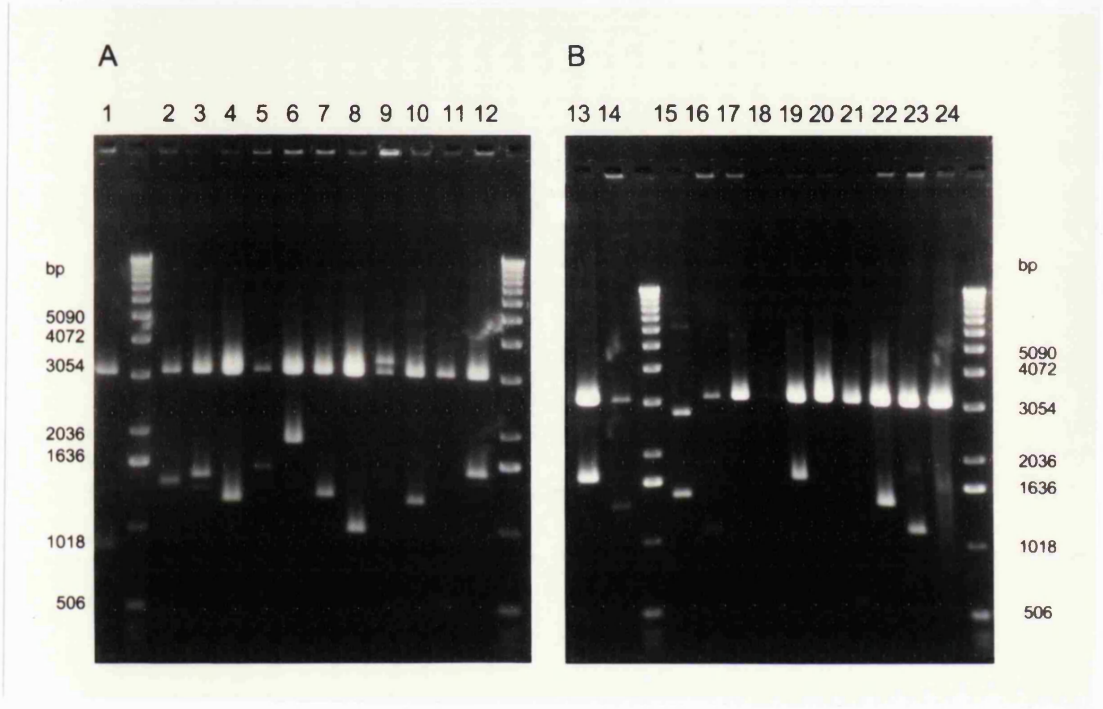
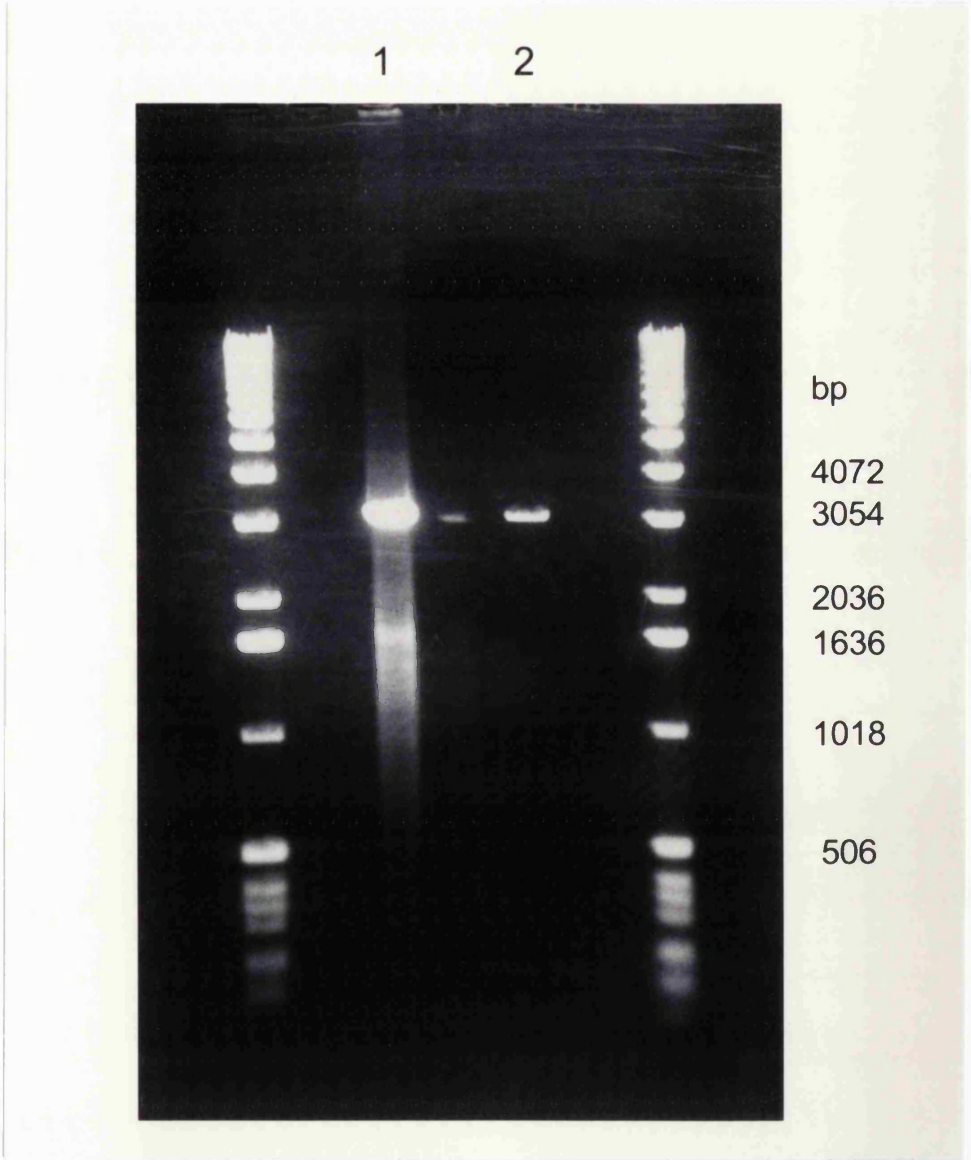


Figure 10.10.3:

Analysis of the cDNA library: In order to assess the approximate size of the cDNA inserts in the plasmid preparation, 1 µg of DNA was digested with MluI and fractionated in a 0.8% agarose gel stained with ethidium bromide (lane 1). The digested pSVSPORT1 plasmid (50 ng) was also run in the gel as a control (lane 2). The digested plasmid is seen to be approximately 3000 bp in size and the cDNA as a smear of 1000 to 5000 bp. The bulk of the cDNA is approximately 1600 bp. This is consistent with the result which was obtained by analysis of the randomly selected clones (average insert size 1493 ± 213 bp; Figure 10.10.2). This is also similar to the average size of cellular mRNA which is quoted as 1500 nucleotides (Alberts et al, 1989).



10.11 POLYMERASE CHAIN REACTIONS

The polymerase chain reaction (PCR) is a technique for amplifying specific segments of DNA or RNA. It uses two oligonucleotide primers which are designed to hybridise to opposite strands in a double stranded DNA sequence. A DNA polymerase extends the primers in opposite directions, resulting in a double stranded product whose ends are defined by the primers. In a repetitive series of cycles, the primers, which are present in great molar excess, tend to amplify not only the original target DNA but also the products of previous reactions. Therefore, the amount of product doubles in each reaction. This exponential increase results in great amplification of the target sequence; after twenty reactions, the theoretical amount of DNA which is generated from one molecule is 2^{20} which is approximately 10^6 molecules. The amplified molecule can be detected, and its size verified, in an agarose gel. This method was invented by Kary Mullis and his colleagues at Cetus Corporation in California. It was originally used to amplify a region of the β-globin gene for the diagnosis of sickle cell anaemia (Saiki et al, 1985).

In order to allow hybridisation of the primers to the target DNA, it is necessary first to separate the two strands in double stranded DNA, by heating to 95^0C. This step is the "denaturing" step. The temperature is then lowered to allow the primers to hybridise to the target DNA; this is the "annealing" step. The temperature is then adjusted to allow the DNA polymerase to extend the 3' ends of the primers. This cycle of denaturing, annealing and extension is repeated in order to generate increasing amounts of DNA. In the initial work, the Klenow fragment of E coli DNA polymerase I was used to amplify the DNA target (Saiki et al, 1985). This enzyme was inactivated by the high temperatures which are required in the denaturing step, so a fresh aliquot of enzyme was added at each cycle. However, a thermostable DNA polymerase was then isolated from *Thermus aquaticus;* this enzyme, *Taq* polymerase, survives the high temperatures and it is only necessary to use one aliquot which is added to the reaction tube at the beginning of the cycling procedure. The use of this enzyme made it possible to carry out the cycling reactions in an automated format (Gelfand, 1989).

In view of the exponential amplification of the target sequence, PCR is capable of detecting target molecules with great sensitivity. However, if the target molecule is very rare, the primers tend to hybridise to other molecules with which they have less sequence homology.

This results in the generation of "non-specific products" which usually appear as a variety of molecules of heterogeneous sizes in the gel. As PCR is typically used to amplify rare target molecules, the design of primers is critical and the conditions have to be optimised for each set of reactions.

The most important factor in determining the specificity of the PCR is the sequence of the primers. Ideally, the primers should be chosen to align to unique segments in the target DNA which are not shared by any other DNA molecule. In practice, this is seldom possible, so other methods which increase the specificity of the PCR have been developed. In the initial work, the Klenow fragment was used at 30^0C, at which temperature the primers could hybridise to targets with which they have relatively little sequence homology; this resulted in the generation of a large amount of non-specific products. The problem was solved by transferring the DNA from the gel to a nylon membrane and hybridising with an internal primer which detected the small amounts of specifically amplified DNA (Saiki et al, 1985). The subsequent use of *Taq* polymerase, whose optimum temperature is 72^0C, greatly increased the stringency of the reactions and this resulted in a great increase in specificity. As the generation of non-specific products was minimised, the yield of specific products was increased. Another advantage of *Taq* polymerase is that it is highly "processive", ie, it is capable of amplifying much longer fragments (up to 10 kb) than the Klenow fragment. A disadvantage of *Taq* polymerase is that it does not have 3'-to-5' exonuclease ("proof-reading") activity, so it has a relatively greater rate of amplification errors than other DNA polymerases. The error rate for *Taq* is estimated at approximately 10^{-4} bases/cycle (Erlich, 1989). This error rate is very small and is unimportant when PCR products are used as a population, for example in the generation of hybridisation probes. However, when individual PCR products are cloned and sequenced, it is important to make allowance for the possibility of amplification errors; this is usually done by sequencing more than one PCR-generated clone.

In view of the great sensitivity of PCR, it is important to avoid contamination by extraneous DNA or by products of previous reactions. Such contamination could result in the generation of artefacts which may appear as non-specific products and which may reduce the yield of specific products. Contamination may also result in the generation of "specific" products which could be misleading. In the present work, precautions against contamination included

the use of dedicated bench space, pipettes and sterile gloves, and the reactions were prepared and amplified in separate rooms. Further, all PCRs included a control in which no template was added, to ensure that any amplified DNA is not being generated from some contaminating material.

The primers were designed to be 20-30 bases in length, with a melting temperature (Tm) of 55-80^{0}C and to contain 45-55% guanidine and cytosine in the total sequence. In general, the primers were designed to amplify sequences of 250 to 600 bp. The possibility of forming non-specific products is increased if the primers hybridise to each other to form primer dimers, or to internal sequences in the PCR product; as the primers and the PCR product are present in much greater concentrations than the target sequence, the polymerase would tend to amplify the primer dimer or the internal primer-product sequences in preference to the desired target sequence. In order to avoid the formation of primer dimers, the following precautions were taken in the design of the primers:
1. The PCR primers were designed to include no more than 4 consecutive hybridising bases, of which no more than two could be guanidine-cytosine hybridisations.
2. Primer dimer formation at the 3' end is particularly disruptive to the formation of specific products, so no more than two consecutive hybridising bases were allowed at the 3' end.

Hybridisation of the 5' end of the primer to the template is not crucial for successful amplification. In contrast, annealing of the 3' end of the primer to the template is particularly important for extension of the primer by the polymerase. The primers were therefore designed to have G or C bases at their 3' ends, to enhance the alignment to the template. In order to avoid the formation of hybridisations between the 3' ends of the primers and the PCR product, the primers were designed such that there were no more than 5 consecutive hybridising bases between either primer and the PCR product.

This primer design was conveniently carried out using the MacVector sequence analysis software on a Macintosh computer. As a useful modification, adenines and thymines were usually removed from the 5' ends of the primers, as they may interfere with TA cloning (see Section 10.12).

At room temperature, the primers may anneal to regions of the template in which there is relatively little sequence homology to the primers. As *Taq* polymerase is active at room

temperature, this phenomenon may result in the generation of non-specific products which could then be amplified during the subsequent PCR cycles. In order to minimise the possibility of annealing of the primers to the template at room temperature, the primers were kept separate from the template until the temperature reached 80^0C. This technique which is known as "hot start PCR" was achieved by using the proprietory barrier Ampliwax (Perkin Elmer). The PCR primers, dNTPs and PCR buffer were first added to the reaction tubes. A bead of Ampliwax was then placed in each tube and the tubes were then heated in the thermal cycler to 80^0C for 10 minutes to melt the Ampliwax. The tubes were then cooled to room temperature, at which the Ampliwax forms a solid barrier above the PCR reagents. The PCR buffer, DNA polymerase and template were then added above the Ampliwax and the temperature cycles were then commenced. During the first denaturing step, Ampliwax melts and forms a vapour above the reactions which are in liquid phase. At the end of the PCR cycles, the reaction tubes were cooled to 4^0C, at which temperature Ampliwax forms a solid barrier above the reactions. This barrier can then be pierced with a pipette tip in order to obtain the reaction products.

The following methods were used to increase the sensitivity and specificity of the PCRs:
1. In order to increase the possibility of detecting a target sequence, the primers were usually designed from a translated region of the cDNA, which is likely to be less variable than the 3' and 5' untranslated regions.

2. Whenever possible, the primers were designed from regions of the translated sequence in which there is homology with the sequence of a related cDNA. For example, the primers which were intended to amplify the synaptic vesicle monoamine transporter (SVMAT) were designed from regions of that molecule which were homologous to the chromaffin granule amine transporter (CGAT; Section 11.9.3). This strategy was intended to increase the statistical chances of detecting a cDNA whose sequence is similar but not identical to known sequences.

3. Hybridisation of the 3' ends of the primers to the target sequence is particularly important to enable extension by the polymerase. Therefore, the 3' ends of the primers were designed to terminate in the first or the second base of a conserved amino acid codon.

4. The possibility of forming non-specific primer-template hybridisations at room temperature was minimised by the use of Ampliwax in a "hot start" technique (see above).

5. Increasing magnesium concentrations tend to favour non-specific primer hybridisation. When it was necessary to perform highly specific reactions, the concentration of magnesium was kept at a minimum, in order to increase the stringency of the reactions. However, in some reactions, it was necessary to use high concentrations of magnesium in order to increase the chances of detecting templates which may not share much homology with the known sequences from which the primers were derived. In general, the concentration of magnesium was optimised for each reaction.

6. In some reactions, reducing the concentrations of the primers reduced the amounts of primer dimers and resulted in generation of increased amounts of the specific PCR product.

7. Nested PCR: This procedure was used to increase the sensitivity of the PCR when a product of the desired size was not evident in the ethidium bromide stained gel. In this method, the products of the PCR were used as templates in a second PCR which utilised a pair of primers whose location in the DNA sequence was internal to the first primer pair. This procedure greatly increased the sensitivity of the reactions.

8. Purification of the PCR product for a second PCR: this procedure was employed when the PCR generated not only products of the desired size but also non-specific reaction products; in such a situation, the use of nested PCR primers may increase the proportion of the desired product in the reaction, but this does not eliminate the non-specific products which were generated in the first PCR. Therefore, the desired PCR product was cut from the ethidium bromide-stained gel under ultraviolet light. The DNA was extracted from the gel using the Qiaex kit (Qiagen; see section 10.10) and was then used as a template in a second PCR which employed either the same set of primers or a nested primer pair.

9. AmpliTaq Gold is a modified, inactive form of *Taq* polymerase; it is activated by heating to 95^0C for a few minutes (Birch, 1996). As polymerase activity is absent at room temperature, there is no extension of misplaced primers which hybridise at low temperature. The use of this enzyme in several experiments in these studies resulted in increased specificity and improved yield of the desirable PCR products.

The reactions were carried out in MicroAmp autoclaved reaction tubes (Perkin-Elmer). These thin-walled tubes are designed to ensure that the contents reach the desired temperature very rapidly. The thermal cycler was a Perkin-Elmer model 2400. This machine has a heated lid which prevents condensation of vapour on the tube caps; this feature makes it unnecessary to use oil for prevention of evaporation.

The PCR template was either GnRH cell total cellular RNA or the GnRH cell cDNA library. DNA was prepared from the library at a concentration of 1 μg/μl and usually contained approximately 10^7 recombinants/μg. In the PCRs which used the cDNA library as template, the reactions consisted of PCR buffer (Perkin Elmer), dNTPs (200 μM each), primers (initially 1 μM each), AmpliTaq or AmpliTaq Gold DNA polymerse (Perkin Elmer; 2.5 units) and template (0.5 μg) to a final volume of 75 μl. These reactions were carried out as 'hot start' using Ampliwax. The reactions which employed AmpliTaq Gold were first incubated at 95^0C for 12 minutes to activate the enzyme. Cycling parameters were as follows:

1. Denaturing at 95^0C for 30 seconds;
2. Annealing (usually 50-60^0C, depending on the Tms of the primer) for 30 seconds;
3. Extension at 72^0C for 1 minute.

Usually, 30 cycles of amplification were employed. The concentration of magnesium was optimised by using magnesium-free PCR buffer (PCR buffer II; Perkin Elmer) and different concentrations of magnesium chloride. Controls included reactions in which either a primer or the template were excluded. The reactions were analysed in ethidium bromide-stained agarose gels which were photographed with Polaroid paper in ultraviolet light. In some of the reactions, the DNA was transferred from the agarose gels to nylon membranes and the PCR products were detected by hybridisation to a specific radiolabelled probe; this detection method is more sensitive and specific than visualisation of DNA in ultraviolet light.

Shortly after its invention, the technique of DNA amplification by PCR was extended to amplify RNA molecules. In a first step, the RNA is reverse-transcribed to cDNA using an

antisense (downstream) primer. Amplification is then carried out using two primers in conventional PCRs. In the initial cycles, the antisense primer anneals to the RNA which is amplified as the target sense strand, but in subsequent cycles, the PCR products increase exponentially and the antisense primer tends to anneal to, and amplify, the sense strand of the PCR product. The introduction of thermostable reverse transcriptases has substantially improved the yield of the first reaction; the higher temperatures presumably denature the secondary structure of RNA and facilitate the generation of the first cDNA strand. The thermostable reverse transcriptase which was used in the present studies was the recombinant DNA polymerase from *Thermus thermophilus.* This enzyme functions as a reverse transcriptase in the presence of manganese but as a DNA polymerase in the presence of magnesium (Myers & Gelfand, 1991). After the completion of the reverse transcription, the manganese is removed using a chelating agent and magnesium is added to the reaction. The chelating agent was EGTA which has much lower affinity for magnesium than for manganese. In the presence of magnesium, the DNA polymerase function catalyses the PCR. Thus, the same enzyme can be used at high temperatures for both the reverse transcription and for the PCR and the two reactions are carried out in the same tube (Myers & Gelfand, 1991).

In this RNA PCR method, the reverse transcription reaction consisted of 250 ng RNA, the downstream primer (0.2 μM), rTth DNA polymerase/reverse transcriptase (Perkin Elmer; 5 units), dNTPs (200 μM each), $MnCl_2$ (1 mM) and reverse transcription buffer to a total volume of 20 μl. Reverse transcription was carried out in the thermal cycler at 60^0C for 15 minutes. The reaction product was then amplified by adding to the reaction tube chelating buffer (Perkin Elmer), $MgCl_2$ (2.5 mM) and the upstream primer (0.2 μM) to a total volume of 100 μl. Cycling parameters were as follows:

1. Denaturing at 95^0C for 1 minute then 95^0C for 10 seconds;
2. Annealing and extension at 65^0C for 15 seconds.

Usually, 35 cycles were carried out followed by final extension at 65^0C for 7 minutes. Controls included reactions in which either a primer or the template were excluded. The reaction products were analysed in ethidium bromide-stained agarose gels.

10.12 CLONING OF PCR PRODUCTS

Two methods were used to clone PCR products:

1. Ligation of blunt-ended DNA:

In this method, the PCR products are ligated to a plasmid which has been digested with a restriction enzyme, *Srf* I, which results in blunt ends, ie, without 3' or 5' overhangs. *Srf* I recognises the sequence 5'-GCCC/GGGC-3' which occurs very rarely in mammalian DNA. The plasmid which is used in this method is designated pCR-Script SK(+). It is derived from the Bluescript SK(+) plasmid by adding a recognition sequence for *Srf* I within the multiple cloning site (Figure 10.12.1). It contains an ampicillin resistance gene, a *lac* promoter for blue-white colour selection of recombinant plasmids (see below), and T3 and T7 RNA polymerase binding sites. In order to prevent re-ligation of the plasmid, the ligation reaction is performed in the presence of the *Srf* I enzyme. This method was conveniently performed by using the Stratagene pCR-Script SK(+) cloning kit.

The ligation reaction was carried out in a volume of 10 µl containing 10 ng of the plasmid, ligation buffer, 1 mM ATP, 5 Units *Srf* I, T4 DNA ligase and the PCR product. The amount of PCR product used was optimised by titrations in separate reactions. In general, 2-4 µl of the PCR product gave satisfactory results. The reaction was incubated at room temperature for one hour. It was then heated to 65^0C for 10 minutes to inactivate the enzymes. The ligate was then introduced by the heat shock method (see below) into *E coli* (strain Epicurian Coli XL1-Blue MRF' Kan) which are included with the kit from Stratagene.

2. TA cloning:

DNA polymerases catalyse the addition of deoxynucleotides to DNA primers in a template-directed manner. The requirement for template instruction distinguishes these enzymes from other nucleotidyl transferases, such as terminal deoxynucleotidyl transferase, which do not require a template. However, *Taq* polymerase carries out blunt-ended addition of nucleotides to the 3' end of a double stranded DNA (Clark, 1988). The requirement for double stranded DNA distinguishes this property of *Taq* polymerase from terminal deoxynucleotidyl transferase, which adds nucleotides to single stranded DNA. Of the four nucleotides, dATP is added much more efficiently by Taq polymerase than the other three nucleotides. The rate of this non-templated addition is slower than template-directed synthesis (Clark, 1988).

This property of *Taq* polymerase has been used to ligate PCR products to a plasmid which has complementary 3' thymidine extensions. The efficiency of this method is greater than blunt-ended ligation of PCR products (Mead et al, 1991). The plasmids which have the 3' T extensions are generated by digesting plasmid vectors which have two asymetrical sites for the restriction enzymes *Xcm* I or *Hph* I, as these enzymes can leave a single 3' T extension in the digested DNA (Mead et al, 1991).

The efficiency with which *Taq* polymerase adds a terminal adenosine is influenced by the identity of the nucleotide at the 3' end of the double stranded DNA. The addition by *Taq* polymerase of a terminal adenosine is facilitated if the terminal nucleotide is cytosine or guanine, whereas the addition of an adenosine is very unlikely if the terminal nucleotide is an adenosine (Hu, 1993). The 5' end of a PCR primer determines the 3' end of the complementary strand of the PCR product. Therefore, PCRs which use primers whose sequences start with a 5' thymidine are unlikely to be 3' adenylated by Taq polymerase (Brownstein et al, 1996). In the present studies, the PCR primers were usually designed to have 5' guanosines or cytidines, to facilitate TA cloning.

Two plasmids were used for TA cloning (Figure 10.12.2). The pCR2.1 plasmid has the resistance genes for both ampicillin and kanamycin. The latter was useful to prevent the growth of bacteria which may contain ampicillin-resistant plasmids from the cDNA library; after the ligation reaction, the transformed bacteria were grown on LB plates containing kanamycin, to which the pSVSPORT1 plasmid is not resistant. This prevented the growth of any plasmids from library DNA which may have been present in the ligate. The pCR2.1 plasmid also has the *lac* gene for blue-white colony screening (see below) and a T7 RNA polymerase site for synthesis of RNA from the insert. The T7 and M13 sites can also be used for sequencing of the insert using appropriate primers. The ColE1 origin ensures replication and maintenance of high plasmid copy number in E coli and the f1 site can be used to generate single stranded DNA. The pCR3.1 plasmid has the immediate early promoter from human cytomegalovirus (CMV) for expression of the insert in mammalian cells. It also has the bovine growth hormone polyadenylation and transcription termination signals, to ensure mRNA stability and effective termination of transcription in mammalian cells. It has resitance genes for ampicillin, kanamycin and neomycin; the latter can be used to select growth of transfected mammalian cells during the generation of stable cell lines. The insert in this plasmid can be sequenced using primers for the T7 and pCR3.1 reverse priming

sites. This plasmid does not have a *lac* gene, so blue-white colony selection is not possible.

PCR products were generated using optimised conditions as described in section 10.11, except that the reactions included a final extension step of 72^{0}C for 10 minutes to increase the likelihood of addition of 3' adenosines by *Taq* polymerase. Immediately after the end of the reactions, 2 pmoles of reaction product was combined with 20 fmoles of the TA cloning vectors pCR2.1 or pCR3.1 (Invitrogen), T4 DNA ligase (4 Weiss units) and ligation buffer (Invitrogen) to a final volume of 10 µl and incubated overnight at 14^{0}C. The ligation reaction was then used to transform competent E coli.

Transformation of E coli with ligates using the heat shock method:

Transformation is the process whereby bacteria absorb and functionally integrate exogenous DNA. Plasmid DNA transforms bacteria much more efficiently than linear DNA. Several methods are available for transforming bacteria. In these studies, the heat shock method (Hanahan, 1983) was used to transform *E coli* strains Epicurian Coli XL1-Blue MRF' Kan (Stratagene), INVαF' or TOP10F' (Invitrogen) with recombinants containing the PCR products. The principle of the procedure is that plasmid DNA can enter E coli in the presence of divalent cations at low temperature. The process is enhanced by sulphydryl reagents and by brief exposure of the DNA/bacterial mixture to high temperature. The efficiency of transformation can be greatly increased if the bacteria are grown in certain conditions which make them "competent" for tranformation. Competence is achieved by exposing the bacteria to ice cold solutions of calcium chloride, DMSO, reducing agents and cobalt chloride. The mechanism of action of these substances is unknown.

In this method, an aliquot of competent *E coli* was placed on ice to thaw and 2 µl of β-mercaptoethanol (0.5 M) was added and stirred gently with the pipette tip. The ligation reaction (2 µl) was then added and stirred gently with the pipette tip. The tube was kept on ice for 30 minutes then tranferred to 42^{0}C for 30 seconds. The tube was then incubated on ice for two minutes and 250 µl of SOC medium added at room temperature. The bacteria were warmed to 37^{0}C in a shaking incubator at 225 rpm for 60 minutes. The LB culture plates which were used in these studies contained an appropriate antibiotic (ampicillin or

kanamycin, 50 μg/ml); for blue-white colour selection, 40 μl of 0.1 M IPTG and 100 μl of 2% Bluogal had been spread on each plate using a sterile rod (see below). The warmed, transformed bacteria were spread on the LB agar culture plate and the bacteria were allowed to grow at 37^0C overnight. Individual white colonies were picked, amplified by overnight growth in liquid LB medium containing an appropriate antibiotic and the plasmid DNA was then extracted using Qiagen miniprep kits (Section 10.10). An aliquot of each miniprep DNA was subjected to PCR using the primers and reaction conditions which were used to generate the product, to confirm the correct identity of the inserts in the cloning vectors. PCR products which had been correctly ligated to the cloning vectors were sequenced either manually or using an automated ABI sequencer.

Blue-white colony selection:

This method was used to identify bacteria which contain recombinant plasmids. It relies on the production by *E coli* of the enzyme β-galactosidase, which catalyses the hydrolysis of β-D-galactosides to their component sugars. A normal substrate for this enzyme is lactose which is hydrolysed to glucose and galactose. The *lac* operon in E coli consists of a promoter which controls the transcription of three downstream genes: *lacZ* which encodes β-galactosidase, *lacY* which encodes galactoside permease and *lacA* which encodes galactoside acetylase (Ullman et al, 1967; Watson et al, 1987). The three genes are transcribed into a single polycistronic mRNA. The three genes are normally inhibited by the "*lac* repressor protein" which is encoded by a separate gene, *lacI* which is located upstream of the *lac* promoter (Figure 10.12.3). The *lac* repressor protein binds to the *lac* promoter and prevents transcription of the *lac* genes. In E coli, lactose or its metabolite allolactose bind to the *lac* repressor protein and remove its suppressive effect. This effect of allolactose can be reproduced by the synthetic analogue IPTG (isopropylthio-β-D-galactoside), which has the advantage that it is not metabolised by the bacteria.

Ullman et al (1967) discovered E coli mutants which were deficient in β-galactosidase activity. However, the mutants generated peptide fragments which, when combined, were able to function as β-galactosidase. They concluded that the E coli mutants were deficient in segments of the gene which encoded the proximal (α) or the distal (ω) parts of the gene.

This method of generating the active β-galactosidase protein using the two inactive fragments is known as α-complementation (Ullman et al, 1967).

Many of the plasmids which are in current use include the proximal part of the gene for β-galactosidase *(lacZ)*. The sequence of this gene is interrupted by the polycloning site which does not disrupt the reading frame for the gene, but results in the harmless inclusion of a small number of amino acids in the amino terminal of β-galactosidase. Plasmids carrying this gene are expressed in bacteria which have been engineered to contain the distal part of the β-galactosidase gene. The protein fragments which are produced by the plasmid and by the bacteria are each inactive, but the two proteins associate to form an enzymatically active protein in the bacteria. These bacteria can be recognised by their blue colour in the presence of the chromogenic β-galactosidase substrates X-gal or Bluo-gal. However, insertion of a fragment of foreign DNA into the polycloning site of the plasmid results in the production of a protein which is incapable of α-complementation. Therefore, bacteria which carry a recombinant molecule do not possess β-galactosidase and appear white; they are easily distinguishable from the blue bacteria which contain self-ligated plasmids or very small inserts (Sambrook et al, 1989).

Most bacteria possess the *lac* repressor protein which inhibits the transcription of the *lac* gene under normal conditions. These bacteria require the presence of IPTG to induce the synthesis of the α protein from the plasmid. However, IPTG is not necessary for the INVαF' strain which does not express the *lac* repressor protein.

Figure 10.12.1:

The pCR-Script SK(+) plasmid was used in blunt-ended ligation of PCR products. It is derived from the Bluescript SK(+) plasmid by adding a recognition sequence for *Srf* I within the multiple cloning site. The plasmid contains an ampicillin resistance gene, a *lac* promoter for blue-white colour selection of recombinant plasmids, and T3 and T7 RNA polymerase binding sites. The ligation reaction is performed in the presence of the *Srf* I enzyme, in order to prevent re-ligation of the plasmid.

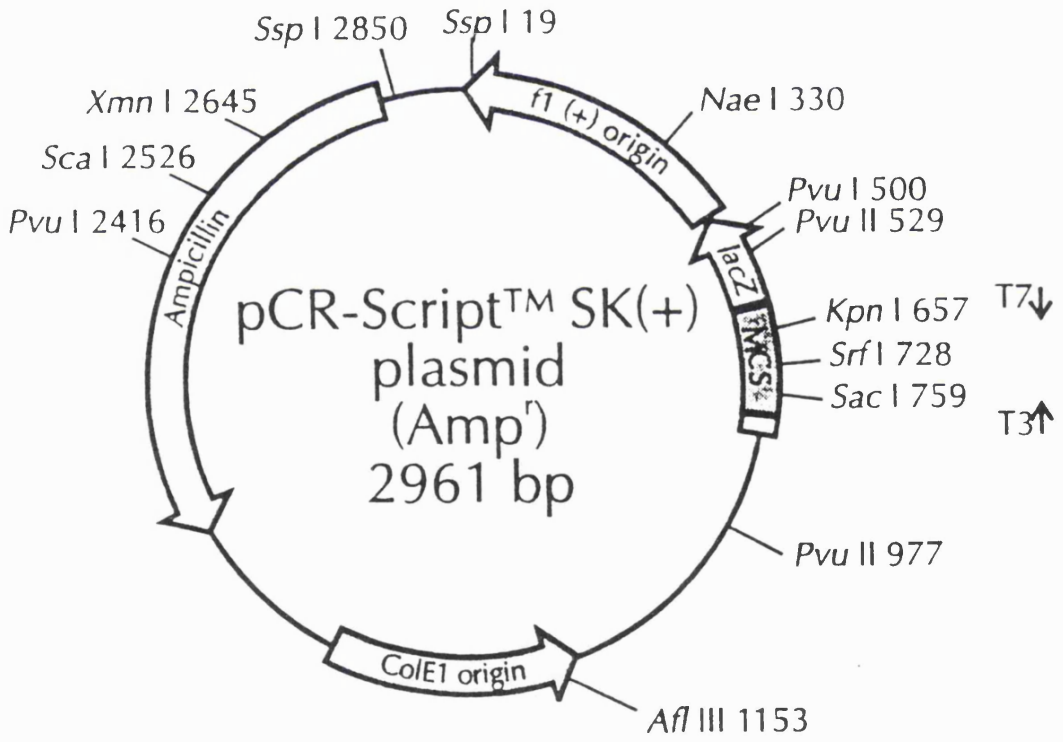
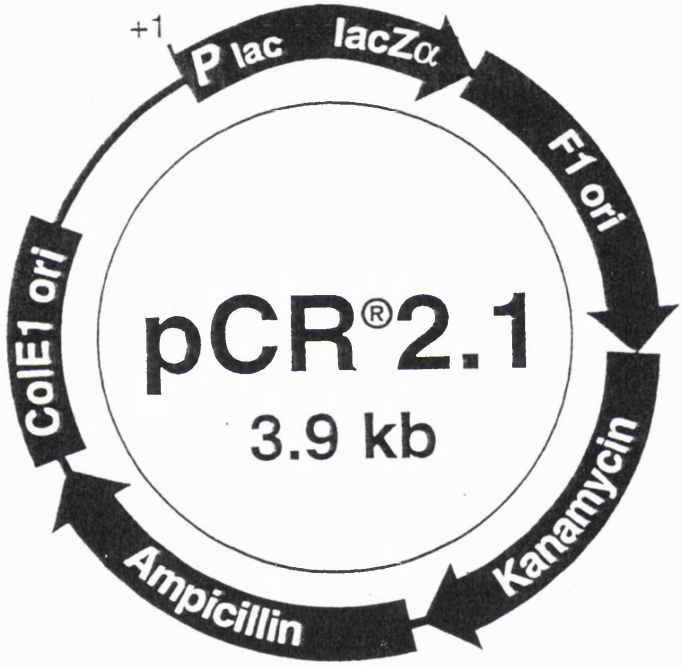


Figure 10.12.2:

The plasmids which were used for TA cloning were purchased from Invitrogen. The *lac* gene in pCR2.1 can be used for blue-white colony screening. The plasmids have the resistance genes for ampicillin and kanamycin for selection in bacteria. The neomycin resitance gene in pCR3.1 can be used to select growth of transfected mammalian cells. This plasmid utilises the immediate early promoter from human cytomegalovirus (CMV) and the bovine growth hormone polyadenylation and transcription termination signals for expression of the insert in mammalian cells. The ColE1 origin ensures replication and maintenance of high plasmid copy number in E coli and the f1 site can be used to generate single stranded DNA.



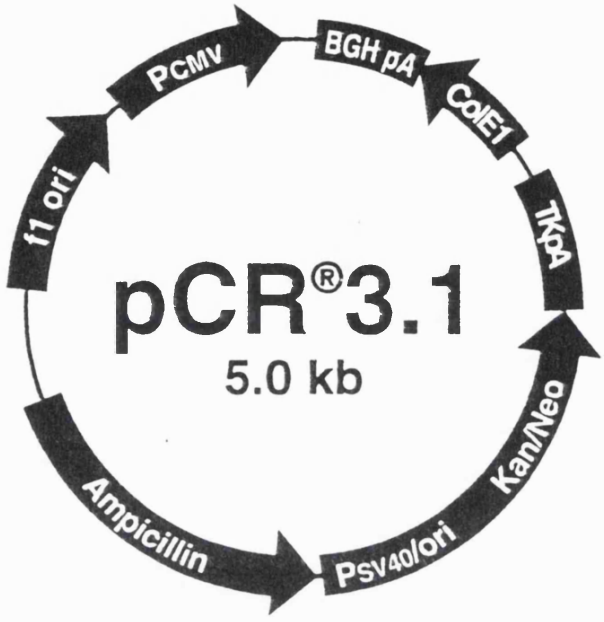
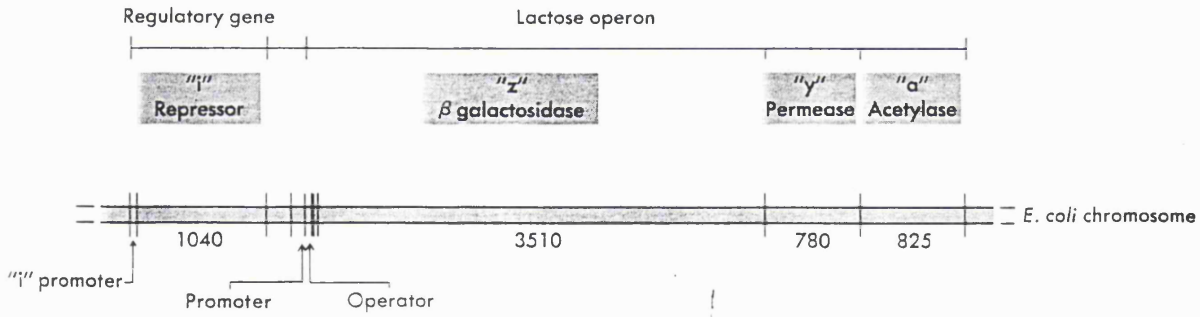


Figure 10.12.3:

The lactose operon and its associated regulatory genes (reproduced from Watson et al, 1987). The *lac* operon in E coli consists of a promoter which controls the transcription of three downstream genes: *lacZ* which encodes β-galactosidase, *lacY* which encodes galactoside permease and *lacA* which encodes galactoside acetylase. The three genes are normally inhibited by the "*lac* repressor protein" which is encoded by a separate gene, *lacI* which is located upstream of the *lac* promoter. The *lac* repressor protein binds to the *lac* promoter and prevents transcription of the *lac* genes. In E coli, lactose or its metabolite allolactose bind to the *lac* repressor protein and remove its suppressive effect. This effect of allolactose can be reproduced by the synthetic analogue IPTG (isopropylthio-β-D-galactoside), which has the advantage that it is not metabolised by the bacteria.



10.13 NUCLEOTIDE SEQUENCE ANALYSIS

In the early part of this work, nucleotide sequence analysis was performed manually. In subsequent stages, sequences were obtained using automated techniques, either within University College London or commercially. Both techniques are based on the di-deoxy chain termination method which was developed by Sanger and his colleagues at the MRC Laboratory of Molecular Biology in Cambridge (Sanger et al, 1977). Many of the subsequent refinements of these techniques, such as the improvements in radiolabels, electropohoresis techniques and computerised methods for DNA sequence analysis were also pioneered at the same Laboratory in Cambridge.

2',3'-Dideoxynucleotide triphosphates (ddNTPs) differ from deoxynucleotide triphosphates (dNTPs) in that they lack a hydroxyl residue at the 3' position of deoxyribose. They can be incorporated by DNA polymerases into a growing DNA chain through their 5' triphosphate groups. However, the absence of a 3'-hydroxyl residue prevents formation of a phosphodiester bond with the succeeding dNTP. Further extension of the growing chain is therefore impossible. Thus, when a small amount of one ddNTP is included with the four dNTPs in a reaction mixture for DNA synthesis, there is competition between extension of the chain and infrequent but specific termination. The products of the reaction are a series of oligonucleotide chains of different lengths. By using the four different ddNTPs in four separate enzymatic reactions, oligonucleotides are generated which terminate at positions occupied by every nucleotide in the template. By running the four reactions in adjacent lanes in a polyacrylamide gel, a pattern of bands is obtained from which the sequence can be read (Sambrook et al, 1989).

There are two types of sequencing strategies. The *random or shotgun sequencing* approach was developed at the MRC Laboratory of Molecular Biology in Cambridge and was used to sequence the human mitochondrial genome (Anderson et al, 1981). Sequence data are collected from subclones containing random segments of the DNA of interest. The strategy utilises a universal primer which is in the vector, and the data are stored and assembled by a computer. In general, it is necessary to sequence five to seven times more nucleotides than the actual length of the DNA of interest. However, the gradual accumulation of these redundant sequences greatly improves the accuracy of the final assembled sequence (Sambrook et al, 1989). This strategy is generally used in large scale projects such as the sequencing of the human genome (Venter et al, 1998). In the present work, DNA was

sequenced by a directed, step-wise strategy, starting with universal primers which are in the plasmid vector, flanking each end of the cDNA insert. Examples of such primers included T7, SP6 and others which are commercially available. The sequences which were obtained from these universal primers were used to design new primers for a subsequent set of reactions. The DNA sequence was therefore obtained from both strands in a progressive manner. The sequences were then assembled and analysed using the AssemblyLign and MacVector sequence analysis software (Oxford Molecular Group). The oligonucleotide primers were generally chosen to be 20-30 nucleotides in length and were designed to have annealing temperatures of 60-80^{0}C. The primers were conveniently designed using the MacVector sequence analysis software on a Macintosh computer, and were synthesised by commercial suppliers.

The template for sequencing was double-stranded plasmid DNA which was prepared using Qiagen kits (Section 10.10). The template was used as the circular recombinant molecule and was denatured by heating in a thermal cycler device.

The Klenow fragment was used as the polymerase in the original method (Sanger et al, 1977). The extension reactions were performed at room temperature and there were many termination artefacts which were attributable to the secondary structure of the DNA template. In the present work, *Taq* polymerase was used as it has the following advantages:

1. The thermal stability of this enzyme makes it possible to denature the DNA by heating it to high temperature. The thermal cycler was used to carry out cycles of denaturing at 95^{0}C followed by annealing of the sequencing primers at an appropriate temperature and then extension of the primer by *Taq* polemerase at 72^{0}C. Approximately 30 cycles were performed, as previously described (Innis et al, 1988).
2. *Taq* polymerase is highly processive and this results in longer sequences (Innis et al, 1988).
3. The absence of 3' to 5' exonuclease activity in *Taq* polymerase reduces the chances of removal of the 'abnormal' ddNTPs from the ends of the growing chains (Innis et al, 1988).

In the original method, [α-^{32}P]dATP was used as the radiolabel to detect the DNA in the gel (Sanger et al, 1977). The high energy β particles (1.71 MeV) which are emitted by this

radionuclide resulted in diffuse bands in the gel and resolution was therefore poor. This was particularly evident in the longer DNA chains which are relatively poorly separated in the upper part of the gel. Subsequently, ^{35}S, which emits low energy β particles was used as the radiolabel and this resulted in much sharper bands in the gel (Biggin et al, 1983). In the present studies, ^{33}P was used as the radiolabel as it emits β particles whose energy is similar to ^{35}S (0.249 and 0.167 MeV, respectively; Evans & Read, 1992). A further advantage of ^{35}S and ^{33}P is that the low energy which is emitted by these radionuclides does not destroy the sugar-phosphate backbone of the DNA, so that the sequencing reactions can be stored for several days if necessary.

In the original work, [α-^{32}P]dATP was included in the extension reaction (Sanger et al, 1977). The nucleotide was incorporated into the growing chains which became radiolabelled throughout their length. This meant that longer DNA chains had greater radioactivity than shorter chains. This presented a problem as the separation of the longer chains is relatively poor in the upper part of the gel. In the present studies, the sequencing primers were labelled at the 5' end with ^{33}P using T4 polynucleotide kinase. The restriction of the radiolabel to the 5' end of the DNA results in chains which are equally radiolabelled, and this makes it easier to resolve the longer DNA chains in the upper part of the gel. ^{33}P was used in the reactions because addition of ^{35}S by T4 polynucleotide kinase is inefficient (Evans & Read, 1992).

For convenience, a commercially available kit was used. In the Cycle Sequencing System (Life Technologies), a sequencing primer (1 pmole) was labelled with ^{33}P using T4 polynucleotide kinase (1 unit) in a 5 μl reaction. The labelled primer was then used in a cycle sequencing reaction which employed 200 ng of double stranded circular DNA template and 1.25 units of *Taq* polymerase. Cycling parameters were: 95^0C for 30 seconds (denaturing), 55^0C for 30 seconds (annealing) and 72^0C for 1 minute (extension); 30 cycles. The reaction products were then electrophoresed in a 6% polyacrylamide gel which resolves DNA fragments of 25 to 400 nucleotides in length (Sambrook et al, 1989). The logarithmic relationship between the length of a fragment of DNA and its mobility produces widely spaced bands at the bottom of the gel and crowded bands at the top. By electrophoresing

aliquots of the sequencing reaction for different lengths of time, it was possible to extend the amount of readable sequence in the gel (Figure 10.13.1). The gels were dried and autoradiographed using Kodak MR-2 film.

Manual DNA sequencing is expensive and highly labour intensive. Automated DNA sequencing techniques were developed at CALTECH and Applied Biosystems in California (Smith et al, 1986). The automated technique is based on the same principles which are described above for manual sequencing using the Sanger chain termination method, and *Taq* polymerase is used in the sequencing reaction which is carried out in a thermal cycler. The major technical difference in the sequencing reactions is that the oligonucleotide primers are tagged with fluorophores which enable detection of the DNA strands. A different coloured fluorophore is used for each of the four reactions which are specific for the four bases in DNA. The reaction mixtures are then combined and electrophoresed in a single lane in a polyacrylamide gel tube. The separated fluorescent bands of DNA are detected by fluorescent detectors which are located near the bottom of the gel and the sequence information is transmitted directly to a computer (Smith et al, 1986). The four original fluorescent dyes which were used to label the primers were designed to have emission maxima which were well resolved from each other. Nevertheless, the emission spectra of the dyes still overlapped substantially, so peaks corresponding to more than one dye were detected by each channel; these peaks were resolved by multicomponent analysis in the computer. A further problem was that the different dyes affected DNA mobility to different degrees; the computer analysis also took account of these mobility shifts (Smith et al, 1986). These problems were subsequently resolved by the introduction of dyes which have narrower emission spectra and which do not affect the mobility of the DNA fragments (Metzker et al, 1996). In order to increase the emission intensity, the primers were labelled with two dyes, utilising the principle of fluorescence energy transfer from an acceptor dye to a donor dye (Metzker et al, 1996). The ABI PRISM 3700 and 3100 sequencers which are in current use utilise polyacrylamide gel capillaries in a fully automated format which can handle up to 1000 sequencing reactions per day with minimal operator time (15 minutes compared with 8 hours in the ABI PRISM 377; Venter et al, 1998).

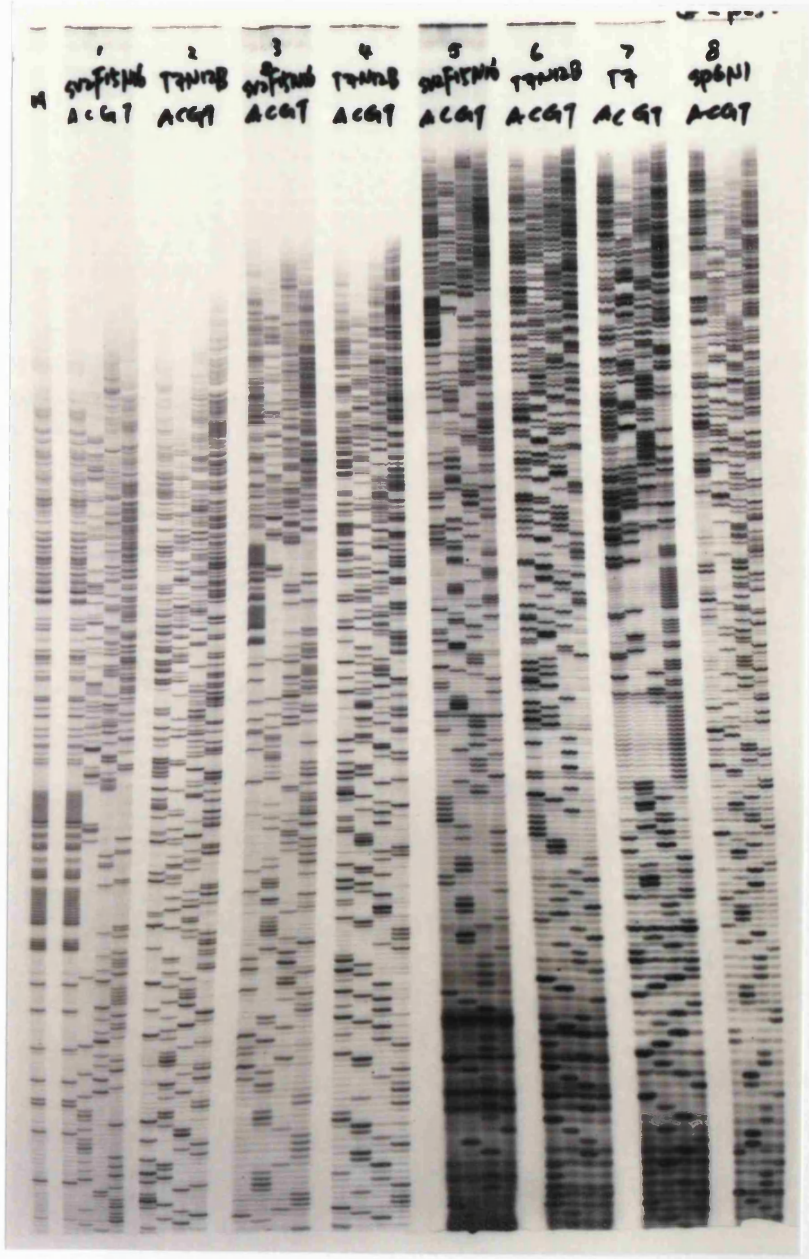
The development of these automated DNA sequencing techniques was crucial to the establishment of the high-throughput DNA sequencing projects which are being used to determine the sequences of large genomes (Adams et al, 1994). These automated techniques

have also greatly facilitated projects which are of a size which is described in this thesis. The advantages of the automated technique are as follows:

1. The steps of drying the gel, autoradiography, reading the X-ray film and feeding the data into the computer are all eliminated and the procedure is therefore much faster.
2. The resolution of the DNA fragments is improved. This is because the detectors are placed near the bottom of the gel, where the separation of the fragments is greatest, due to the logarithmic relationship between the length of a fragment of DNA and its mobility.
3. Running the four samples in a single lane in the gel eliminates problems of uneven gel thickness or composition which can make it difficult to resolve bands in adjacent lanes.

Figure 10.13.1:

Autoradiogram of DNA sequencing reactions which were performed manually. The sequencing primers were labelled with ^{33}P using T4 polynucleotide kinase. The labelled primers were then used in cycle sequencing reactions which employed double stranded circular DNA template and *Taq* polymerase. The reaction products were then electrophoresed in a 6% polyacrylamide gel. The logarithmic relationship between the length of a fragment of DNA and its mobility produces widely spaced bands at the bottom of the gel and crowded bands at the top. By electrophoresing aliquots of the sequencing reactions for different lengths of time, it was possible to extend the amount of readable sequence in the gel. The gel was dried and autoradiographed using Kodak MR-2 film.



10.14 SCREENING THE cDNA LIBRARY BY HYBRIDISATION

Classically, a cDNA library is screened by colony hybridisation (Grunstein & Hogness, 1975). The main disadvantage of this method is that it usually results in identification of an incomplete cDNA; it is then necessary to use the incomplete clone as a probe to screen more colonies, and this should eventually result in the identification of a full-sized clone or of several incomplete clones which can be ligated to form a full-sized molecule. This procedure is time consuming. In the present work, the library was screened for cDNAs of identified size. The principle of this procedure is to divide the library into pools of bacteria, extract the plasmid DNA and electrophorese it in a gel, then to test the DNA from each pool for the presence of the clone of interest, by hybridisation to an approriate probe. When a positive pool is identified, sub-pools are prepared from this positive pool, containing smaller numbers of clones. The hybridisation signal can be expected to be stronger in the sub-pool, due to enrichment of the target molecule. Progressive cycles of pools containing diminishing numbers of clones will ultimately result in identification of an individual clone. Alternatively, once a positive sub-pool has been identified as containing a clone of an appropriate size, colony hybridisation can be used to obtain the individual positive clone. The advantage of this method over the classical procedure is that the size of the cDNA insert can be determined from the start in the gel, so it is more likely to yield a full-sized clone.

The procedures which are used for membrane hybridisation were developed in the Universities of Illinois, Harvard and Edinburgh. Nygaard & Hall (1963) discovered that DNA can bind to nitrocellulose filters. In order to prevent the re-annealing of double-stranded DNA during the hybridisation procedure, Gillespie & Spiegelman (1965) denatured the DNA then fixed it to the nitrocellulose filters; they then carried out the hybridisation procedure on the filters. Denhardt (1966) found that the non-specific attachment of DNA probes to nitrocellulose membranes could be reduced by including Ficoll, albumin and ploy vinylpyrrolidine in the hybridisation buffer, and by pre-incubating the membranes with the hybridisation buffer (Denhardt, 1966). Southern (1975) developed a method for transferring DNA fragments which had been separated in a gel to nitrocellulose filters.

10.14.1 Preparation of plasmid pools

When grown in liquid medium in a shaking incubator, bacteria which contain a small cDNA insert multiply at a faster rate than those which contain a large insert. This results in skewing of the bacterial population towards small insert size. In the present work, the bacterial pools

were grown in suspension in a semi-solid medium. Under such conditions, the bacterial colonies grow to a uniform size, which is independent of their growth rate in liquid medium. This is probably because the bacterial colonies become nutrient-limited in the three-dimensional space and thus the slower growing bacterial colonies catch up with the faster growing colonies until the nutrients are depleted and growth stops (Kriegler, 1991). The semi-solid medium consisted of tryptone 20 g/l, yeast extract 10 g/l, NaCl 10 g/l and low melting temperature agarose (Sea-Prep agarose, FMC) 3 g/l, pH 7. The medium was autoclaved and when it cooled to 37^0C, ampicillin was added to 50 µg/ml.

The library consists of $2.1x10^6$ recombinants which have been amplified to $1.8x10^9$ bacteria/ml (Section 10.10). An aliquot of the library which had been frozen in liquid nitrogen was placed on ice. A chip was removed with a sterile blade and placed in a sterile 1.5 ml tube on ice. The bacteria were diluted serially in LB medium containing ampicillin 50 µg/ml, to a concentration of $1.5x10^6$ bacteria/ml. One hundred µl (150,000 bacteria) were added to each of twenty 50 ml tubes containing 40 ml of the semi-solid medium and mixed thoroughly. In order to confirm the number of bacteria which had been added to each of the 50 ml tubes, an aliquot of 25 µl was removed from one of the 50 ml tubes, spread on an LB/ampicillin plate and grown at 37^0C overnight. The 50 ml tubes were placed on ice for an hour for the agarose to gel, then transferred to a static incubator at 37^0C and left overnight. The following morning, the bacterial suspensions were transferred to warm centrifuge tubes and centrifuged at 6000 rpm, 37^0C for 20 minutes in a DuPont 5B Superspeed centrifuge. The supernatant was discarded and the bacteria were resuspended in 5 ml PBS. After two washes in 5 ml PBS, 1.6 ml of the bacterial suspension was transferred to a set of 1.5 ml tubes and used for extraction of plasmid DNA using Qiagen kits (Section 10.10). The remainder of the bacteria were suspended in one ml of bacterial stocking solution (20% glycerol in LB medium). An aliquot was removed, diluted serially and the bacteria grown on LB/ampicillin plates, in order to estimate the number of bacteria in the pool following amplification. The bacteria were then stored at -80^0C.

10.14.2 Digestion and transfer of DNA from library pools

The principle of the procedure is to release the plasmid from the insert using an appropriate restriction enzyme, then to separate the plasmid from the insert by electrophoresis in an

agarose gel. The DNA is denatured *in situ* and the fragments are then transferred to a membrane using techniques which were developed by Southern (1975) and refined subsequently (Reed & Mann, 1985). The DNA fragments retain their relative positions when they are transferred from the gel to the membrane. Originally, nitrocellulose was used as the membrane but its main disadvantage is that it becomes brittle and may be difficult to handle. Nylon membranes are much tougher and DNA can be irreversibly linked to them by exposure to ultraviolet light (Khandjian, 1987). Two types of nylon membranes are available: neutral and positively charged. The latter bind DNA with greater affinity but they also result in greater non-specific binding of the radiolabelled probe. In the present work, neutral membranes were used.

The plasmid DNA which had been extracted from the pools was digested with *Mlu* I, for which there is a restriction site in the *Sal* I adapter at one end of the cDNA and another site in the pSVSPORT1 plasmid at the other end of the multiple cloning site (Section 10.10). This treatment releases the insert from the plasmid, enabling assessment of the size of the cDNA insert. An aliquot of DNA (1 μg) from each pool was digested in this manner and electrophoresed in a 0.8% SeaKem agarose gel which contained ethidium bromide 500 μg/l. The gel included a positive control (0.1 ng of the DNA from which the probe had been prepared) and DNA size markers (1 kb ladder, Life Technologies) which had been labelled with ^{32}P using T4 polynucleotide kinase. Approximately 25,000 dpm of the size markers were loaded per lane. After completion of the electrophoresis, the gels were photographed in ultraviolet light. In order to facilitate the transfer of DNA from the gel to a solid support, the gels were placed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) which partially hydrolyses the DNA to smaller fragments. After 30 minutes in denaturing solution, the gels were rinsed with de-ionised water then placed in neutralisation solution (1.5 M NaCl, 1 M Tris, pH 7.5) for a total of 60 minutes. The gels were then rinsed with de-ionised water and placed in transfer buffer (10x SSPE/10x SSC) until ready for the transfer. The DNA was transferred from the gels to nylon membranes (Maximum Strength Nytran, Schleicher & Schuell, 0.45 μm pores) using a Posiblot Pressure Blotter (Stratagene), according to the manufacturer's instructions. The DNA was cross-linked to the nylon membranes by exposure to ultraviolet light in a Stratalinker UV Crosslinker (Stratagene). The membranes were wrapped in cellophane and kept at 4^0C until ready for hybridisation.

10.14.3 Preparation of a radiolabelled probe using random hexamers

The hybridisation probe was usually a PCR product which had been ligated to a plasmid vector and amplified in E coli. Plasmid DNA was extracted from these bacteria and digested with an appropriate enzyme to separate the plasmid from the insert. The digested DNA was then electrophoresed in an agarose gel and photographed with minimum exposure to ultraviolet light. Using a clean blade, the insert was cut from the gel and the DNA extracted from the agarose using a Qiaex kit (Section 10.10).

The aim of the procedure was to produce a probe with very high specific activity, in order to maximise the sensitivity of detecting rare clones in the library. The hybridisation probes which were used in this work were therefore labelled with ^{32}P, as this radionuclide has the highest emission energy among isotopes which are commonly used in biological research laboratories. Spatial resolution (which is best provided by isotopes which emit lower energy such as ^{33}P and ^{35}S) was not an important consideration in the preparation of the probe.

Three methods are commonly used for labelling DNA probes: end labelling with terminal deoxynucleotidyl transferase, nick translation and random priming. The highest specific activities are achieved by random priming (Feinberg & Vogelstein, 1983), so this method was used in the present studies. In this method, the double stranded DNA is denatured and allowed to anneal to a high concentration of primers which are composed of a random sequence of six bases (random hexamers). The primers are then extended in the presence of a radiolabelled nucleotide, using the Klenow fragment of E coli DNA polymerase I. This enzyme is used as it does not possess the 5' to 3' exonuclease activity which is inherent in the whole enzyme. The result is a mixture of molecules of varying lengths which are uniformly labelled with radioactivity (Feinberg & Vogelstein, 1983).

For convenience, a commercially available kit was used (Random Primer DNA Labelling System, Life Technologies). The DNA template was denatured by boiling in a water bath for 5 minutes, then placed immediately on ice to prevent rapid re-annealing of the DNA strands. The labelling reaction consisted of the denatured DNA template (25 ng), dATP, dTTP and dGTP (1 nmole each), [$\alpha^{32}P$]dCTP (3000 Ci/mmole; 50 μCi), random hexamers (0.27 OD_{260} units/ml), Klenow fragment of E coli DNA polymerase (3 units) and its reaction

buffer, to a total volume of 50 µl. The reaction was incubated at room temperature overnight then 5 µl of 0.5 M EDTA was added to stop the reaction. The probe was then purified from unincorporated [α^{32}P]dCTP by passage through a Sephadex G-50 column.

To determine the specific activity of the probe, 1 µl of the reaction mixture was added to 249 ul water and 5 µl of this diluted reaction mix spotted on each of two Whatman GF/C filters and allowed to dry in air. One of the filters was placed in a vial with scintillation liquid and the total radioactivity (probe and unincorportaed nucleic acid) counted. The other filter was washed three times with 50 ml ice cold 10% trichloroacetic acid/1% sodium pyrophosphate, followed by one wash in ethanol at room temperature and allowed to dry in air. This filter thus contained the TCA-precipitable material which represents the probe. Approximately 70% of the radioactivity was incorporated in TCA-precipitable material. After passage on the Sephadex G-50 column, the TCA-precipitable material usually represented 97% of total radioactivity, indicating that the probe had been highly purified. The specific activity of the probe was usually $3x10^9$ dpm/µg.

10.14.4 Hybridisation of DNA to a radiolabelled probe

The principle of this procedure was to hybridise the probe at low stringency, in order to increase the chances of detecting a rare clone in the library; the membranes were then washed at increasing stringency, to reduce non-specific binding of the probe to the membranes. These aims were achieved as follows:

1. Pre-hybridisation: The non-specific binding of a probe can be reduced by pre-incubating the membranes with the hybridisation buffer; this does not affect the specific hybridisation of the probe to complementary sequences in the membrane (Denhardt, 1966). The detergent sodium dodecyl sulphate also reduces non-specific binding.

2. Temperature: Hybridisation is more stringent at higher temperature as the kinetic energy denatures DNA duplexes. As previously recommended following empirical studies (McConaughy et al, 1969), hybridisation was carried out at 20-25^{0}C below the Tm of the probe, whereas the higher stringency washes were performed at 15^{0}C below the Tm of the probe. The Tm of the probe was calculated using the MacVector programme which uses the

following formula:

$Tm = 81.5^0C + 16.6(\log_{10}[Na^+]) + 0.41(\%GC) - 0.62(\%formamide) - (675.0 / length)$

3. Ionic strength: Hybridisation is less stringent in the presence of high concentrations of sodium which neutralises the phosphate groups in the backbone of the DNA, allowing DNA strands to anneal more easily. The hybridisation was therefore carried out at high sodium concentration (0.9 M NaCl) whereas the higher stringency washes were carried out in the presence of low sodium concentration (0.01 M NaCl).

4. Formamide ($HCONH_2$): Organic solvents such as formamide and dimethylsulphoxide denature nucleic acid duplexes by disrupting hydrogen bonds (Helmkamp & Tso, 1961). The Tm of duplexes is reduced in a linear manner by increasing concentrations of formamide (McConaughy et al, 1969; Casey & Davidson, 1977). Formamide is fully miscible with water, so its inclusion in the hybridisation buffer compensates for lowering the temperature. It was found empirically that hybridisation at lower temperature in the presence of formamide reduces non-specific binding of the probe and yields better results than hybridisation in aqueous solutions at higher temperature (Bonner et al, 1967; McConaughy et al, 1969). Formamide slows the rate of hybridisation, so the membranes were exposed to the probe overnight.

5. Hybridisation volume: Hybridisation is faster at greater concentrations of the probe; the hybridisation volume was therefore minimised and sealing bags were used as they enable use of minimal volumes of buffer while keeping the membranes entirely covered.

The hybridisation buffer which was used in these experiments (Hybrisol I; Oncor) contained 50% formamide and 0.9 M NaCl. The following solutions were also used:
20x SSPE: 3 M NaCl; 0.2 M NaH_2PO_4; 0.02 M EDTA; pH 7.4
20x SSC: 3 M NaCl; 0.3 M sodium citrate; pH 7.0

The nylon membranes with immobilised DNA were wetted in 6x SSC for 5 minutes then placed in a hybridisation bag. Approximately 100 $\mu l/cm^2$ hybridisation buffer was added, air bubbles were removed and the bag was sealed with a heating device. The bags were placed in a sandwich box and incubated overnight at 42^0C. Following overnight incubation, the

hybridisation bag was cut in a corner and the buffer removed completely. An aliquot of the probe (10^7 dpm for each membrane) was boiled for 5 minutes to denature the DNA then transferred immediately to ice to stop rapid re-annealing. The denatured probe was then added to 10 ml hybridisation buffer (10^6 dpm/ml buffer), mixed and added to the hybridisation bag which was sealed and placed in a water bath at 42^0C. This temperature is 20-25^0C below the Tm of most probes which were used in this study. The following morning, the membranes were removed from the bags and washed as follows:

A. Low stringency wash:

Three times in 6x SSPE, 0.1% sodium dodecyl sulphate for 15 minutes at room temperature

B. High stringency wash:

Twice in 0.1x SSPE (0.01 M NaCl), 0.5% sodium dodecyl sulphate for 30 minutes. The temperature of this wash was chosen to be 15^0C below the Tm of the probe.

The membranes were allowed to dry and a small amount of radioactivity was spotted in a corner to aid orientation. The membranes were then wrapped in cellophane and exposed to Kodak XAR film with two intensifier screens at -80^0C for 24 hours.

Figure 11.1.12 is a composite of autoradiograms of gels which had been hybridised to a probe consisting of a PCR product which is derived from an α_{1B} adrenoceptor. The Figure shows that one of the pools contains a clone of the appropriate size. The Figure also shows the amplification of the hybridisation signal in sub-pools of diminishing size.

10.14.5 Colony hybridisation

As described above, the library was screened by preparing pools containing diminishing numbers of bacteria. The procedure of colony hybridisation was sometimes used to obtain an individual clone from a positive sub-pool containing a cDNA of an appropriate size. This procedure was developed by Grunstein & Hogness (1975) at Stanford University in California. The principle of the procedure is that bacterial colonies are transferred from a culture plate to a membrane. The colonies on the membrane are then lysed *in situ* and their DNA fixed to the membrane which is hybridised to an appropriate probe. Bacteria carrying the clone of interest are detected by autoradiography.

The bacteria from a positive sub-pool were diluted in LB medium and grown on LB ampicillin plates (approximately 100 bacteria on each 15 cm plate). Bacteria containing the PCR product (the probe which had been used to screen the library) were grown on a separate plate and used as a positive control. The bacteria were grown overnight at 37^0C. The following morning, the plate was covered with a nylon membrane (Maximum Strength Nytran; Schleicher & Schuell; 0.45 μm pores) which had been cut to an appropriate shape. The membrane was left on the plate until it had wetted through. The membrane and the underlying plate were marked for orientation by stabbing them with an 18 guage needle. For each membrane, four pieces of Whatmann 3MM paper were cut to an appropriate size and shape and saturated with one of the following solutions:

10% SDS (sodium dodecyl sulphate)

Denaturing solution (0.5 M NaOH and 1.5 M NaCl)

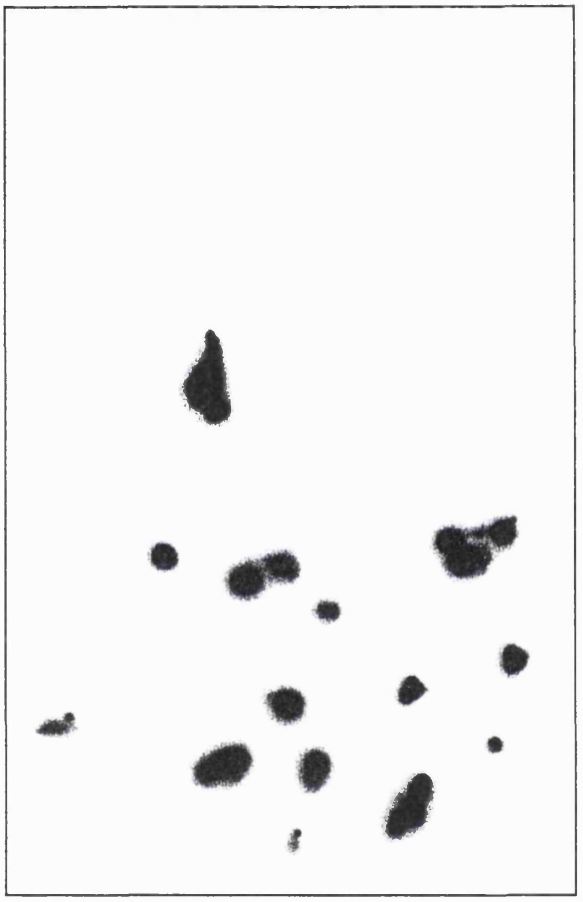
Neutralisation solution (1.5 M NaCl and 1 M Tris base, pH 7.5)

2x SSC (0.3 M NaCl; 0.03 M sodium citrate; pH 7.0)

Excess liquid was poured off to avoid diffusion of the lysed bacterial colonies. Using blunt-ended forceps, the membrane was peeled from its plate and placed, colony side up, on the 10% SDS paper. Air bubbles were removed from under the membrane. After 3 minutes, the membrane was placed on the denaturing solution paper for 5 minutes, then on the neutralisation solution paper for 5 minutes and finally on the 2xSSC paper for 5 minutes. The membrane was dabbed on a dry paper towel after exposure to each solution. At the end of the procedure, the membranes were allowed to dry in air on a paper towel and the DNA was crosslinked to the nylon by exposure to ultraviolet light in the Stratalinker UV Crosslinker (Stratagene). The membrane was wrapped in cellophane and kept at 4^0C until ready for hybridisation to a probe which had been radiolabelled with ^{32}P by random priming, as described above. Positive colonies were detected by autoradiography (Figure 10.14.1). The X-Ray film was aligned to the bacterial culture plate, the positive colony was picked with a sterile toothpick and grown in liquid medium in a shaking incubator. The identity of the cDNA insert was then analysed by restriction analysis followed by hybridisation or by sequencing.

Figure 10.14.1:

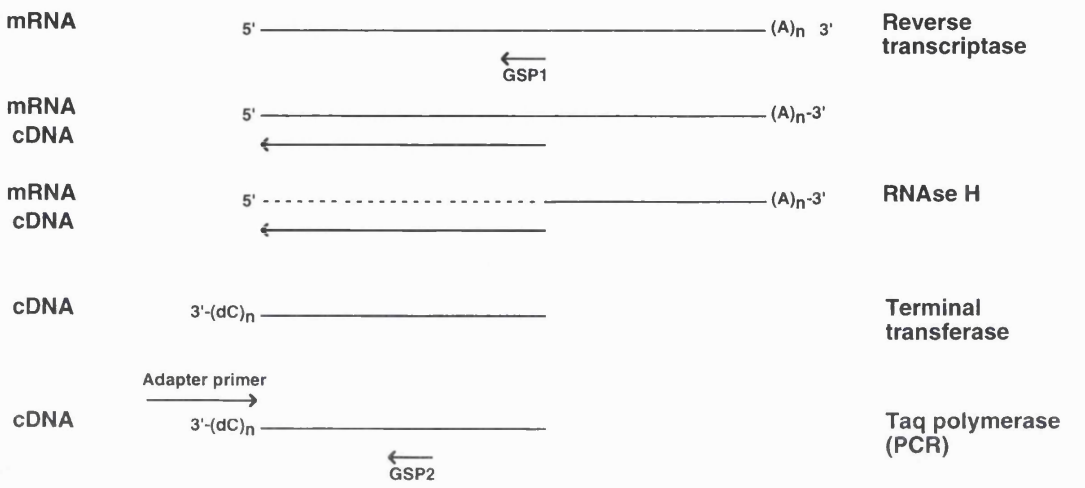
Colony hybridisation: Bacterial colonies were transferred from a culture plate to a membrane. The colonies on the membrane were lysed *in situ* and their DNA fixed to the membrane which was hybridised to an appropriate probe. Bacteria carrying the clone of interest were detected by autoradiography.



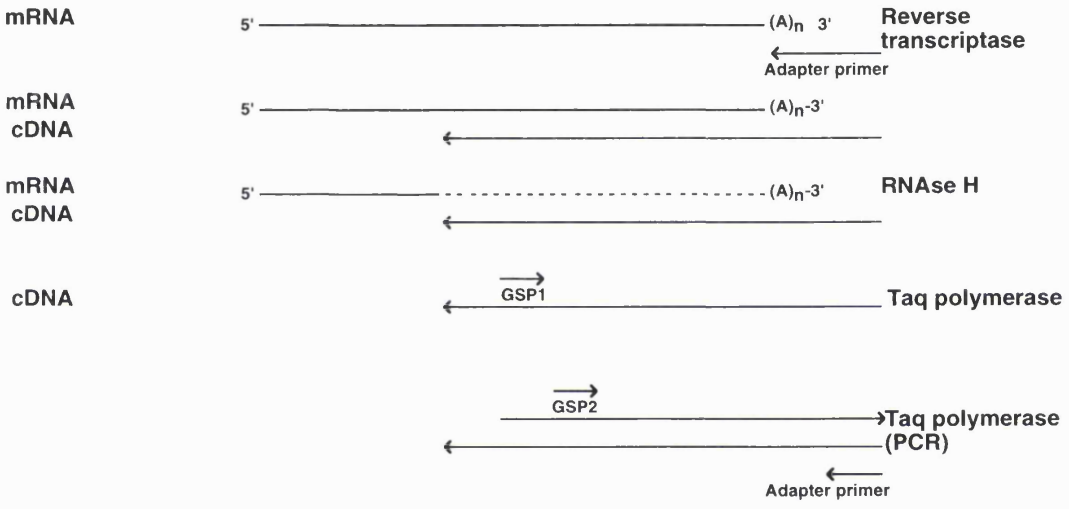
10.15 RAPID AMPLIFICATION OF cDNA ENDS (RACE)

As summarised above, screening a library by colony hybridisation usually results in identification of incomplete cDNAs. In most procedures for construction of libraries, cDNAs are synthesised using an oligonucleotide poly(dT) primer which anneals to the poly(A) tail at the 3' end of messenger RNA. In view of the secondary structure which is inherent in RNA molecules, the reverse transcriptase does not transcribe RNA molecules with equal efficiency, and many of the cDNAs lack the 5' ends. Despite the development of strategies for increasing the efficiency of reverse transcriptases, obtaining the 5' ends of cDNAs remains a challenging task. Less commonly, the 3' ends of cDNAs may also be absent. This may be due to annealing of the oligonucleotide poly d(T) primer to a region in the coding sequence which is rich in A residues, and which is upstream of the poly(A) tail. Frohman et al (1988) in California developed a strategy for obtaining the ends of cDNAs using a procedure which is based on knowledge of a single, short sequence of the cDNA, without knowledge of its 3' or 5' ends. The procedures can be summarised as follows:

A. 5' RACE: To obtain the 5' end of the cDNA of interest, the RNA is reverse transcribed using a gene-specific primer (GSP1). This generates the (-) strand of the cDNA. The RNA is degraded using RNAseH, the cDNA is purified and a homopolymeric tail is added to the 3' end of the cDNA using terminal deoxynucleotidyl transferase. The (+) strand is synthesised using a primer which anneals to the homopolymeric tail, and which has an "adapter" sequence at its 5' end. The resulting cDNA is then amplified by PCR using a second gene-specific primer (GSP2) and a primer which anneals to the adapter sequence. The second gene-specific primer is located in a position which is upstream of the first gene-specific primer; this internal location increases the specificity and efficiency of the amplification, as GSP2 should only amplify the cDNA of interest, and should not bind to cDNAs which were generated by mismatch of GSP1 to irrelevant RNA molecules.



B. 3' RACE: To obtain the 3' end of the cDNA, the RNA is reverse transcribed using an oligonucleotide d(T) primer which has an "adapter" sequence at its 5' end. This generates the (-) strand of cDNAs with adapter sequences. The RNA is degraded using RNAseH and the (+) strand is synthesised using a gene-specific primer (GSP1) which anneals to the (-) strand. The resulting cDNA is then amplified by PCR using a second (internal) gene-specific primer (GSP2) and a primer which anneals to the adapter sequence. The adapter sequence is used for amplification instead of the d(T) sequence because long stretches of d(T) sequence do not align sufficiently at the high annealing temperatures which are required for specific amplification during the PCR.



The identity of the RACE products can be confirmed by Southern blotting. The RACE product is then cloned and sequenced. It can either be ligated to the rest of the cDNA or its sequence can be used to generate PCR products which are themselves ligated to the rest of the cDNA. The latter approach is preferable for the following reasons:

1. It eliminates the homopolymeric tail which may interfere with translation;
2. It minimises sequence errors which may result from multiple PCR amplification cycles;
3. It confirms that the RACE product is indeed present in the original RNA (Frohman, 1990).

Subsequent to the report by Frohman et al (1988), the RACE procedures which are summarised above were also described by other investigators and given other names such as

"one-sided PCR" and "anchored PCR" (Ohara et al, 1989; Loh et al, 1989).

For convenience, RACE procedures were performed using the RACE System from Life Technologies. The kit includes reverse transcriptase, E coli RNAseH, terminal deoxynucleotidyl transferase, adapter primers, nucleotides and buffers. The kit also includes DNA purification spin cartidges which are based on the procedure described by Vogelstein & Gillespie (1979) which was described in Section 10.10.

10.16 EXPRESSION OF cDNA USING LIPOSOMES

Liposomes are vesicles which form spontaneously when isolated natural cell membrane phospholipids are shaken in water. They were discovered by Alec Bangham in Cambridge (reviewed by Bangham, 1995). Bangham was a practising haematologist who was intrigued by the observation that white blood cells and platelets aggregated towards the edge of a blood smear on a microscope slide and in centrifuged whole blood. The latter had long been known as a "buffy coat". In contrast, red blood cells do not ordinarily stick to one another. He left clinical haematology for an appointment at the Institute of Animal Physiology in Babraham and discovered that the "stickiness" of dispersed phospholipids was related to the electric charge which they presented at the surface. Electron microscopic examination of dispersed phospholipids revealed that they were made of concentric rings of unilamellar and multilamellar structures of bimolecular dimension. This appearance was interpreted as representing closed membrane systems in water. Bangham and his colleagues did extensive studies on the ionic permeabilities of liposomes and the effects of ionophores on their artificial systems, and their work established the validity of liposomes as models for cell membranes. There were many practical applications of the scientific work, particularly in the use of liposomes as vehicles for the delivery of genes, drugs and cosmetics. As drug delivery systems, the initial optimism was not realised because following administration *in vivo,* the original lecithin liposomes were rapidly removed by macrophages. This led to the development of newer generations of liposomes which contain hydrophilic substances such as polyethylene glycol ("stealth liposomes") and this makes them less readily recognisable by the immune system (Lasic & Papahadjopoulos, 1995). Such liposomes have been used for delivery of drugs and genes in humans (Crystal, 1995; Lasic, 1996).

Liposomes which carry opposite surface charges tend to aggregate, so positively charged liposomes aggregate and may fuse with negatively charged cell membranes (Stamatatos et al, 1988). These properties have been utilised to deliver genes into mammalian cells. Liposomes which are intended to deliver nucleic acids are composed of polycationic lipids, the first of which was N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA; Felgner et al, 1987). The first cationic liposomes for gene delivery consisted of a mixture of DOTMA and dioleoylphosphatidylethanolamine (DOPE) which is a neutral lipid which allows fusion of the lipid bilayers. These DOTMA liposomes were marketed by Life Technologies under the trade name Lipofectin (Felgner & Ringold, 1989). DNA spontaneously forms complexes with DOTMA and this traps the DNA among the liposomes. The cationic liposomes bind and

fuse with negatively charged lipid membranes such as the plasma membrane, and DNA/liposome complexes are also accumulated by endocytosis (Felgner et al, 1987; Zabner et al, 1995). Cellular uptake of the lipids is temperature dependent and is inhibited by depletion of ATP, which is consistent with properties of endocytosis (Wrobel & Collins, 1995). Both the DNA and the cationic liposomes enter the cells and a substantial proportion of the DNA is located in cytoplasmic vesicles, especially around the nucleus (Zabner et al, 1995). In order for the DNA to be transcribed in the nucleus, it may be important for the DNA to dissociate from the cationic lipid (Zabner et al, 1995). The transfections are preferably performed in serum free media, possibly because of the presence of negatively charged serum components which may interact with the cationic liposomes (Felgner & Ringold, 1989). In many cell types, the efficiency of transfection with cationic liposomes far exceeds the efficiency of transfection with calcium phosphate or DEAE dextran (Felgner et al, 1987; Felgner & Ringold, 1989).

Using high resolution X-Ray scattering, it was shown that when DNA is added to cationic liposomes, the liposomes form multiple sheets of phospholipid bilayers, with the DNA sandwiched as intercalated monolayers (Radler et al, 1997). When appropriate surfactant molecules are added to the DNA-cationic lipid mixture, the multilamellar sheet structure is transformed into a cylindrical arrangement, with the DNA occupying the centre of the cylinder and the phospholipid forming a unilayer surrounding it; these cylindrical micelle structures were observed to be organised in hexagonal groups (Figure 10.16.1). The hexagonal cylinder arrangement was more efficient in fusing with model cell membranes (anionic lipid globules) than the multilamellar sheet structure. Following fusion with the anionic membrane, the lipid/DNA structure was lost; the lipid diffused into the anionic membrane and the DNA entered the model cells (Koltover et al, 1998).

In addition to DOTMA, several other cationic lipids were synthesised for use in liposomes which are intended to introduce DNA into mammalian cells. Lipofectamine (Life Technologies) is a 3:1 liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and DOPE. Lipofectamine was used for some of the transfections in the present work.

COS-7 cells, GT1-1 cells or DDT_1 MF-2 cells were grown in Nunc 175 cm^2 flasks (approximately 10^7 cells/flask). The transfection procedure was started three to four days

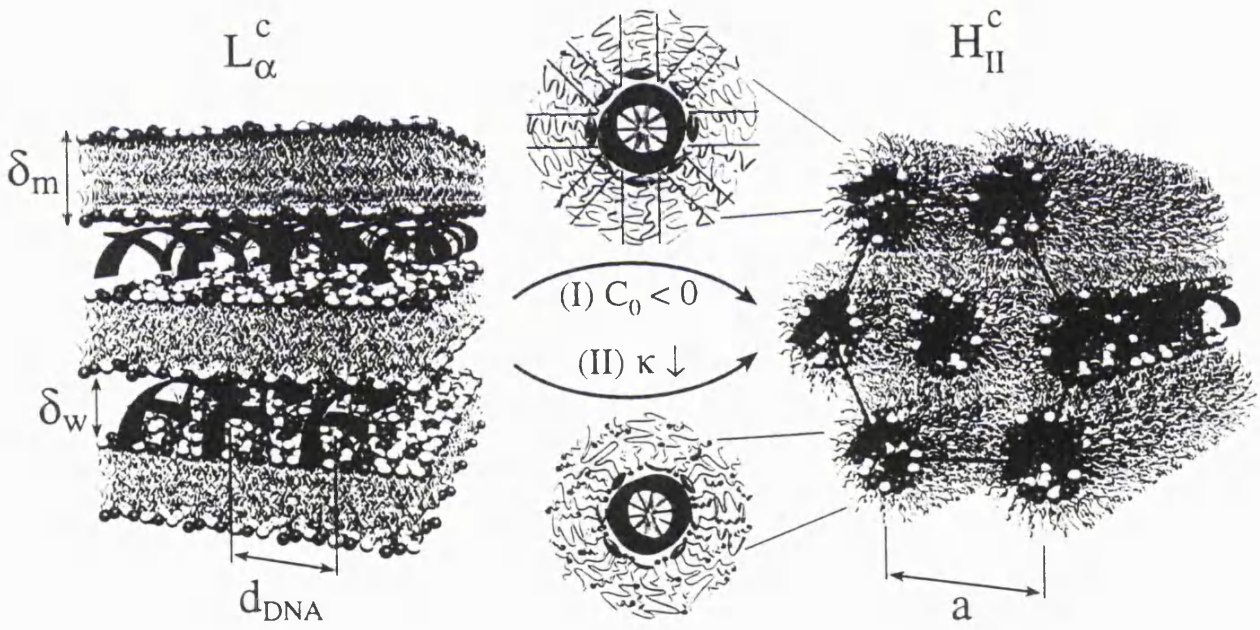
later, while the cells were still sub-confluent. On the first day, the cells were dispersed, diluted in culture medium to a density of 10^5 cells/ml and incubated in 6-well plates which had been coated with poly-D-lysine and laminin ($2x10^5$ cells/well; 22,000 cells/cm^2). The transfection was performed 20-24 hrs later, at which time the cells were 60-70% confluent. Serum-free medium (Opti-MEM, Life Technologies) 200 µl was mixed with liposomes (Lipofectamine, Life Technologies) and plasmid DNA (1 µg/µl). In preliminary experiments, the amounts of Lipofectamine and DNA were optimised for each cell line. The suspension was mixed gently and incubated at room temperature for 45 minutes to allow the formation of DNA-liposome complexes. The suspension was then diluted to 1 ml (by adding 800 µl of serum-free medium). The cells were washed once with 2 ml serum-free medium and the DNA/liposome suspension (1 ml) was then added to the cells which were incubated at 37^0C in 5% CO_2. Twenty four hours later, the transfection mixture was removed and 2 ml of culture medium was added to each culture well. Assays for binding or uptake were performed 24 hours later.

Lipofectamine was found to inhibit Transport-P in GT1-1 GnRH peptidergic neurones. This can be seen in the Table below, from which it is clear that Lipofectamine inhibits the paradoxical increase in accumulation of [^{3}H]prazosin at unlabelled prazosin 10^{-6} M. This inhibitory effect is presumably attributable to the cationic nature of Lipofectamine which would result in neutralisation of the intracellular acidity which is required for the function of Transport-P (Section 11.3.2).

Lipofectamine concentration	*[^{3}H]prazosin cpm/well*	
	Control	*Unlabelled prazosin 10^{-6} M*
None	710 ± 26	1453 ± 70
2 ul/ml	684 ± 27	829 ± 26
3 ul/ml	641 ± 15	689 ± 20

Figure 10.16.1:

Schematic model of DNA-liposome complexes based on high resolution X-ray scattering. When DNA is added to cationic liposomes, the liposomes form multiple sheets of phospholipid bilayers, with the DNA sandwiched as intercalated monolayers (left). When appropriate surfactant molecules are added to the DNA-cationic lipid mixture, the multilamellar sheet structure is transformed into a cylindrical arrangement, with the DNA occupying the centre of the cylinder and the phospholipid forming a unilayer surrounding it; these cylindrical micelle structures were observed to be organised in hexagonal groups. Reproduced from Koltover et al, 1998.



10.17 EXPRESSION OF cDNA BY ELECTROPORATION

When cells are exposed to an electric field, a large membrane potential may develop across the two poles of the cell, resulting in damage to the cell membrane and formation of membrane pores. These membrane pores have been visualised using rapid freezing microscopy techniques (Chang et al, 1992). The proportion of cells which die depends on the intensity and the duration of the electric field. If the electric field is applied as a very short pulse, some cells may be able to recover, presumably by resealing the pores. Providing that the electroporation conditions are mild, the cells which recover do not seem to undergo detectable alterations in their functional or structural features. Molecules in the external medium may diffuse into the cells while the membranes are porous. Electroporation can therefore be used as a method for introducing molecules into cells. The variety of substances which have been introduced into cells by electroporation ranges from small molecules such as catecholamines to much larger molecules such as DNA. In general, electroporation of mammalian cells with DNA results in more efficient expression of proteins than chemical methods (Chang et al, 1992).

COS-7 cells or GT1-1 immortalised GnRH neurones were grown in 175 cm^2 flasks (approximately $2x10^7$ cells/flask) for 2-4 days and culture media were changed at 48 hour intervals. The cells were then dispersed in the presence of trypsin, DNAseI and EDTA and washed twice in 10 ml culture medium (Section 10.1). The cells were then concentrated to a density of $1.2x10^8$ cells/ml of culture medium. An aliquot of the cell suspension (250 µl; $3x10^7$ cells) was placed in Bio-Rad cuvettes (inter-electrode distance 0.4 cm) and 10 µg of plasmid DNA dissolved in TE buffer (1 µg/µl) was added. In control experiments, TE buffer without DNA was added. The suspension was then mixed and electroporated at room temperature in a Bio-Rad Gene Pulser II device with a Capacitance Extender Plus and Pulse Controller Plus. Electroporation parameters were set as follows:

GT1-1 GnRH neurones: 190 V, 1000 uF;

COS-7 kidney cells: 170 V, 950 uF.

After 60 seconds, culture medium was added to the cuvette and the cell suspension was transferred to a total of 25 ml culture medium ($1.2x10^6$ electroporated cells/ml). The cells were then incubated in 12-well culture plates (2 ml/well) which had been coated with poly-D-

lysine and laminin. The cells were grown at 37^0C in the presence of 5% CO_2 in air. Binding and uptake studies were carried out two days after electroporation using the methods which are described in Sections 10.2 and 10.3.

Electroporation had no effect on Transport-P in the GT1-1 GnRH peptidergic neurones. This can be seen in the Table below, from which it is clear that electroporation (in the absence of DNA) has no effect either on the paradoxical increase in accumulation of [^{3}H]prazosin at unlabelled prazosin 10^{-6} M, or on the inhibition of uptake by desipramine.

	[^{3}H]prazosin cpm/well	
	Control cells	*Electroporated cells*
Control	1800 ± 86	1598 ± 66
Desipramine 10^{-5} M	1302 ± 24	1154 ± 24
Unlabelled prazosin 10^{-6} M	3528 ± 70	3345 ± 135
Desipramine + unlabelled prazosin	899 ± 15	851 ± 31

10.18 SCREENING THE cDNA LIBRARY BY EXPRESSION

In this procedure, pools were prepared from the cDNA library, each containing approximately 1000 bacteria. The bacteria were amplified overnight in a semi-solid medium to ensure representative amplification of the clones (see Section 10.14). Some of the bacteria from each aliquot were stored and the plasmid DNA extracted from the remainder using Qiagen kits (Section 10.10). The plasmid DNA from the pools was expressed in COS-7 cells by electroporation, as this expression method has no effect on Transport-P (Section 10.17). The GnRH neurone α_{1B} adrenoceptor cDNA was used as the positive control in the electroporation procedure, as it is cloned in the same pSVSPORT1 plasmid in which the cDNA library had been constructed. The negative control was the TE buffer in which the DNA had been dissolved. Each electroporation cuvette contained approximately $2x10^6$ cells and approximately 10 μg DNA in 250 μl culture medium. The cells were then electroporated at 170 V and 950 μF. After 60 seconds, the cells were transferred to 4 ml culture medium, mixed by pipetting and placed in 6-well plates (2 ml/well) which had been coated with poly-

D-lysine (0.5 $\mu g/cm^2$) and laminin (0.05 $\mu g/cm^2$). The cells were then allowed to grow at 37^0C in a humidified atmosphere containing 5% CO_2 in air. Forty eight hours later, the cells were examined for expression of Transport-P, by assaying for the uptake of [^{3}H]prazosin in the presence of unlabelled prazosin 10^{-6} M; at this concentration of unlabelled prazosin, Transport-P is activated and binding of [^{3}H]prazosin to α_1 adrenoceptors is completely blocked (Section 10.3 and Figures 11.1.8 & 11.1.15). The duplicate well from each electroporation was used to assay for the uptake of prazosin 10^{-6} M in the presence of desipramine 10^{-4} M, in order to ensure that accumulation of prazosin is indeed sensitive to antidepressants, as is the case with Transport-P. Assay for the positive control (the α_{1B} adrenoceptor) was by measuring the binding of [^{3}H]prazosin (B_0) in one well, and by confirming that the increased [^{3}H]prazosin binding is displacable by unlabelled prazosin (at 10^{-6} M) in the duplicate well. A positive pool was defined as one which causes increased accumulation of prazosin (at 10^{-6} M) which is reversible by desipramine (at 10^{-4} M). When a positive pool is found, a representative number of bacteria from that pool are subdivided into progressively smaller fractions, until the bacterium of interest is identified.

11. RESULTS

11.1 α_1 ADRENERGIC RECEPTORS IN GnRH NEURONES

[^{3}H]Prazosin binds to peptidergic neurones and is displaceable by unlabelled prazosin in concentrations up to 10^{-7} M. However, at greater concentrations of prazosin, there is a paradoxical accumulation of [^{3}H]prazosin which was attributed to Transport-P. The work which is described in this Section was aimed to test the hypothesis that in GnRH peptidergic neurones, the displacement of [^{3}H]prazosin by unlabelled prazosin is due to the presence of α_1 adrenergic receptors in the GnRH neurones.

11.1.1 Properties of prazosin binding in GnRH neurones and in other cells

Figures 11.1.1 and 11.1.2 compare the accumulation of prazosin in GnRH neurones to the accumulation of noradrenaline in SK-N-SH noradrenergic neurones. The lower panels demonstrate the accumulation of radiolabelled ligand, and the upper panels demonstrate accumulation of total ligand (labelled and unlabelled) by correcting for the fall in specific activity consequent upon isotope dilution. At a concentration of 2.1×10^{-10} M, [^{3}H]prazosin associates with the peptidergic neurones and is displaced by unlabelled prazosin in concentrations up to 3.33×10^{-7} M (binding at 37^{0}C: B_0: 4285 ± 50 dpm/well, unlabelled prazosin 3.33×10^{-7} M: 3791 ± 38 dpm/well; Figure 11.1.1B). However, at greater concentrations of unlabelled prazosin, there was a paradoxical increase in the binding of [^{3}H]prazosin, as previously described (Al-Damluji et al, 1993). At 0^{0}C, accumulation of prazosin is less than at 37^{0}C but [^{3}H]prazosin still binds and is displaced by unlabelled prazosin (binding at 0^{0}C: B_0: 2885 ± 88 dpm/well, unlabelled prazosin 3.33×10^{-7} M: 2185 ± 47 dpm/well; Figure 11.1.1B). In addition, cooling the cells to inhibit transport processes, abolished the paradoxical increase in accumulation of [^{3}H]prazosin (Figure 11.1.1B). Accumulation of prazosin in the peptidergic neurones at 0^{0}C was linear with concentration up to at least 10^{-6} M, indicating that it represented non-specific accumulation (Figure 11.1.1A). Hence, in these experiments, non-specific uptake was defined as uptake at 0^{0}C. Specific uptake was obtained by subtracting non-specific uptake from total uptake. At

concentrations of prazosin greater than 10^{-7} M, the specific accumulation of prazosin was non-linear (Figure 11.1.1A).

SK-N-SH noradrenergic neurones are known to possess the pre-synaptic re-uptake process for noradrenaline ($Uptake_1$; Section 10.1.2.2). At 37^0C, [^{3}H]noradrenaline binds to noradrenergic neurones and is displaced by unlabelled noradrenaline. There was no increase in [^{3}H]noradrenaline binding at concentrations of unlabelled noradrenaline greater than 10^{-7} M (Figure 11.1.2B). At 0^0C, accumulation of noradrenaline was less than at 37^0C (B_0 214 ± 23 dpm/well *vs* 3832 ± 73 dpm/well). Accumulation of noradrenaline in noradrenergic neurones at 0^0C was linear with concentration throughout, indicating that it represented non-specific accumulation (Figure 11.1.2A). The concentration-dependance of specific accumulation of noradrenaline was parabolic and tended to saturate at noradrenaline concentrations greater than 10^{-7} M (Figure 11.1.2A).

In DDT_1 MF-2 smooth muscle cells which are known to express native α_{1B} adrenoceptors, [^{3}H]prazosin bound to the cells and was displaced by unlabelled prazosin in the concentration range 10^{-10} to 10^{-7} M (K_D $4.8x10^{-10}$ M). There was no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M (Figure 11.1.3).

SK-N-SH noradrenergic neurones and COS-7 kidney cells took up only small amounts of prazosin, and this was unaffected by 10^{-5} M desipramine. Further, there was no increase in the uptake of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M in either type of cell (Figure 11.1.4).

11.1.2 Molecular identification of α_{1B} adrenoceptors in GnRH neurones

PCR primer pairs were derived from regions which are homologous in the sequences of the α_1 adrenergic receptor cDNAs (details in Table 11.1.1 and Figure 11.1.5). Using GnRH neurone RNA as template, it was possible to detect a PCR product of the appropriate size in reactions which used the A1BF2/A1BB9 primer pair in a first PCR, followed by the

A1BF1/A1BB1 nested primer pair in a second PCR (Figure 11.1.6). Further, using the GnRH neurone cDNA library as template, it was possible to detect PCR products of the appropriate sizes in reactions which used the A1BF2/A1BB9, A1BF4/A1BB4 and A1BF5/A1BB5 primer pairs (Figure 11.1.6). These PCR products were cloned using the TA technique and sequenced using methods which are described in Sections 10.12 and 10.13. Analysis of the translated sequences of these three PCR products indicated that the sequences were 99.2% identical to the translated sequence of the mouse α_{1B} adrenoceptor (GenBank accession number Y12738; Figure 11.1.7).

GT1-1 is a mouse cell line. The mouse, rat and human α_{1B} adrenoceptor genes contain a large intron whose splice site is in the sixth transmembrane domain (Figure 11.1.7; Ramarao et al, 1992; Gao & Kunos, 1993; Zuscik et al, 1999). This splice site is flanked by the A1BF4/A1BB4 PCR primer pair. The sequence of the PCR product which was generated by this primer pair contained no intronic sequences, demonstrating that the reactions amplified mRNA rather than contaminating genomic DNA.

11.1.3 Prazosin binding in COS cells transfected with α_{1b} cDNA

In COS-7 cells which had been transfected by electroporation with the hamster smooth muscle α_{1b} adrenoceptor cDNA, [^{3}H]prazosin bound to the cells and was displaced by unlabelled prazosin in the concentration range 10^{-10} to 10^{-7} M. The IC_{50} of prazosin for these binding sites was $2x10^{-9}$ M and the K_D was $5x10^{-10}$ M. There was no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M (Figure 11.1.8). In these experiments, non-specific binding was defined as the amount of prazosin accumulated in control cells which had been electroporated in the absence of DNA. Specific binding of prazosin to COS-7 cells which had been transfected with α_{1b} adrenoceptor cDNA reached saturation at approximately 10^{-8} M.

11.1.4 Effects of α_1 agonists on [^{3}H]prazosin binding in DDT_1 MF-2 cells

In DDT_1 MF-2 smooth muscle cells which express native α_{1B} adrenoceptors, bound

[^{3}H]prazosin was displaced by the selective α_1 adrenergic agonists methoxamine and phenylephrine (K_D $2x10^{-4}$ M and $1.9x10^{-5}$ M, respectively) and by the non-selective endogenous agonists adrenaline and noradrenaline (K_D $6.2x10^{-6}$ M and 10^{-5} M). None of these agonists caused an increase in the binding of [^{3}H]prazosin (Figure 11.1.9).

11.1.5 Over-expression of α_{1B} adrenoceptors in GnRH neurones

In GT1-1 GnRH cells which had been transfected by electroporation with hamster smooth muscle α_{1b} adrenoceptor cDNA, [^{3}H]prazosin (at $2.5x10^{-10}$ M) binding was greater than in control (mock-transfected) cells (B_0: control transfection 4262 ± 107 dpm/mg protein; with α_{1b} transfection 7416 ± 69 dpm/mg protein; Figure 11.1.10). In both groups of cells, [^{3}H]prazosin was displaced by unlabelled prazosin in concentrations up to $3.33x10^{-7}$ M (control 3080 ± 64 dpm/mg protein; with α_{1b} transfection 3120 ± 104 dpm/mg protein; Figure 11.1.10). At greater concentrations of unlabelled prazosin, the binding of [^{3}H]prazosin increased in both the control cells and in the cells which had been transfected with α_{1b} adrenoceptor cDNA. There was no difference in the increase between the two groups of cells (Figure 11.1.10).

11.1.6 Effect of clonidine on [^{3}H]prazosin binding in GnRH neurones

The α_2 adrenergic agonist clonidine displaced [^{3}H]prazosin (at $2.1x10^{-10}$ M) from GT1-1 cells (B_0: 3610 ± 94 dpm/mg protein; clonidine 10^{-3} M: 2487 ± 50 dpm/mg protein; Figure 11.1.11). There was no increase in the binding of [^{3}H]prazosin at concentrations of clonidine up to 10^{-3} M (Figure 11.1.11).

11.1.7 Isolation & expression of the α_{1b} adrenoceptor from GnRH neurones

The sequence of the PCR products was similar, but not identical to the known α_{1B} subtype of α_1 adrenoceptors (Figure 11.1.7). In order to study the functional properties of this new

alpha-1 adrenoceptor, the cDNA library was screened for a full-sized, functional clone, using the methods which are described in Section 10.14. The cDNA library consists of $2x10^6$ recombinants. The library was divided into pools, each containing 63,000 bacteria. The bacteria were amplified by overnight growth in a semi-solid medium, to ensure representative amplification of the clones. The plasmid DNA was extracted and digested with *Mlu* I which separates the plasmid from the insert. One μg of digested DNA from each pool was then electrophoresed in 0.8% agarose and transferred to a nylon membrane. One of the α_{1B} adrenoceptor PCR products was labelled with ^{32}P using random hexamers and used as a probe in a hybridisation procedure. One of the pools (pool 14) was positive, demonstrating a hybridisation band of 1800 bp (Figure 11.1.12). This pool was tested with the PCR primers which had been used previously; all the primers yielded PCR products of the appropriate sizes, indicating that the pool contains a clone which is likely to have the full coding sequence of the α_{1B} adrenoceptor. Sub-pools were prepared from pool 14, containing 6,750 bacteria and treated in the same manner. One of these sub-pools (sub-pool 37) was positive, demonstrating the same hybridisation band which was now amplified, due to enrichment of the target molecule. The procedure was repeated using further sub-pools, containing diminishing numbers of clones. Finally, colony hybridisation was used to obtain an individual, positive clone. The translated sequence of this clone is identical to the sequences of the PCR products. The clone has a shorter carboxylic terminal tail than the hamster and mouse α_{1B} adrenoceptors (Figure 11.1.13); this is likely to be due to a cloning artefact, as the poly(A) tail was not present in the clone. Membrane-spanning domains were determined with the Kyte-Doolittle algorithm using a function in the MacVector software on a Macintosh computer (Figure 11.1.14).

In COS-7 cells which had been transfected by electroporation with the GnRH neurone α_{1b} adrenoceptor cDNA, [^{3}H]prazosin bound to the cells and was displaced by unlabelled prazosin in the concentration range 10^{-10} to 10^{-7} M. The K_D of prazosin for these binding sites was $4x10^{-10}$ M. There was no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M (Figure 11.1.15). In these experiments, non-specific binding was defined as the amount of prazosin accumulated in control cells which had been

electroporated in the absence of DNA. Specific binding of prazosin to COS-7 cells which had been transfected with the GnRH neurone α_{1b} adrenoceptor cDNA reached saturation at approximately 10^{-8} M.

Following expression in COS-7 cells, the affinities of a range of adrenergic agonists, antagonists and antidepressants for the GnRH neurone α_{1B} adrenoceptor were identical to their affinities for the hamster smooth muscle α_{1B} adrenoceptor (not shown).

11.1.8 Comment

In previous work, we had demonstrated that immortalised GnRH neurones possess α_1 adrenoceptors, as indicated by binding of prazosin and its displacement by unlabelled prazosin (Al-Damluji et al, 1993). The present work confirms that these neurones express an α_1 adrenoceptor gene and demonstrates that its molecular sub-type is α_{1B}. The presence of these receptors in GnRH neurones is sufficient to account for the displacement of [^{3}H]prazosin by unlabelled prazosin in the concentration range 10^{-9} to 10^{-7} M. However, a simple binding process at these receptors would obviously not explain the paradoxical increase above that concentration range. I therefore investigated the possibility that this prazosin paradox in peptidergic neurones may be due to some post-binding event at α_1 adrenoceptors, such as internalisation of ligand-receptor complexes. COS-7 cells transfected with hamster α_{1b} adrenoceptor cDNA expressed abundant α_{1B} adrenoceptors, as indicated by the great increase in [^{3}H]prazosin binding (Figure 11.1.8). However, there was no prazosin paradox in these transfected cells; the accumulation of prazosin was in accord with simple equilibrium and reached saturation at approximately 10^{-8} M. It therefore seemed unlikely that the prazosin paradox is due to some function of α_1 adrenoceptors.

Nevertheless, the α_{1B} adrenoceptors in these COS-7 cells were not in a native environment; I therefore studied DDT_1 MF-2 cells which express native α_{1B} adrenoceptors and which are known to internalise these receptors upon exposure to the agonists adrenaline, noradrenaline and phenylephrine (Cornett & Norris, 1982; Fratelli & DeBlasi, 1987; Cotecchia et al,

1988). As in the transfected COS-7 cells, there was no prazosin paradox in DDT_1 MF-2 cells (Figure 11.1.3). In any case, receptor internalisation seemed an unlikely explanation since prazosin is an α_1 adrenoceptor antagonist but internalisation of ligand-receptor complexes usually follows binding of an agonist to its receptor. I therefore examined the effects of a series of adrenergic agonists which included both the endogenous non-selective neurotransmitters adrenaline and noradrenaline, and synthetic selective agonists. All these agonists displaced [^{3}H]prazosin from α_{1B} adrenoceptors in DDT_1 MF-2 cells, but the prazosin paradox was not seen in any of these experiments (Figure 11.1.9). The conclusion is that receptor internalisation is not a factor in these experiments.

The prazosin paradox was observed in peptidergic neurones but DDT_1 MF-2 are smooth muscle cells which may not possess the specialised neuronal components which are required for function of neuronal receptors. The α_{1B} adrenoceptors were therefore over-expressed in the GnRH peptidergic neurones, to see if this would enhance the prazosin paradox in these neurones. However, the prazosin paradox was unaffected by over-expression of α_{1B} adrenoceptors, indicating that it is mediated by a different component of the GnRH neurones (Figure 11.1.10).

The translated sequence of the PCR products was similar, but not identical to the sequence of the known α_{1B} adrenoceptor. In order to assess the possible relevance of these sequence differences to the phenomena which are under study, the cDNA library was screened for the α_{1b} adrenoceptor clone. Despite the extensive sequence homology between the cloned α_1 adrenoceptors, the screening procedure yielded only one clone whose sequence was that of an α_{1B} adrenoceptor; this was consistent with the results of the PCR experiments in which it was concluded that GnRH neurones possess only α_{1B} adrenoceptors (Section 11.1.2). Electroporation of COS-7 cells with this GnRH neurone α_{1b} adrenoceptor clone resulted in expression of α_{1B} adrenoceptors but did not result in expression of the functional properties of Transport-P (Figure 11.1.15).

From all of the foregoing, it is clear that the prazosin paradox is unlikely to be due to some function of α_1 adrenoceptors. The paradoxical increase in binding of [^{3}H]prazosin in the presence of greater concentrations of unlabelled prazosin is likely to be due, instead, to an unusual uptake process whose functional properties are distinct from those of α_1 adrenoceptors. Further, the structural properties of ligands for Transport-P are different from those of ligands for α_1 adrenoceptors (see Section 11.5 and Table 11.5.2).

The sequence of the GT1-1 GnRH neurone α_{1B} adrenoceptor is almost identical to previously cloned α_{1B} adrenoceptors (Figure 11.1.13), and the minor differences are likely to have no functional significance. Thus, there are no differences between the GnRH neurone sequence and the hamster smooth muscle sequence within any of the membrane-spanning domains, which are the sites of ligand binding (Savarese & Fraser, 1992); accordingly, the affinities of adrenergic agonists and antagonists were identical for the two receptors. The third intracellular loop plays a major role in coupling these receptors to G proteins (Cotecchia et al, 1990; Luttrell et al, 1993; Hawes et al, 1994); this region of the GnRH neurone α_{1B} adrenoceptor is identical to the hamster smooth muscle receptor (Figure 11.1.13). Coupling to G proteins also appears to be mediated by the sequence NPIIY which is located near the end of the seventh membrane spanning domain (Wang et al, 1997); this region is conserved in the GnRH neurone α_{1B} adrenoceptor. The carboxyl terminus tail of the hamster smooth muscle α_{1B} adrenoceptor is important for desensitisation and internalisation of the receptor following exposure to adrenaline (Lattion et al, 1994); these effects are mediated by phosphorylation of serine residues which are located in positions 404, 408 and 410, in the carboxyl terminus tail (Diviani et al, 1997); these residues are also present in the GnRH neurone receptor. The GnRH neurone α_{1B} adrenoceptor clone ends at the arginine in position 459; however, mutagenesis studies have demonstrated that hamster smooth muscle α_{1B} adrenoceptors which are truncated at residues 412, 425 or 469 have full physiological functions, including normal ligand affinities and activation of G proteins, and are normally phosphorylated, desensitised and internalised following exposure to adrenaline (Diviani et al, 1997; Wang et al, 2000). Therefore, the terminal amino acids which are

missing from the GnRH neurone α_{1B} adrenoceptor clone are unlikely to be functionally important. It is possible to obtain the missing 3' ends of clones by the technique of 3' RACE (Section 10.15); however, this was deemed unnecessary for the purpose of the present work.

In our previous work on GT1-1 cells, we were unable to detect the presence of α_2 adrenoceptor binding sites using the ligand [^{3}H]idazoxan (RX781094; Al-Damluji et al, 1993). However, Lee et al (1995) subsequently used another ligand, RX821002, and demonstrated the presence of α_{2A} binding sites in a related cell line, GT1-7 cells. They corroborated the finding by demonstrating RNA and immunoreactivity which is appropriate for α_{2A} adrenergic receptors in the GT1-7 cells. At this stage, it is unclear whether these apparent differences are due to technical factors or to some genuine difference between the GT1-1 and the GT1-7 cells; in the mouse and rat brain, many cells which are immunoreactive to GnRH do not possess α_{2A} adrenoceptors (Lee et al, 1995). It is therefore possible that the GT1-1 and the GT1-7 cell lines may have been derived from subsets of GnRH neurones which differ in the expression of α_2 adrenoceptors. In any case, presence of α_2 adrenoceptors does not account for the functional properties of Transport-P for the following reasons: a) The structural properties of ligands for Transport-P are different from the properties of ligands for α_2 adrenoceptors (see Section 11.5 and Table 11.5.2); b) The α_2 adrenergic agonist clonidine does not cause a paradoxical increase in accumulation of [^{3}H]prazosin in the GnRH neurones (Figure 11.1.11). The displacement of [^{3}H]prazosin by clonidine is attributable to the known agonist action of clonidine at α_1 adrenoceptors (Nichols & Ruffolo, 1991).

In the present study, cooling the cells to 0^0C was used as an experimental control, in order to inhibit uptake without affecting binding to the α_1 adrenoceptors. The same experimental control was used to study the uptake of noradrenaline in noradrenergic neurones, in order to

enable comparison of Transport-P in peptidergic neurones to $Uptake_1$ in noradrenergic neurones. The results distinguish Transport-P from $Uptake_1$; in noradrenergic neurones, there was no increase in the accumulation of [^{3}H]noradrenaline in the presence of concentrations of unlabelled noradrenaline greater than 10^{-7} M. Uptake of noradrenaline is linear up to 10^{-7} M and begins to saturate at greater concentrations of the amine (Figure 11.1.2). Further, there is no evidence for specific accumulation of prazosin in the noradrenergic neurones (Figure 11.1.4), which further distinguishes Transport-P from $Uptake_1$. Inability to accumulate prazosin in SK-N-SH noradrenergic neurones is explicable by the presence in prazosin of phenolic methoxyl groups which inhibit $Uptake_1$ (Iversen, 1967). In contrast, Transport-P is unaffected by phenolic methoxyl groups (Section 11.5 and Table 11.5.2). Non-neuronal cells such as muscle and glia possess an uptake process for amines ($Uptake_2$) which is insensitive to antidepressants and reserpine but is blocked by steroid hormones (Iversen, 1965; Russ et al, 1996; Sections 7.5.10 & 7.5.11). The work reported here suggests that Transport-P does not exist in non-neuronal cells. Thus, there was no antidepressant-blockable prazosin paradox in DDT_1 MF-2 smooth muscle cells or in COS-7 kidney cells (Figures 11.1.3 & 11.1.4). The absence of a desipramine-sensitive prazosin paradox in noradrenergic neurones, in kidney cells and in smooth muscle cells suggests that Transport-P is a specialised function of peptidergic neurones.

Under the present culture conditions, the GT1-1 GnRH cells express the α_1 adrenoceptors at relatively low levels, possibly due to the absence of physiological factors which are known to induce the expression of α_1 adrenoceptors, such as glucocorticoids and gonadal steroids (Sakaue & Hoffman, 1991; Petitti et al, 1992; Karkanias et al, 1996). *In vivo,* GnRH neurones are densely innervated by noradrenergic nerve terminals which modulate reproductive function via their action on α_1 adrenoceptors in the hypothalamus (Barraclough & Wise, 1982; Kalra & Kalra, 1983; Rosie et al, 1994; Le et al, 1997; Herbison, 1997). Previously, we demonstrated the existence of α_1 adrenoceptor binding sites in GnRH neurones (Al-Damluji & Krsmanovic, 1992; Al-Damluji et al, 1993); the present work identifies these receptors as being of the α_{1B} sub-type. In the rat, GnRH neurones are located in the pre-optic area of the hypothalamus, and this part of the hypothalamus has been

shown to contain α_{1B} adrenoceptors (Petitti et al, 1992; Karkanias et al, 1996). The density of these α_{1B} adrenoceptors in the pre-optic area is modulated by gonadal steroids, suggesting that at least some of the α_{1B} adrenoceptors in the pre-optic area are located on GnRH neurones (Petitti et al, 1992; Karkanias et al, 1996). Thus, the finding in the present study that immortalised GnRH neurones possess α_{1B} adrenoceptors is consistent with pharmacological evidence obtained in the native state in rat brain slices. Identification of the molecular subtype of α_1 adrenoceptors in GnRH neurones should help to further future work on the molecular mechanisms of the adrenergic control of puberty and reproduction.

Figure 11.1.1:

Uptake of prazosin in peptidergic neurones (GT1-1 GnRH cells). The lower panel demonstrates the uptake of radiolabelled ligand; the upper panel demonstrates uptake of total ligand (labelled and unlabelled), ie, it is corrected for the fall in specific activity of [^{3}H]prazosin due to isotope dilution. Non-specific uptake was defined as uptake at 0^0C. "B_0" is an abbreviation for binding of the radiolabelled ligand in the absence of unlabelled ligand. Standard error bars were smaller than the sizes of the symbols.

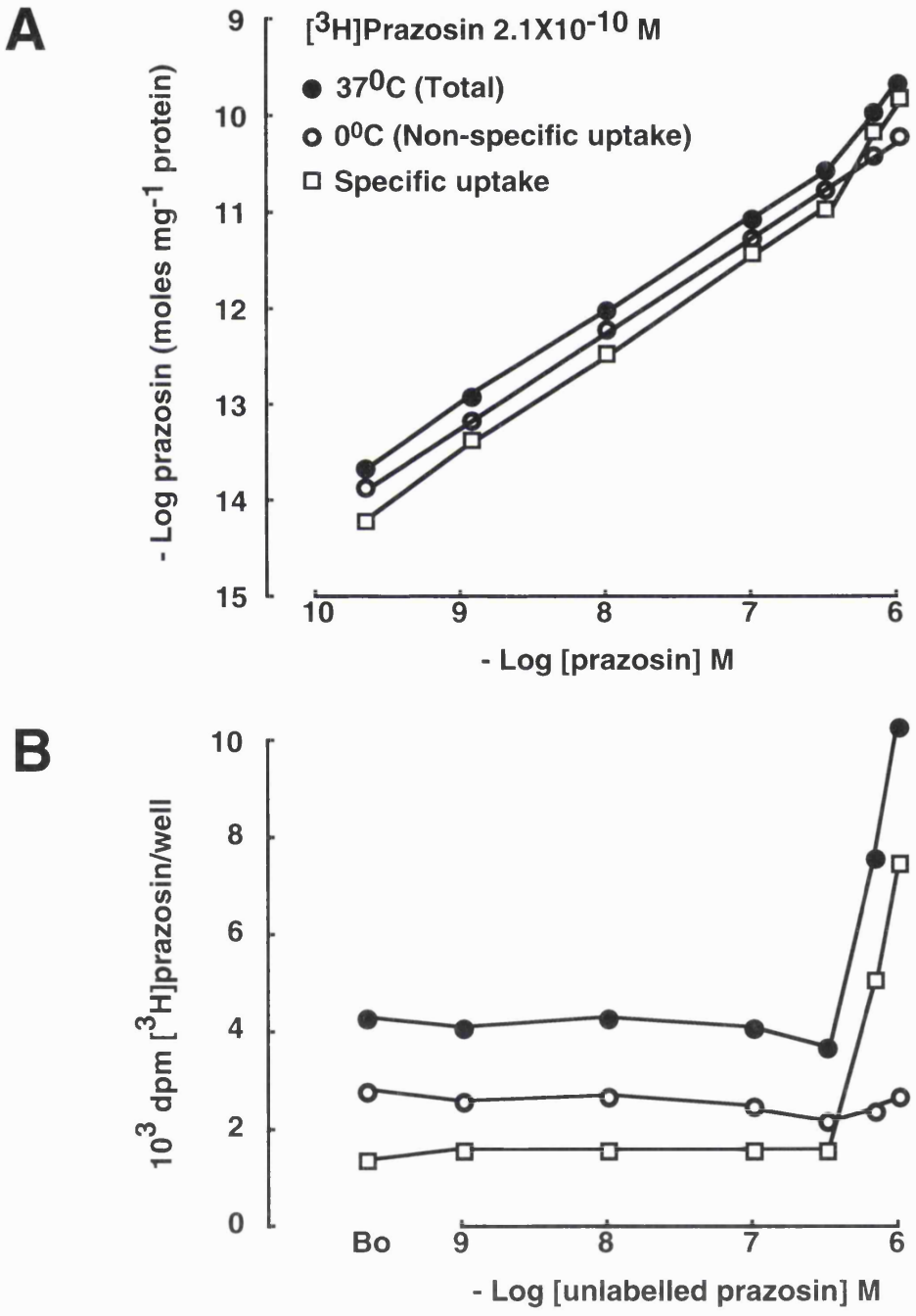


Figure 11.1.2:

Uptake of noradrenaline in SK-N-SH noradrenergic neurones. As in Figure 11.1.1, the lower panel demonstrates uptake of the radiolabelled ligand and the upper panel demonstrates uptake of total ligand (labelled and unlabelled).

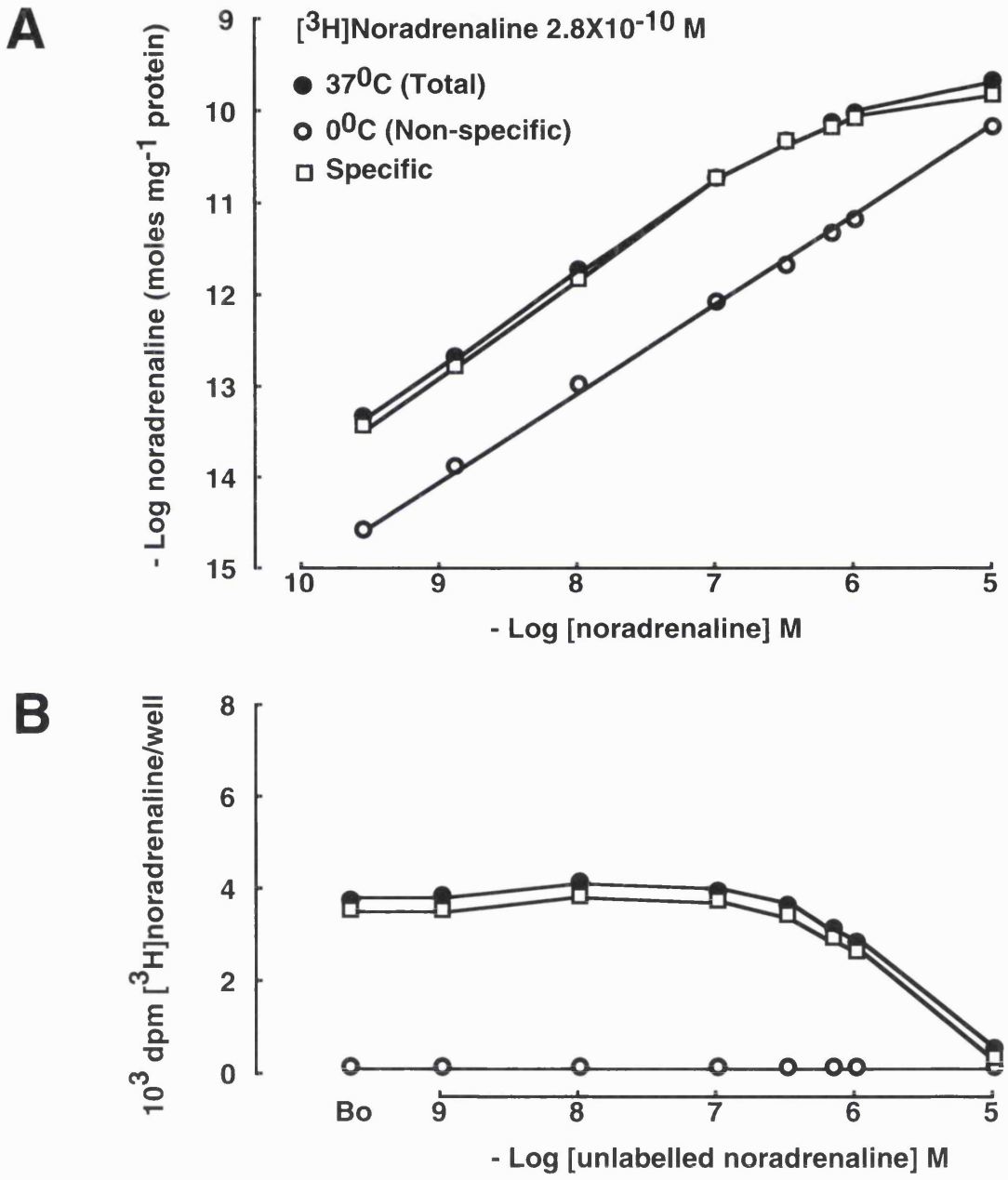


Figure 11.1.3:

[^{3}H]Prazosin binding in DDT_1 MF-2 cells which express native α_{1B} adrenoceptors. The concentration dependence of the binding of [^{3}H]prazosin and its displacement by unlabelled prazosin (K_D $4.8x10^{-10}$ M) is seen in these cells. There is no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M. [^{3}H]Prazosin binding in COS-7 cells and in SK-N-SH cells is shown for comparison.

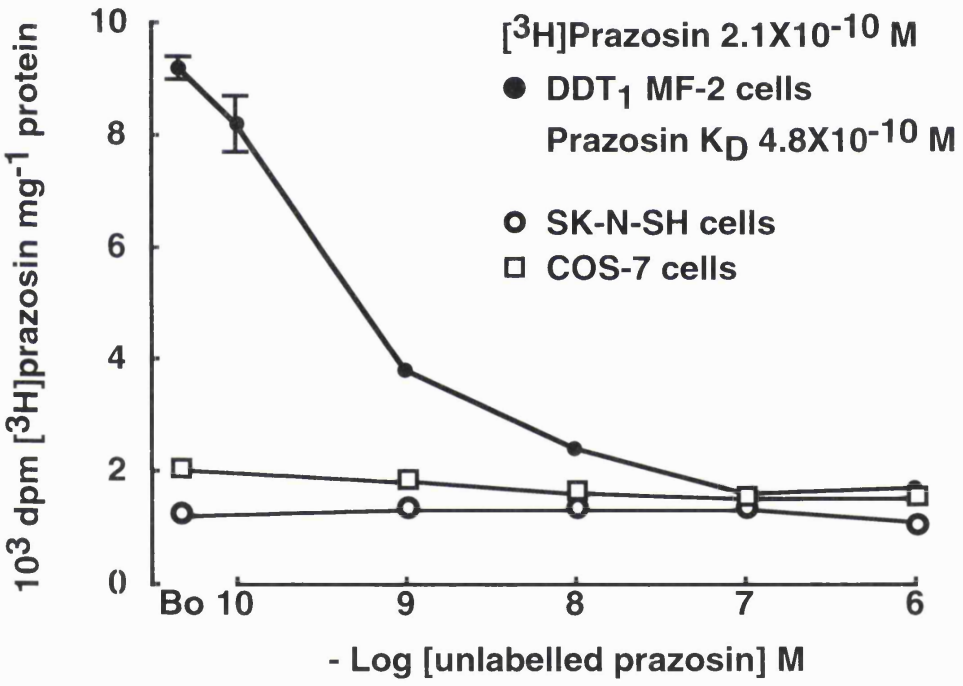
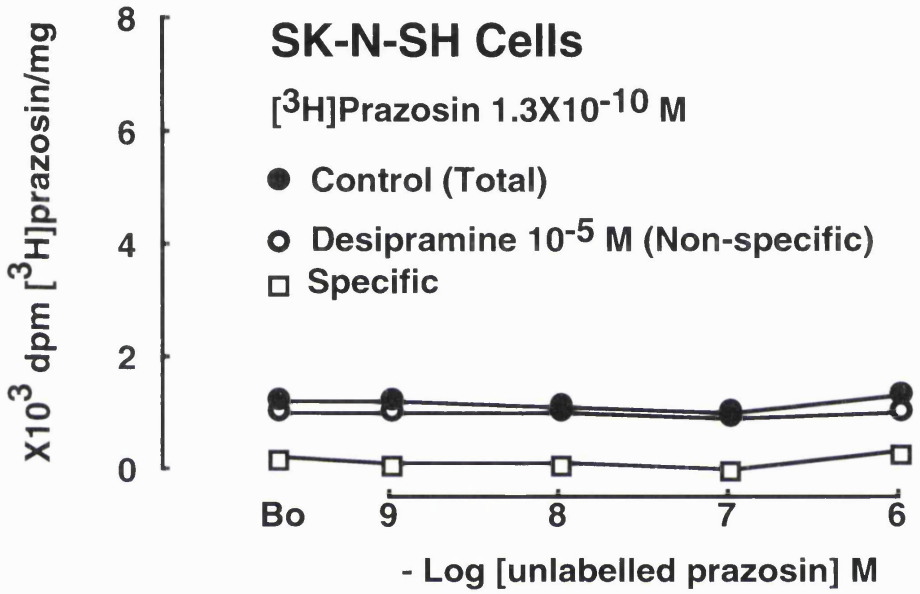


Figure 11.1.4:

[^{3}H]Prazosin binding in SK-N-SH noradrenergic neurones and in COS-7 kidney cells. Binding in these two cell lines is at very low levels and is unaffected by desipramine. There is no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M in these cell lines.



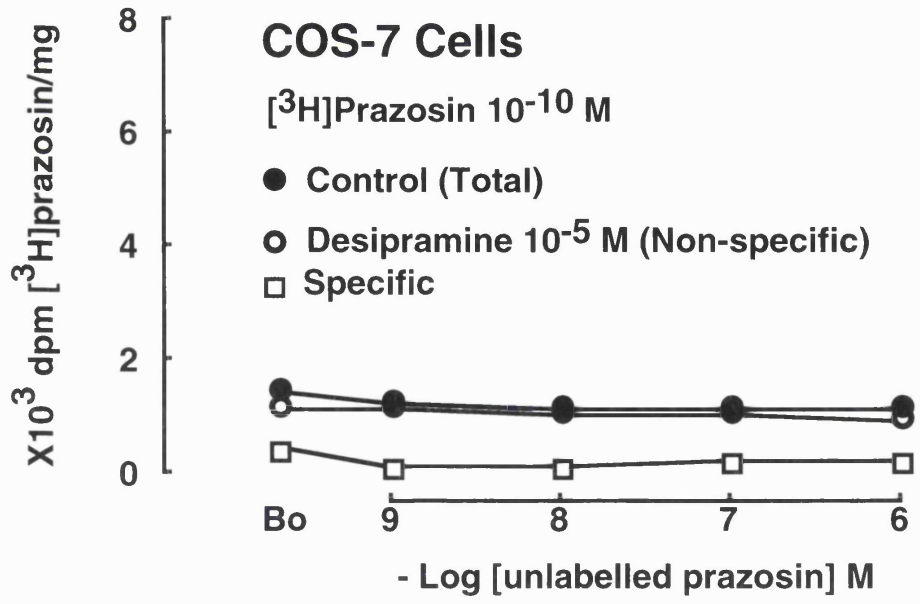


Figure 11.1.5:

Positions of the PCR primers in the sequence of the rat α_{1b} adrenergic receptor cDNA (Lomasney et al, 1991; Genbank accession number M60655). The translated region is from 15 to 1559 and the splice site is at 962-963.

Figure 11.1.6:

The products (shown by arrows) of polymerase chain reactions, using as templates reverse-transcribed (RT) RNA from GnRH neurones or a cDNA library which was constructed from these GnRH neurones. The primers used are described in Table 11.1.1. In panel "A" (RT-RNA as template), the first PCR employed the A1BF2/A1BB9 primer pair and this was followed by a second PCR which employed the nested primer pair A1BF1/A1BB1. In panels B-D, the template was the cDNA library. The primers were A1BF2/A1BB9 in lane B, A1BF4/A1BB4 in lane C and A1BF5/A1BB5 in lane D. Specific products of the predicted sizes were detected in all these reactions. Size markers are shown in parallel lanes in each panel. The experiments which are shown in panels A and B were performed by the Author's trainee, Susan White, according to a protocol which had been written by the Author. All the PCR primers were designed by the Author.

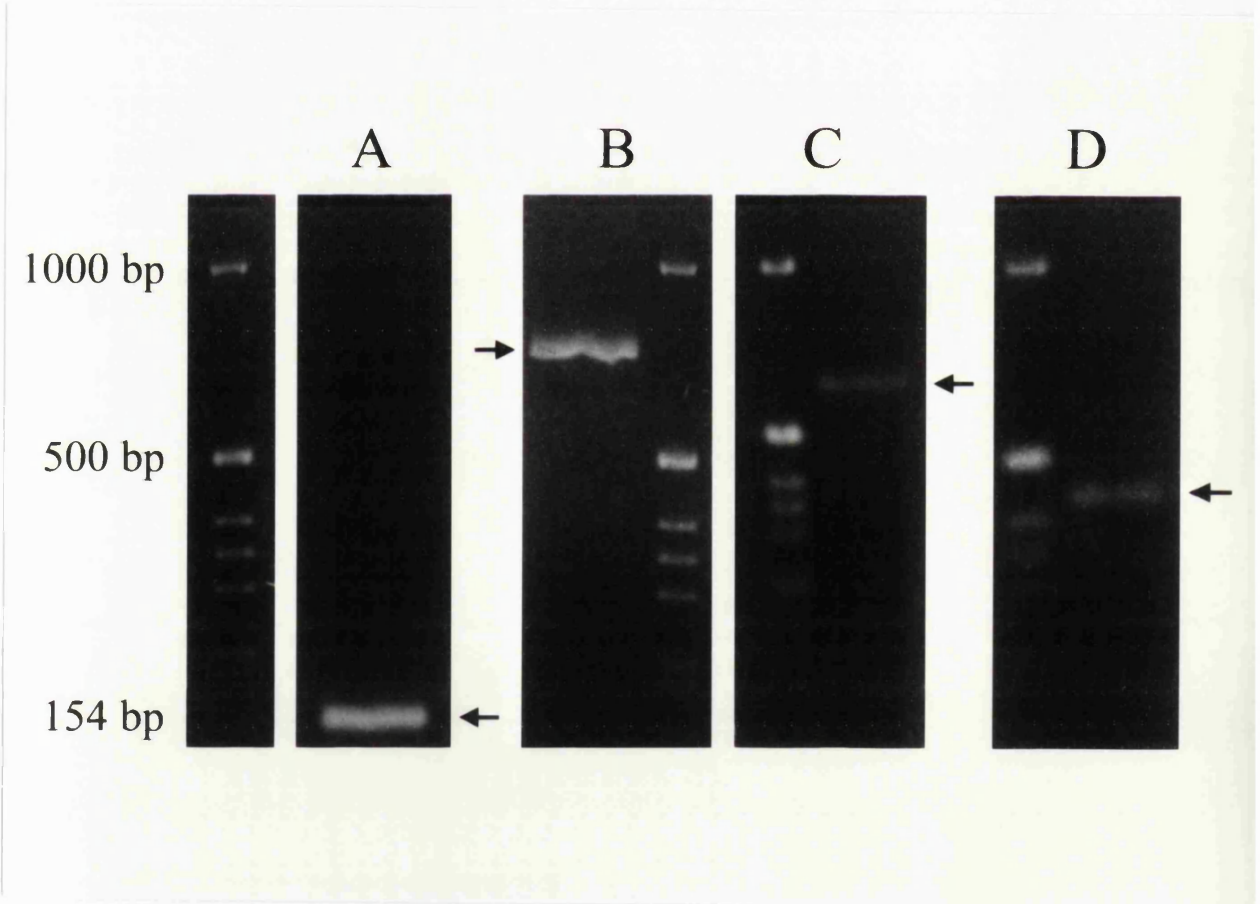


Figure 11.1.7:

The translated sequences of the PCR products which are shown in Figure 11.1.6 have been assembled into a contig and are compared to the sequence of the mouse α_{1B} adrenoceptor (GenBank accession number Y12738). The sequences of the overlapping PCR products were almost identical to the mouse α_{1B} adrenoceptor; sequence differences are indicated by asterisks and the splice site by an arrow.

```
                                                                  * *
Mouse α1B       MNPDLDTGHNTSAPAHWGELKDANFTGPNQTSSNSTLPQLDVTRAISVGC      50
A1BF5/A1BB5           HNTSAPAHWGELKDANFTGPNQTSSNSTLPQLDVTRAISVGLV

                                                        *
Mouse α1B       LGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTDLPFSATL  104
A1BF5/A1BB5     LGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFSATL
& A1BF2/A1BB9

Mouse α1B       EVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLV    156
A1BF5/A1BB5     EVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLV
& A1BF2/A1BB9

Mouse α1B       TRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSL    208
A1BF2/A1BB9     TRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSL
& A1BF4/A1BB4

Mouse α1B       GSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNF    259
A1BF2/A1BB9     GSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNF
& A1BF4/A1BB4

Mouse α1B       HEDTLSSTKAKGHNPRSSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFI    311
A1BF2/A1BB9     HEDTLSSTKAKGHNPRSSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFI
& A1BF4/A1BB4

                      ↓
Mouse α1B       ALPLGSLFSTLKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFMRILG    363
A1BF4/A1BB4     ALPLGSLFSTLKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFMRILG

                                  ?
Mouse α1B       CQCRGGRRRRRRRRLGACAYTYRPWTRGGSLERSQSRKDSLDDSGSCMS      412
A1BF4/A1BB4     CQCRGGRRRRRRRRLGXCAYTYRPWTRGGSLERSQSRKDSLDD

Mouse α1B       GSQRTLPSASPSPGYLGRGTQPPVELCAFPEWKPGALLSLPEPPGRRGRLDS   464

Mouse α1B       GPLFTFKLLGEPESPGTEGDASNGGCDTTTDLANGQPGFKSNMPLAPGHF     514
```

Figure 11.1.8:

Prazosin binding in COS-7 cells transfected with hamster smooth muscle α_{1b} adrenoceptor cDNA. [^{3}H]prazosin is displaced by unlabelled prazosin (IC_{50} $2x10^{-9}$ M, K_D $5x10^{-10}$ M) and there is no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M. In the control transfection, COS cells were electroporated in the absence of DNA.

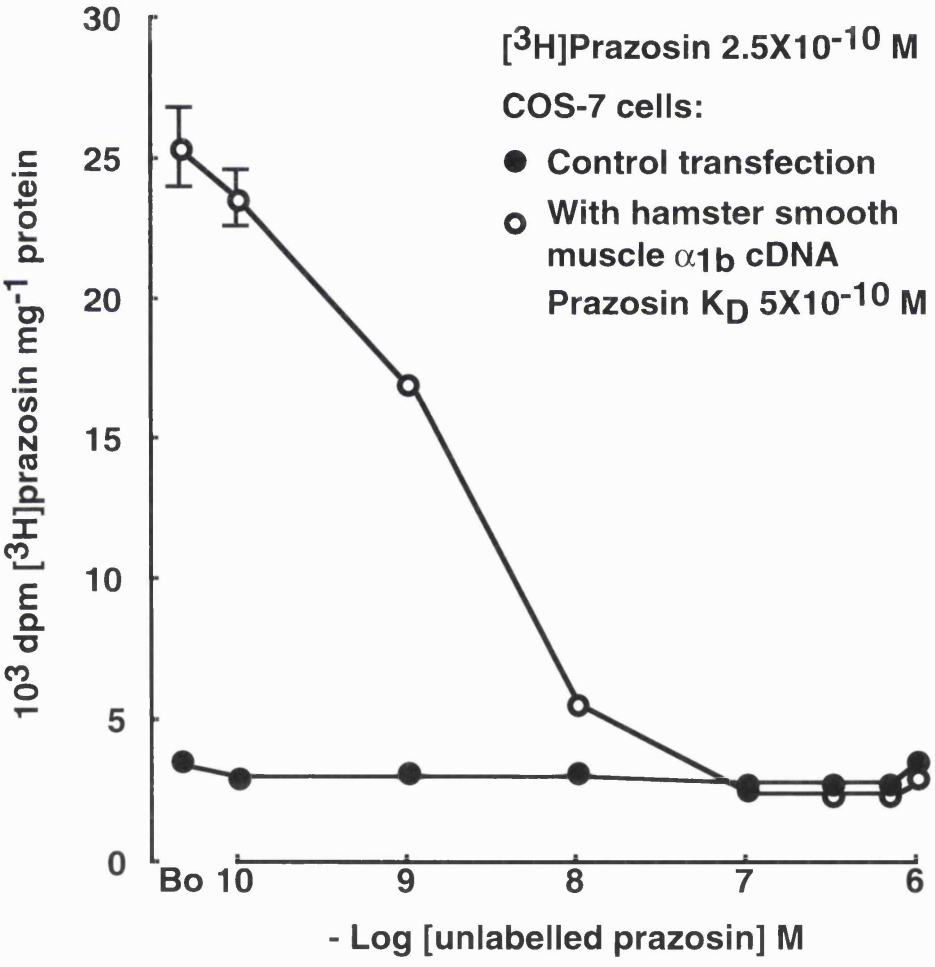


Figure 11.1.9:

Effects of adrenergic agonists on [^{3}H]prazosin binding in DDT$_1$ MF-2 cells. [^{3}H]prazosin was displaced by the selective α_1 adrenergic agonists methoxamine and phenylephrine (K_D $2x10^{-4}$ M and $1.9x10^{-5}$ M, respectively) and by the non-selective endogenous agonists adrenaline and noradrenaline (K_D $6.2x10^{-6}$ M and 10^{-5} M). None of these agonists caused an increase in the binding of [^{3}H]prazosin.

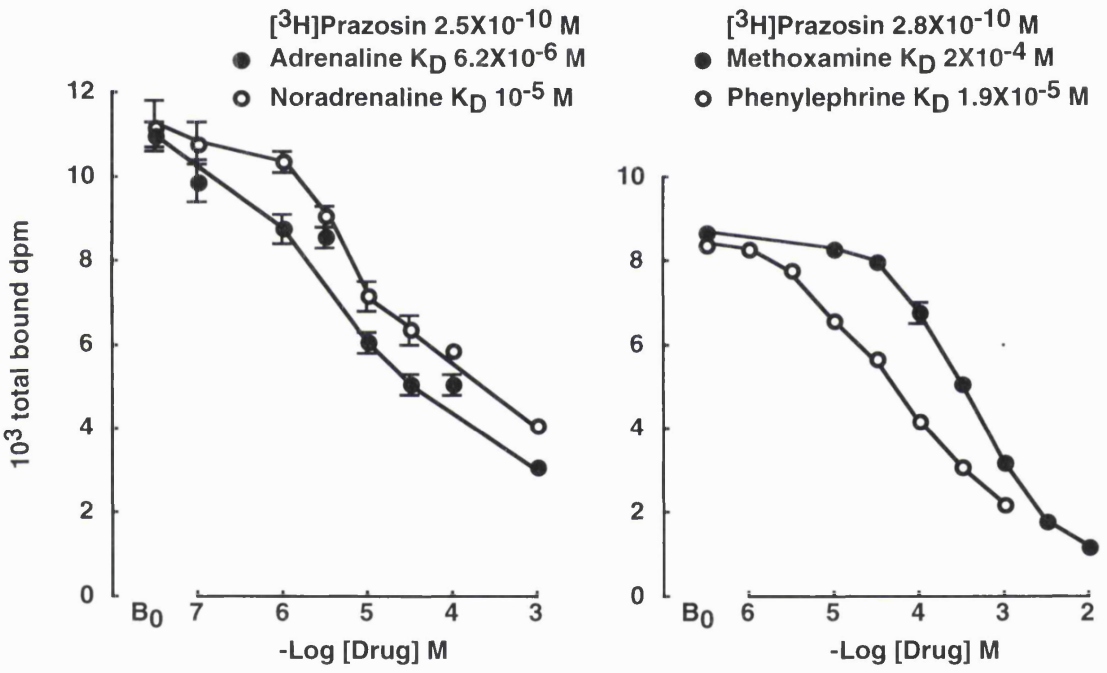


Figure 11.1.10:

Over-expression of α_{1B} adrenoceptors in peptidergic neurones which possess Transport-P.

GT1-1 GnRH cells were transfected with hamster smooth muscle α_{1b} adrenoceptor cDNA.

In the control transfection, GT1-1 cells were electroporated in the absence of DNA. There was no difference in the prazosin paradox between control and transfected cells.

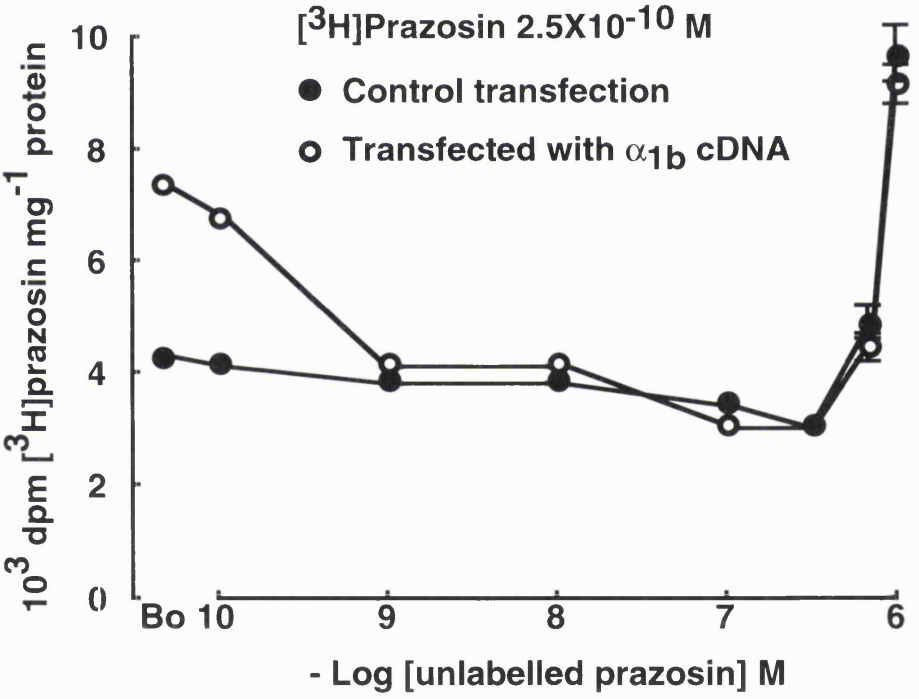


Figure 11.1.11:

Effect of clonidine on the binding of [^{3}H]prazosin in GT1-1 GnRH cells. The α_2 adrenergic agonist clonidine displaced [^{3}H]prazosin from GT1-1 cells. There was no increase in the binding of [^{3}H]prazosin at concentrations of clonidine up to 10^{-3} M

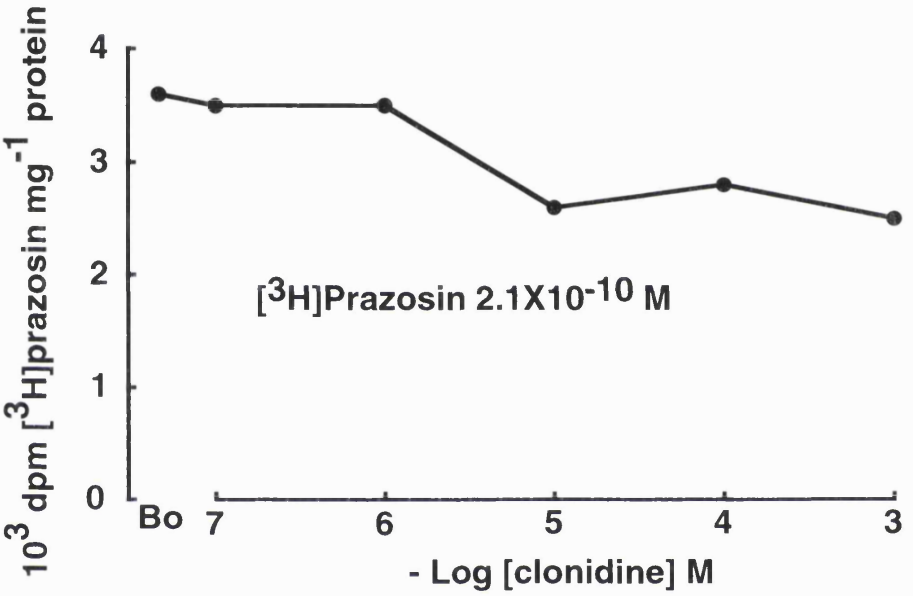


Figure 11.1.12:

Screening the cDNA library for the GnRH neurone α_{1b} adrenergic receptor cDNA. The cDNA library consist of $2x10^6$ recombinants. The library was divided into bacterial pools, each containing 63,000 bacteria. The bacteria were amplified and the DNA was extracted and digested to separate the plasmid from the insert. One µg of digested DNA from each pool was then run in an agarose gel and transferred to a nylon membrane. One of the α_{1B} adrenoceptor PCR products was labelled with ^{32}P using random primers and used as a probe in a hybridisation procedure. One of the pools (pool 14) was positive, demonstrating a hybridisation band of 1800 bp. Sub-pools were prepared from pool 14, containing 6,750 bacteria and treated in the same manner. One of these sub-pools (sub-pool 37) was positive, demonstrating the same hybridisation band which was now amplified, due to enrichment of the target molecule. The procedure was repeated using further sub-pools, containing diminishing numbers of clones. Finally, colony hybridisation was used to obtain an individual, positive clone.

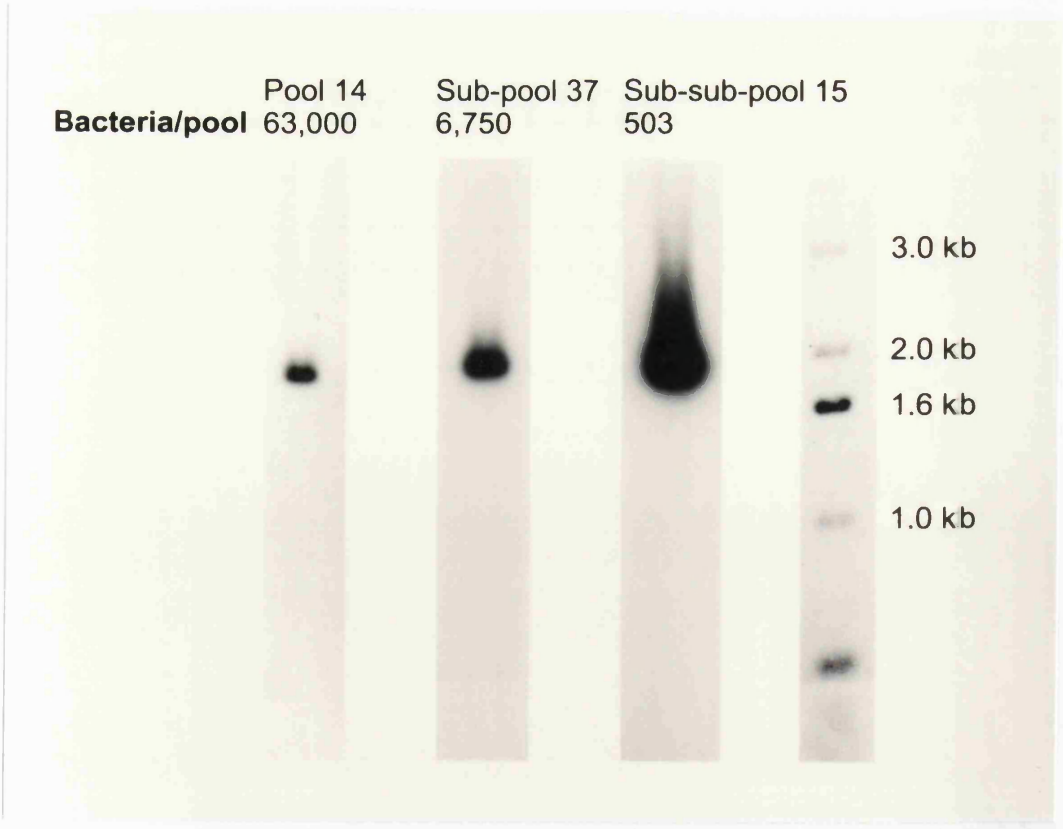


Figure 11.1.13:

Sequence of the mouse GT1-1 GnRH neurone α_{1B} adrenoceptor, compared to the sequences of the hamster and mouse α_{1B} adrenoceptors (Cotecchia et al, 1988 and GenBank accession number Y12738, respectively). Sequence differences are indicated by asterisks and the splice site by an arrow. Membrane-spanning domains are underlined. The GnRH sequence is identical to the sequences of the PCR products in Figure 11.1.7.

```
Hamster α1B   MNPDLDTGHNTSAPAQWGELKDANFTGPNQTSSNSTLPQLDVTRAISVGLV   51
Mouse α1B     MNPDLDTGHNTSAPAHWGELKDANFTGPNQTSSNSTLPQLDVTRAISVGC
GnRH α1B      MNPDLDTGHNTSAPAHWGELKDANFTGPNQTSSNSTLPQLDVTRAISVGLV
                                 *                          * *

Hamster α1B   LGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFSATL  105
Mouse α1B     LGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTDLPFSATL
GnRH α1B      LGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIADLLLSFTVLPFSATL
                                                          *

Hamster α1B   EVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLV   157
Mouse α1B     EVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLV
GnRH α1B      EVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPTLV

Hamster α1B   TRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSL   209
Mouse α1B     TRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSL
GnRH α1B      TRRKAILALLSVWVLSTVISIGPLLGWKEPAPNDDKECGVTEEPFYALFSSL

Hamster α1B   GSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNF   260
Mouse α1B     GSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNF
GnRH α1B      GSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNF

Hamster α1B   HEDTLSSTKAKGHNPRSSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFI   312
Mouse α1B     HEDTLSSTKAKGHNPRSSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFI
GnRH α1B      HEDTLSSTKAKGHNPRSSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFI

Hamster α1B   ALPLGSLFSTLKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFMRILG   364
Mouse α1B     ALPLGSLFSTLKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFMRILG
GnRH α1B      ALPLGSLFSTLKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFMRILG
                        ↑
Hamster α1B   CQCRSGRRRRRRRRLGACAYTYRPWTRGGSLERSQSRKDSLDDSGSCMS   413
Mouse α1B     CQCRGGRRRRRRRRLGACAYTYRPWTRGGSLERSQSRKDSLDDSGSCMS
GnRH α1B      CQCRGGRRRRRRRRLGGCAYTYRPWTRGGSLERSQSRKDSLDDSGSCMS
                 *              *

Hamster α1B   GSQRTLPSASPSPGYLGRGAQPPLELCAYPEWKSGALLSLPEPPGRRGRLDS   465
Mouse α1B     GSQRTLPSASPSPGYLGRGTQPPVELCAFPEWKPGALLSLPEPPGRRGRLDS
GnRH α1B      GSQRTLPSASPSPGYLGRGTQPPVELCAFPEWKPGALLSLPEPPGR
                                  *  *  *  *

Hamster α1B   GPLFTFKLLGEPESPGTEGDASNGGCDATTDLANGQPGFKSNMPLAPGHF   515
Mouse α1B     GPLFTFKLLGEPESPGTEGDASNGGCDTTTDLANGQPGFKSNMPLAPGHF
                                          *
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Figure 11.1.14:

Hydropathy plot of the GT1-1 GnRH neurone α_{1B} adrenoceptor. Membrane-spanning domains were determined with the Kyte-Doolittle algorithm using a function in the MacVector software on a Macintosh computer.

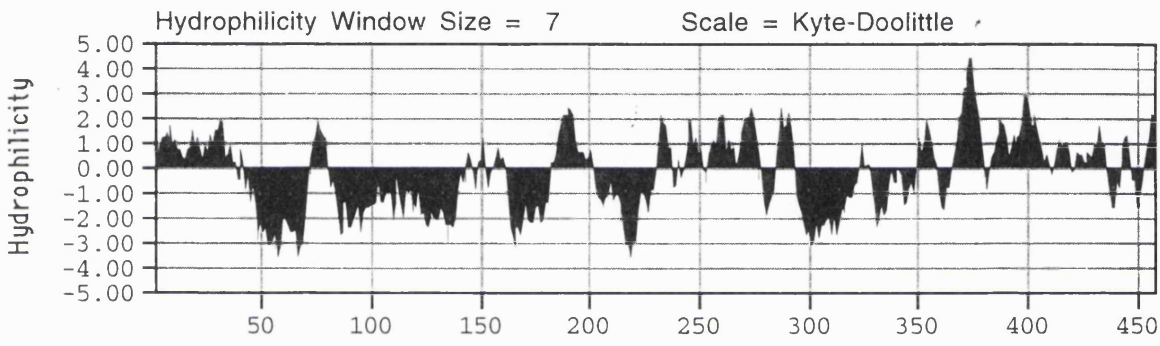


Figure 11.1.15:

Binding of prazosin in COS-7 cells electroporated with GnRH neurone α_{1b} adrenoceptor cDNA. [^{3}H]prazosin is displaced by unlabelled prazosin (K_D 4x10^{-10} M) and there is no increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin up to 10^{-6} M. In the control transfection, COS cells were electroporated in the absence of DNA.

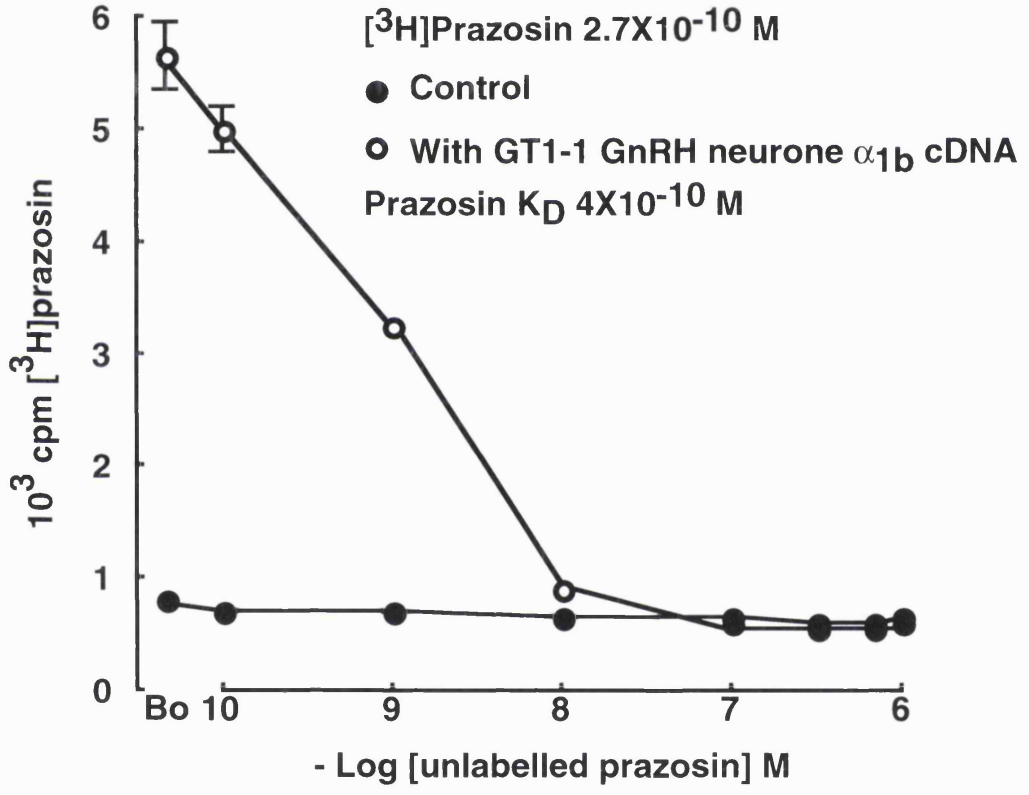


Table 11.1.1:
Design of PCR primers based on the sequence of the rat alpha-1B adrenergic receptor (Lomasney et al, 1991, Genbank accession number M60655).

Analysis settings:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
200 - 1100	20 to 25	55-80	500 - 900	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
A1BF2	204-225	22	forward	54.5	57.4
5'- ATCTTGGTCATCCTGTCGGTGG -3'					
A1BB9	948-926	23	backward	47.8	56.3
5'- TGAAGAAGGGGAGCCAACATAAG -3'					

Optimal annealing temperature: 59.0°C
PCR product:
745 base pairs (204-948)
54.6% G + C content
Tm: 81.4°C

Analysis settings:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
10 - 600	24 - 40	50 - 80	400 - 550	NS	45 - 55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
A1BF5	12-35	24	forward	45.8	59.0
5'- AAATGAATCCCGATCTGGACACCG -3'					
A1BB5	439-416	24	backward	50.0	59.0
5'- TCAATGGAGATGGCACATAGGCTC -3'					

Optimal annealing temp 59.9°C
PCR product:
428 base pairs (12-439)
56.8% G + C content
Tm 81.6°C

Table 11.1.1 (continued):
Design of PCR primers based on the sequence of the rat alpha-1B adrenergic receptor (Lomasney et al, 1991, Genbank accession number M60655).

Analysis settings:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
600 - 1550	22 to 40	55-80	500 - 950	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
A1BF4	604-625	22	forward	54.5	57.1
5'- TCACCGAAGAACCCTTCTACGC -3'					
A1BB4	1236-1215	22	backward	54.5	55.5
5'- TGTCATCCAGAGAGTCCTTCCG -3'					

Optimal annealing temperature: 59.7°C
PCR product:
633 base pairs (604-1236)
58.3% G + C content
Tm: 82.7°C

Analysis settings:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
20 - 1550	20 - 40	69 - 80	150 - 300	NS	45 - 55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
A1BF1	744-775	32	forward	53.1	69.3
5'- GAAATGTCCAACTCCAAGGAGCTGACCCTGAG -3'					
A1BB1	903-874	30	backward	50.0	69.5
5'- CCAAGGTTTTGGCTGCTTTCTTTTCCCTGG -3'					

Optimal annealing temp 59.2°C
PCR product:
160 base pairs (744-903)
50.0% G + C content
Tm 76.2°C

11.2 EFFECTS OF ANTIDEPRESSANTS ON TRANSPORT-P

The work which is described in this Section aimed to test the hypothesis that the paradoxical accumulation of [^{3}H]prazosin at greater concentrations of unlabelled prazosin is due to the presence of an uptake process in post-synaptic peptidergic neurones. This was done by examining the effects of antidepressants which are known to inhibit the uptake of amines in pre-synaptic nerve terminals.

11.2.1 Antidepressant binding sites in peptidergic neurones

[^{3}H]Prazosin (at $2x10^{-9}$ M) was accumulated in GnRH neurones in a time-dependent manner, reaching equilibrium at approximately 30 minutes. In the presence of unlabelled prazosin (at 10^{-5} M), the GnRH cells, rather than displacing the isotopically labelled compound, accumulated greater amounts of [^{3}H]prazosin (Figure 11.2.1). GnRH cells also accumulated [^{3}H]imipramine (at $2x10^{-9}$ M) and apparent equilibrium was reached by 30 minutes. However, addition of unlabelled imipramine reduced the amount of [^{3}H]imipramine associated with the GnRH cells (Figure 11.2.1).

Unlabelled imipramine displaced [^{3}H]imipramine (at $1.7x10^{-9}$ M) from GnRH cells and there was no increase of the radioligand at high concentrations of unlabelled imipramine (Figure 11.2.2). [^{3}H]Imipramine was displaced by unlabelled imipramine (K_D $8.4x10^{-6}$ M; B_{max} $7.9x10^{-10}$ mol/mg protein), desipramine (K_D $4.7x10^{-6}$ M; B_{max} $5.1x10^{-10}$ mol/mg protein), nortriptyline (K_D $9.7x10^{-6}$ M; B_{max} $1.2x10^{-9}$ mol/mg protein), and amitriptyline (K_D $1.5x10^{-5}$ M; B_{max} $1.9x10^{-9}$ mol/mg protein). Scatchard plots revealed that the displacement curves could be fitted to a single site model for each of the four antidepressants (Figure 11.2.3).

11.2.2 Effects of antidepressants on the accumulation of prazosin

In the GnRH cells at equilibrium (60 minutes), [^{3}H]prazosin (at $2.6x10^{-9}$ M) was displaced by unlabelled prazosin in concentrations of 10^{-9} to 10^{-7} M (B_0: 34,088 ± 908 dpm; unlabelled prazosin 10^{-7} M: 25,248 ± 520 dpm). However, at concentrations of unlabelled

prazosin greater than 10^{-7} M, there was an increase in the accumulation of [^{3}H]prazosin (Figure 11.2.4; unlabelled prazosin 10^{-6} M: 93,676 ± 2,442 dpm). Imipramine 10^{-5} M reduced the accumulation of [^{3}H]prazosin $2.6x10^{-9}$ M (B_0: 23,372 ± 594 dpm; Figure 11.2.4). Imipramine also abolished the increase of [^{3}H]prazosin at concentrations of unlabelled prazosin greater than 10^{-7} M: in the presence of imipramine, only displacement of [^{3}H]prazosin by unlabelled prazosin was seen (Figure 11.2.4). Desipramine and amitriptyline had similar effects to imipramine (Figure 11.2.5).

In GnRH cells, the tricyclic antidepressants inhibited the accumulation of prazosin (10^{-6} M) in a dose dependent manner with very similar potencies (Figure 11.2.6A; Table 11.2.1). In addition to the tricyclic antidepressants, accumulation of prazosin (10^{-6} M) in GnRH neurones was also inhibited by newer antidepressant compounds including the "serotonin selective re-uptake inhibitors" fluoxetine, fluvoxamine and paroxetine, and by the "atypical antidepressants" bupropion, mianserin and trimipramine (Table 11.2.1). During the course of this project, it became clear that non-specific binding could be reduced by reducing the concentration of [^{3}H]prazosin from $2x10^{-9}$ M to $2x10^{-10}$ M, and this was accompanied by lower IC_{50} values for inhibitory compounds (Section 10.3).

The inhibitory effect of imipramine could be reversed by increasing concentrations of prazosin in the range 10^{-6} to $3x10^{-6}$ M (Figure 11.2.6B).

Figure 11.2.7 compares the effect of desipramine on the accumulation of prazosin in GnRH neurones to its effect on the accumulation of noradrenaline in SK-N-SH noradrenergic neurones. The lower panels demonstrate the accumulation of radiolabelled ligand, and the upper panels demonstrate accumulation of total ligand (labelled and unlabelled) by correcting for the fall in specific activity consequent upon isotope dilution. At a concentration of $1.9x10^{-10}$ M, [^{3}H]prazosin associates with the peptidergic neurones and is displaced by unlabelled prazosin in concentrations up to $3.33X10^{-7}$ M. However, at greater concentrations of unlabelled prazosin, the paradoxical increase in the binding of [^{3}H]prazosin was seen. In the presence of desipramine, accumulation of prazosin is reduced (Figure 11.2.7). In

addition, desipramine abolished the paradoxical increase in accumulation of [^{3}H]prazosin. Accumulation of prazosin in the peptidergic neurones in the presence of desipramine was linear with concentration up to at least 10^{-6} M, indicating that it represented non-specific accumulation (Figure 11.2.7). Hence, in these experiments, non-specific uptake was defined as uptake in the presence of desipramine. Specific uptake was obtained by subtracting non-specific uptake from total uptake. At concentrations of prazosin greater than 10^{-7} M, the specific accumulation of prazosin was non-linear (Figure 11.2.7). SK-N-SH noradrenergic neurones (which possess the pre-synaptic re-uptake processes for noradrenaline) accumulated [^{3}H]noradrenaline which was displaced by unlabelled noradrenaline. There was no increase in [^{3}H]noradrenaline binding at concentrations of unlabelled noradrenaline greater than 10^{-7} M (Figure 11.2.7). In the presence of desipramine, accumulation of noradrenaline was reduced and was linear with concentration throughout, indicating that it represented non-specific accumulation (Figure 11.2.7). The concentration-dependance of specific accumulation of noradrenaline was hyperbolic and tended to saturate at noradrenaline concentrations greater than 10^{-7} M (Figure 11.2.7).

11.2.3 Comment

Antidepressants inhibit the paradoxical increase in accumulation of [^{3}H]prazosin at concentrations of unlabelled prazosin greater than 10^{-7} M. As antidepressants inhibit the pre-synaptic re-uptake of amines, the findings which are described in this Section are consistent with the hypothesis that the prazosin paradox is due to cellular uptake of the radioligand.

The work which is described in this Section provided the first evidence for the existence of specific antidepressant binding sites in post-synaptic (peptidergic neurones). Imipramine is known to dissolve in lipid bilayers by a process that is not saturable in concentrations up to $7x10^{-4}$ M (Romer & Bickel, 1979). In contrast, [^{3}H]imipramine bound to the GnRH neurones in nanomolar concentrations and was displaced by low concentrations of unlabelled antidepressants (Figure 11.2.3). This indicated the presence of specific, saturable antidepressant binding sites which are presumably the transporter molecules which are responsible for the uptake of prazosin in GnRH neurones. The antidepressants inhibited the uptake of amines in the peptidergic neurones in a competitive manner. These findings

extended the previous observations on desipramine (Al-Damluji et al, 1993) and they represent an additional potential site of action of antidepressants.

In the GnRH neurones, [^{3}H]prazosin was displaced by unlabelled prazosin in concentrations of 10^{-9} to 10^{-7} M, and the paradoxical increase was evident at higher concentrations of unlabelled prazosin. In the presence of antidepressants, only displacement of [^{3}H]prazosin by unlabelled prazosin was seen, presumably representing inhibition of binding of [^{3}H]prazosin to α_1 adrenoceptors in the GnRH neurones (Figures 11.2.4 and 11.2.5). Imipramine inhibited the accumulation of [^{3}H]prazosin $2x10^{-9}$ M (Figure 11.2.4), indicating that uptake takes place at these low extracellular concentrations of prazosin. Although imipramine may bind to α_1 adrenoceptors (U'Prichard et al, 1978b), the inhibitory effect of imipramine on the uptake of [^{3}H]prazosin $2x10^{-9}$ M is unlikely to represent displacement from α_1 adrenoceptors, as displacement of [^{3}H]prazosin by unlabelled prazosin is evident in the presence of imipramine. Imipramine also abolished the paradoxical increase in the binding of [^{3}H]prazosin at concentrations of unlabelled prazosin greater than 10^{-7} M; this is consistent with the hypothesis that the paradoxical increase is due to cellular uptake of the radioligand. The inhibitory effect of imipramine on the uptake of prazosin 10^{-6} M is unlikely to represent displacement from α_1 adrenoceptors; at a concentration of 10^{-6} M unlabelled prazosin, [^{3}H]prazosin is completely displaced from α_1 adrenoceptors (Figures 11.1.8 and 11.1.15). The action of imipramine therefore represents inhibition of uptake, rather than inhibition of binding to α_1 adrenoceptors.

Prazosin and imipramine appear to be substrates for the same uptake process in the GnRH neurones. Thus, [^{3}H]prazosin and [^{3}H]imipramine were accumulated by GnRH neurones by a desipramine sensitive process (Figures 11.2.3 and 11.2.6) and imipramine inhibited the uptake of prazosin 10^{-6} M in these cells (Figure 11.2.6). Further, the inhibitory effect of imipramine could be reversed by increasing concentrations of prazosin (Figure 11.2.6B), indicating that blockade of uptake by the antidepressants is competitive. This competitive action suggests that prazosin and the antidepressants may act on the same transporter

molecule in the GnRH neurones. In pre-synaptic noradrenergic nerve terminals, tricyclic antidepressants inhibited the uptake of noradrenaline in a competitive manner (Maxwell et al, 1969; 1974), and the tricyclics and noradrenaline were subsequently shown to act on the same cloned transporter molecule (Pacholczyk et al, 1991; Giros et al, 1994).

Although the antidepressants and prazosin are likely to act on the same site in the GnRH cells, unlabelled imipramine did not increase the binding of [^{3}H]imipramine (Figure 11.2.2). This indicates that the antidepressants do not activate the uptake process. This is attributable to the structural properties of the antidepressant molecules, as is discussed in Section 11.5.

It is difficult to compare the potencies of antidepressants in inhibiting Transport-P to their potencies in inhibiting other neuronal processes. This is because Transport-P differs from other neuronal transporters and receptors in that it is activated by its substrate. Thus, the IC_{50} of imipramine for inhibiting the uptake of prazosin depends on the concentration of prazosin in the extracellular space (Figure 11.2.6B). Nevertheless, it is possible to compare the concentrations of antidepressants which are observed *in vivo* to the concentrations which were required to inhibit the uptake of prazosin *in vitro*. The recommended therapeutic plasma concentrations for antidepressant treatment with imipramine (0.9-1.8 μM), amitriptyline (0.1-1.0 μM), desipramine (0.4-0.7 μM) and nortriptyline (0.2-0.6 μM; DeVane & Jarecke, 1992; Gram et al, 1984) are within the range of concentrations in which these compounds inhibit the uptake of prazosin 10^{-6} M in GnRH neurones (Figure 11.2.6A; Table 11.2.1). In rats, brain concentrations of antidepressants are over 10-fold greater than their concentrations in plasma (DeVane & Jarecke, 1992). Experiments in rats have suggested that Transport-P may exist in hypothalamic peptidergic neurones *in vivo* (Al-Damluji et al, 1993). It therefore seems possible that in the doses used *in vivo,* the antidepressants may influence the function of Transport-P.

The possible therapeutic significance of these findings is described in Section 12.4.

Figure 11.2.1:

Effects of unlabelled amines on the accumulation of ^{3}H-labelled amines in GT1-1 GnRH neurones: comparison of prazosin and imipramine.

A. Time course of the accumulation of prazosin in GT1-1 GnRH neurones. The neurones were incubated at $37^{0}C$ with [^{3}H]prazosin ($2x10^{-9}$ M), with or without unlabelled prazosin 10^{-5} M.

B. Time course of the accumulation of imipramine in GT1-1 GnRH neurones. The cells were incubated at $37^{0}C$ with [^{3}H]imipramine $2x10^{-9}$ M, with or without unlabelled imipramine 10^{-5} M.

Addition of unlabelled prazosin caused a greater amount of [^{3}H]prazosin to be associated with the cells. In contrast, addition of unlabelled imipramine displaced [^{3}H]imipramine and did not cause an increase in the binding of [^{3}H]imipramine to the cells.

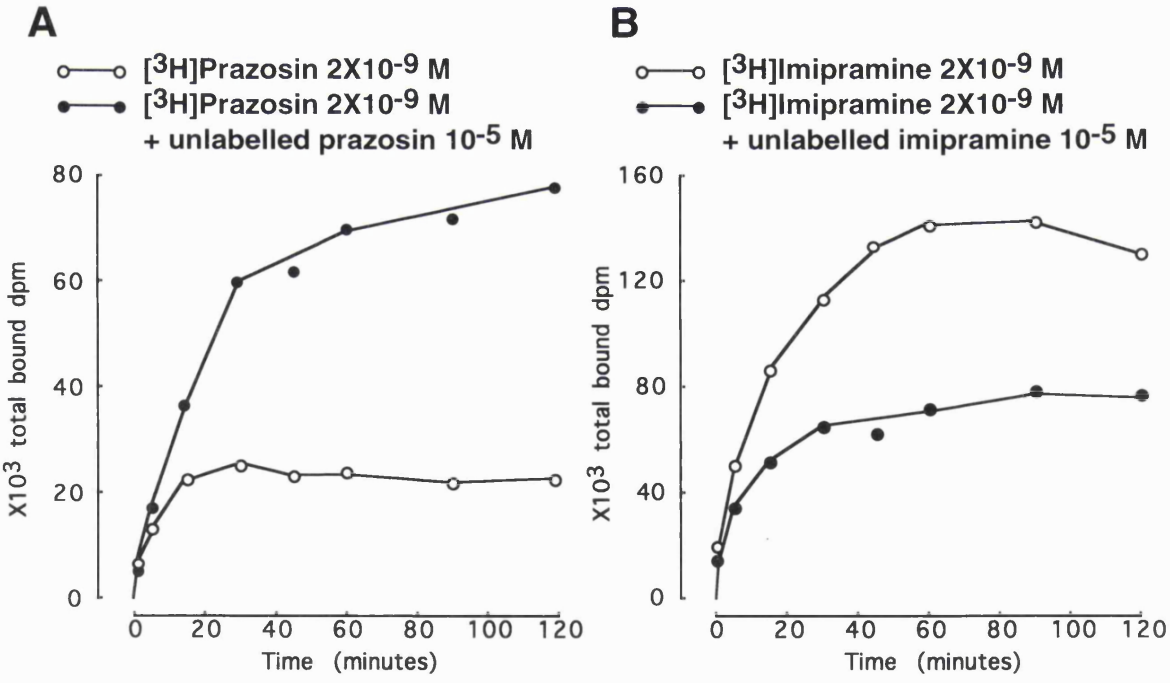


Figure 11.2.2:

Displacement of [^{3}H]imipramine by unlabelled imipramine in GnRH cells.

Only displacement of the radioligand by the unlabelled compound is seen, indicating the presence of specific binding sites in these cells. The data represent total bound dpm.

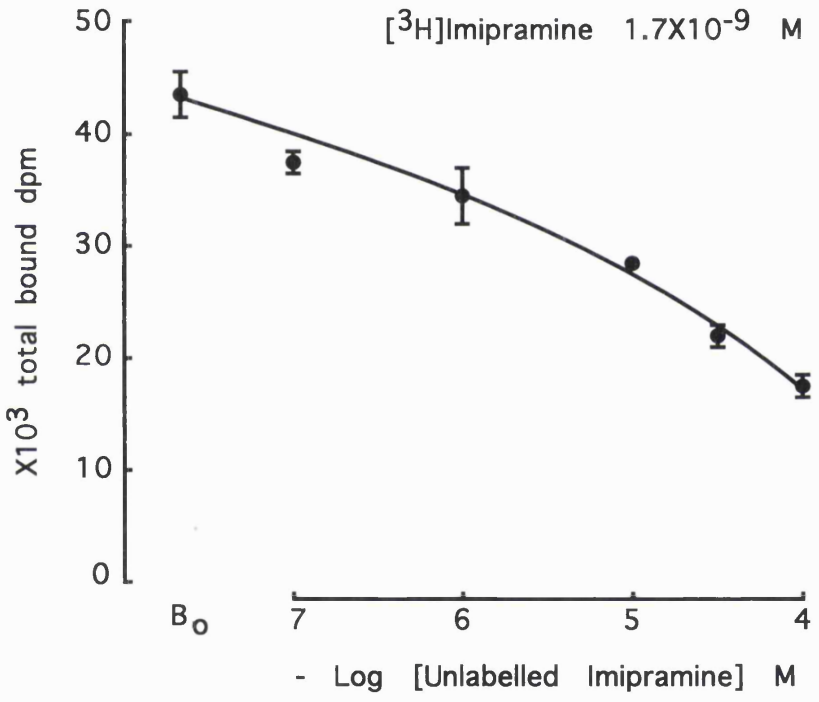


Figure 11.2.3:

Displacement of [^{3}H]imipramine (at 1.6x10^{-9} M) by unlabelled tricyclic antidepressants in GT1-1 GnRH neurones:

A. Displacement isotherms: the data represent specific binding expressed as femtomoles [^{3}H]imipramine/mg protein. Non-specific binding was defined as the remaining dpm in the presence of unlabelled imipramine 10^{-4} M and represented 35% of total bound dpm (see Figure 11.2.2).

B. Scatchard plots of the displacement curves in the upper panel. The data fit a single binding site model for tricyclic antidepressants in GnRH neurones.

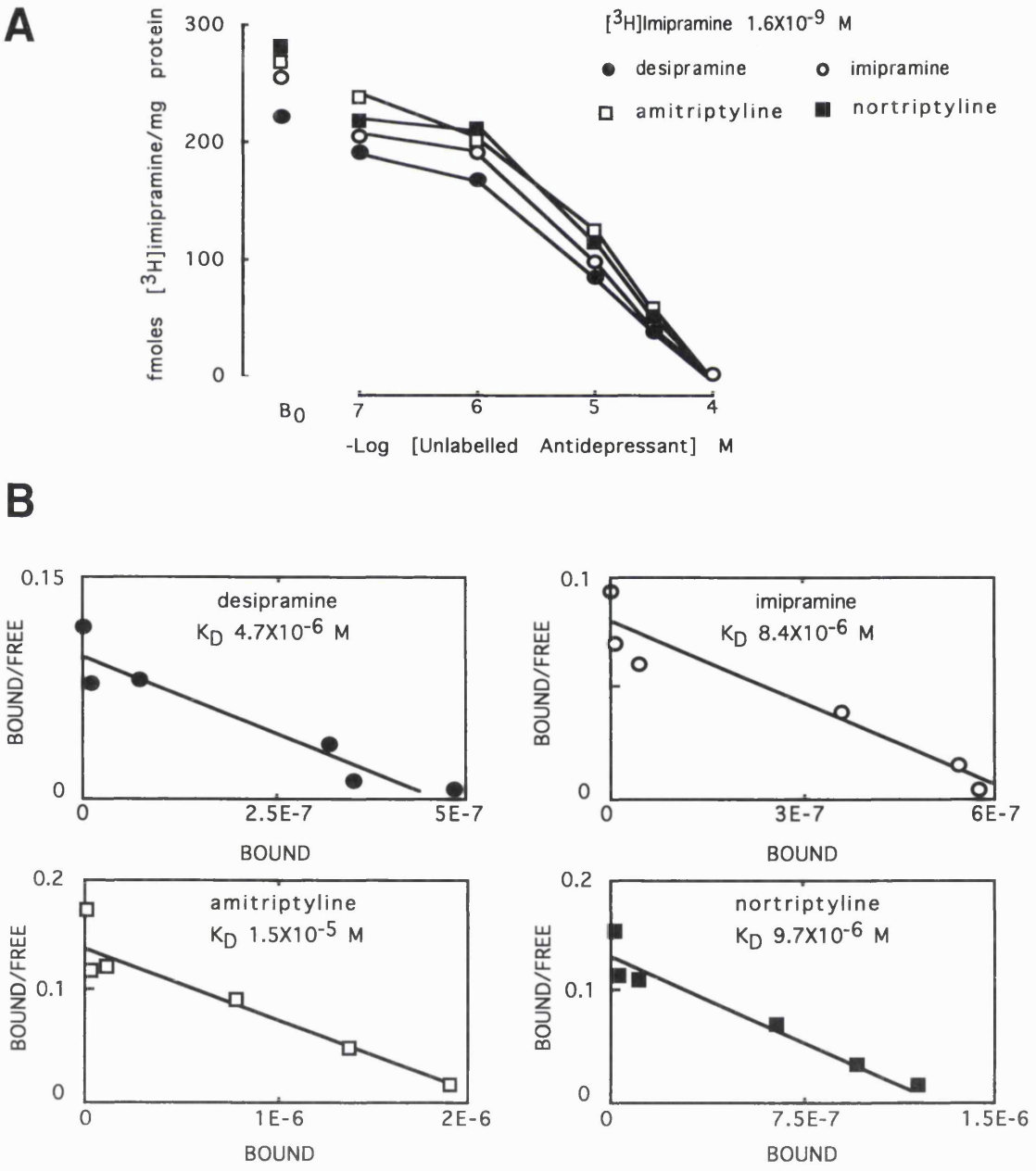


Figure 11.2.4:

Effect of imipramine on [^{3}H]prazosin binding in GT1-1 GnRH neurones:

In the control experiment, displacement of the radioligand (at 2.6x10^{-9} M) at equilibrium (60 minutes) was observed at unlabelled prazosin concentrations of 10^{-9} to 10^{-7} M. At concentrations of unlabelled prazosin greater than 10^{-7} M, the paradoxical increase in [^{3}H]prazosin binding was evident. In the presence of imipramine 10^{-5} M, the prazosin paradox was abolished and only displacement was seen.

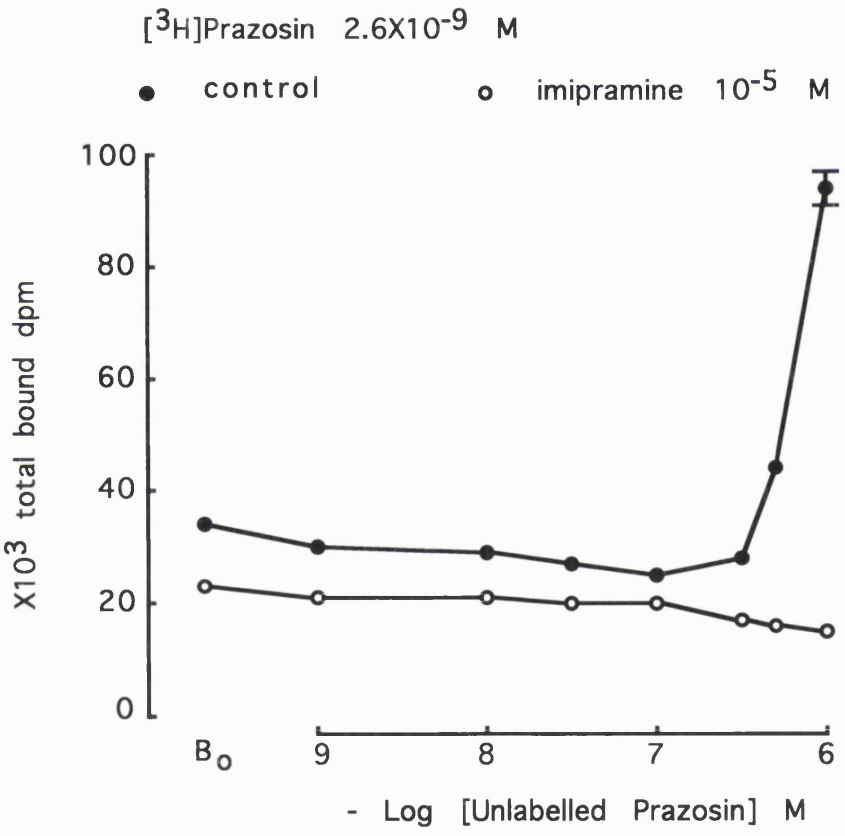


Figure 11.2.5:

Effects of tricyclic antidepressants on [^{3}H]prazosin binding in GT1-1 GnRH neurones: In the control experiment, displacement of the radioligand at equilibrium (60 minutes) was observed at unlabelled prazosin concentrations of 10^{-9} to 10^{-7} M. At concentrations of unlabelled prazosin greater than 10^{-7} M, the paradoxical increase in [^{3}H]prazosin binding was evident. In the presence of tricyclic compounds, the prazosin paradox was abolished and only displacement was seen.

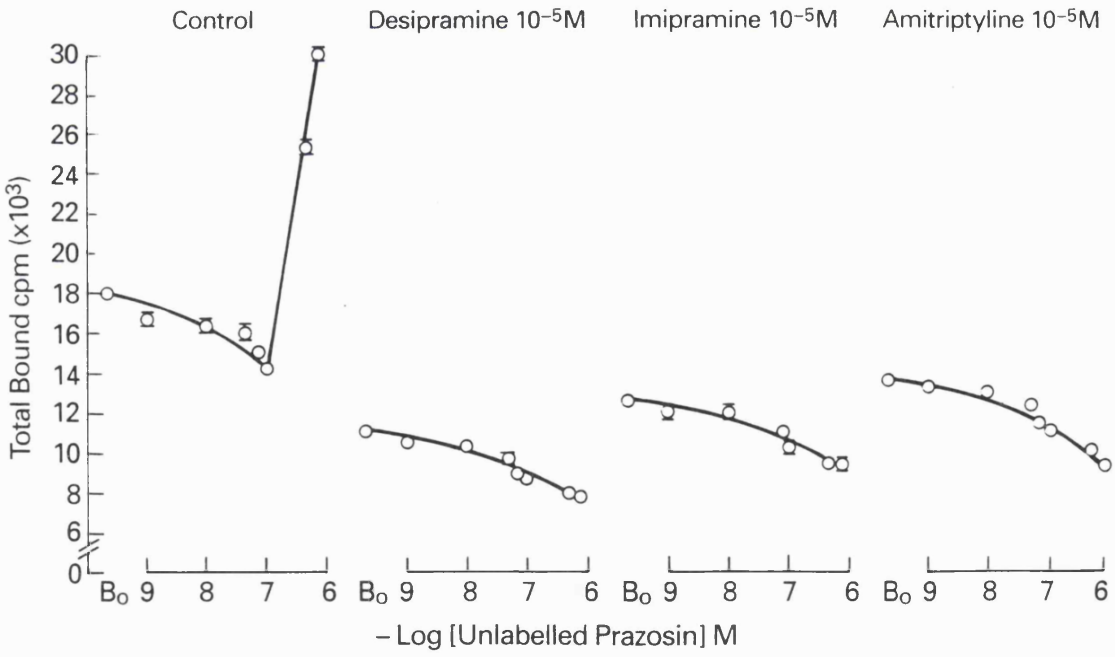


Figure 11.2.6:

Inhibition of the uptake of prazosin by antidepressants in GT1-1 GnRH neurones:

A. The cells were incubated with [^{3}H]prazosin 2x10^{-9} M and unlabelled prazosin 10^{-6} M, with or without the indicated concentrations of the antidepressants.

B. Effects of increasing concentrations of prazosin on the inhibitory effect of imipramine: reversal of the inhibitory effect of imipramine by increasing concentrations of the substrate (prazosin) indicates that the inhibitory effect of imipramine is competitive.

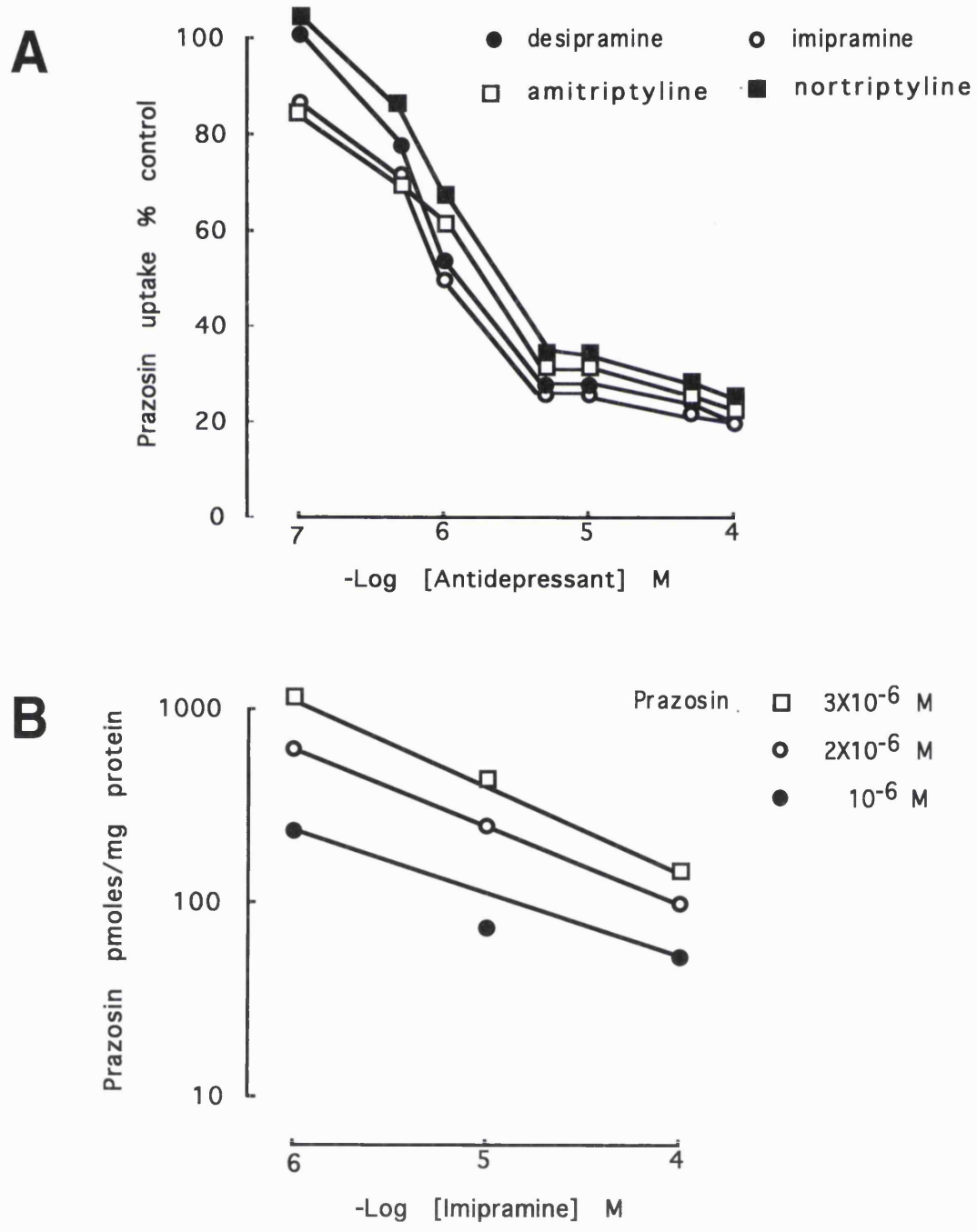


Figure 11.2.7:

Comparison of the uptake of prazosin in peptidergic neurones (GT1-1 GnRH cells; left) to the uptake of noradrenaline in SK-N-SH noradrenergic neurones.

The lower panels demonstrate the uptake of radiolabelled ligand; the upper panels demonstrate uptake of total ligand (labelled and unlabelled), ie, it is corrected for the fall in specific activity of the radioligand due to isotope dilution. Non-specific uptake was defined as uptake in the presence of desipramine. "B_0" is an abbreviation for binding of the radiolabelled ligand in the absence of unlabelled ligand.

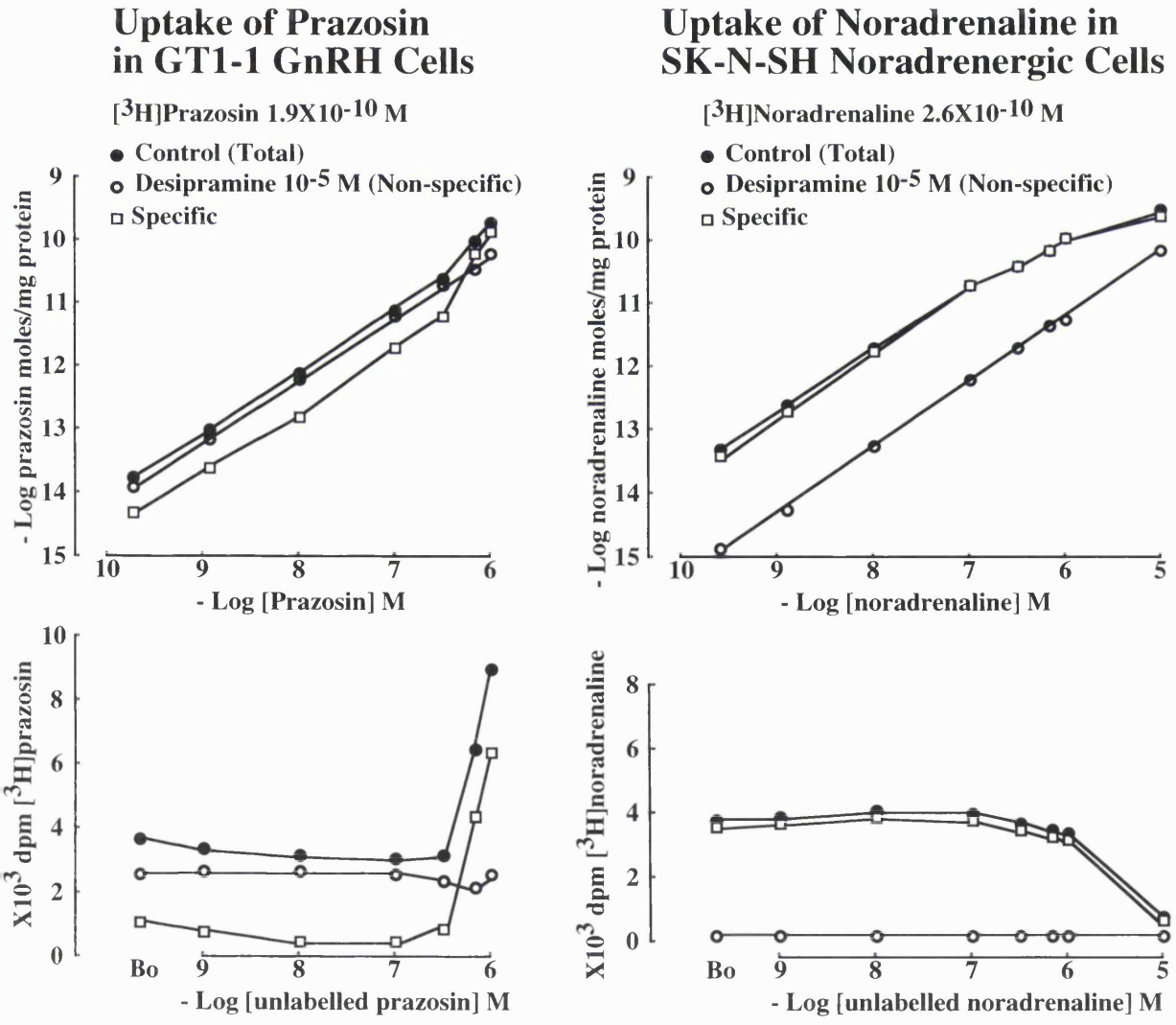


Table 11.2.1:

Potencies of antidepressants at inhibiting the uptake of prazosin 10^{-6} M in GT1-1 GnRH neurones.

Trimipramine was the most potent compound in this series. Relative potencies were calculated as follows: (IC_{50} trimipramine/IC_{50} test compound)x100%.

Compound	IC_{50}	Relative potency %
Amitriptyline	$9.5x10^{-7}$ M	28
Bupropion	$2.3x10^{-6}$ M	12
Desipramine	$5.3x10^{-7}$ M	51
Fluoxetine	$9.5x10^{-7}$ M	28
Fluvoxamine	$1.5x10^{-6}$ M	18
Imipramine	$4.6x10^{-7}$ M	59
Mianserin	$9.0x10^{-7}$ M	30
Nortiptyline	$1.7x10^{-6}$ M	16
Paroxetine	$1.3x10^{-6}$ M	21
Trimipramine	$2.7x10^{-7}$ M	100

11.3 THE SOURCE OF ENERGY FOR TRANSPORT-P

The work which is described in this Section aimed to test the hypothesis that the paradoxical accumulation of [^{3}H]prazosin at greater concentrations of unlabelled prazosin is due to an energy-dependent uptake process in post-synaptic peptidergic neurones. This was done by examining the effects of pharmacological manipulations which are known to influence electrochemical gradients which provide the energy for membrane transport systems in mammalian cells.

11.3.1 Effects of sodium ions and inhibitors of P-ATPase

In SK-N-SH noradrenergic neurones, exclusion of sodium from the extracellular space inhibited the uptake of (-)-noradrenaline (at 10^{-6} M) as effectively as desipramine 10^{-5} M (Figure 11.3.1). At 60 minutes, desipramine inhibited the uptake of (-)-noradrenaline by 85% and exclusion of sodium inhibited uptake by 82% (control 47.2 ± 0.6; desipramine 7.0 ± 0.3; sodium absent 8.3 ± 0.1 pmoles/mg protein; Figure 11.3.1). In contrast, in GnRH cells studied in an identical manner, exclusion of sodium caused only 30% inhibition of prazosin uptake; the uptake of prazosin that was observed in the GnRH cells in the absence of sodium could be inhibited by desipramine (Figure 11.3.1; uptake of prazosin at 60 minutes: control 395.4 ± 12.1; desipramine 61.8 ± 1.5; sodium absent 274.9 ± 7.0; sodium absent + desipramine 46.7 ± 0.3 pmoles/mg protein).

In the SK-N-SH cells, the uptake of (-)-noradrenaline (at 10^{-6} M) was inhibited by the P-ATPase inhibitor, sodium orthovanadate (Na_3VO_4) in a concentration-dependent manner with an IC_{50} of $3.6x10^{-5}$ M (Figure 11.3.2). This value is similar to the IC_{50} which was reported for the inhibitory effect of sodium orthovanadate on the P-ATPase sodium pump in erythrocytes (IC_{50} $4x10^{-5}$ M; Cantley et al, 1978). In contrast, in the GnRH cells which were studied in an identical manner, sodium orthovanadate had no consistent effect on the uptake of prazosin (Figure 11.3.2; prazosin uptake: control 332.9 ± 8.1 pmoles/mg; vanadate 10^{-4} M 324.4 ± 8.9 pmoles/mg). In these experiments, desipramine 10^{-5} M inhibited the uptake of amines in both the SK-N-SH neurones and the GnRH neurones, confirming that active amine uptake was taking place (Figure 11.3.2). In these experiments, the entry of vanadate into the cells was facilitated by exclusion of phosphate from the Krebs-Ringer-HEPES buffer (Cantley et al, 1978). In another experiment, sodium metavanadate

($NaVO_3$) inhibited the uptake of (-)-noradrenaline in the SK-N-SH cells in a concentration-dependent manner with an IC_{50} of $4.5x10^{-5}$ M (not shown). Sodium metavanadate had no effect on the uptake of prazosin in the GnRH cells which were studied in an identical manner. In both of these experiments, the uptake of amines was inhibited by desipramine 10^{-5} M (not shown).

11.3.2 Effects of protons and inhibitors of V-ATPase

The V-ATPase inhibitor, bafilomycinA1, inhibited uptake of (-)-noradrenaline (at 10^{-6} M) into SK-N-SH neurones. BafilomycinA1 also inhibited the uptake of prazosin in the GnRH neurones (Figure 11.3.3). At the highest concentration that was used (10^{-6} M), the inhibitory effect of bafilomycinA1 on the uptake of prazosin in the GnRH neurones was similar to the effect of desipramine 10^{-5} M (Figure 11.3.3). In the concentrations used in this study, bafilomycinA1 does not inhibit P-ATPase ion pumps, and F-ATPase pumps, including mitochondrial ATPase, are completely resistant to this drug (Bowman et al, 1988).

The monovalent (H^+/Na^+) carboxylic ionophore, monensin, inhibited the uptake of (-)-noradrenaline (at 10^{-6} M) in the SK-N-SH neurones (IC_{50} $2.5x10^{-7}$ M) and the uptake of prazosin (at 10^{-6} M) in GnRH neurones (IC_{50} 10^{-6} M; Figure 11.3.4). In these experiments, the accumulation of amines was inhibited by desipramine 10^{-5} M, confirming that active uptake was taking place.

The organic base, chloroquine, inhibited the uptake of (-)-noradrenaline (at 10^{-6} M) in the SK-N-SH neurones (IC_{50} $8x10^{-6}$ M) and the uptake of prazosin (at 10^{-6} M) in the GnRH neurones (IC_{50} $6.8x10^{-6}$ M; Figure 11.3.5). In contrast, chloroquine had no effect on the association of prazosin (at 10^{-6} M) with COS-7 cells (Figure 11.3.5). In these experiments, desipramine 10^{-5} M inhibited the uptake of (-)-noradrenaline (at 10^{-6} M) in the SK-N-SH neurones and the uptake of prazosin (at 10^{-6} M) in the GnRH cells (not shown). In these experiments, as in Figure 11.1.4, desipramine 10^{-5} M had no effect on the accumulation of prazosin (at 10^{-6} M) in the COS-7 cells.

Extracellular pH was varied from 7.40 to 8.10 by increments of 0.10 units. Increases in extracellular pH in the range 7.50 to 8.10 were associated with reduction in the uptake of prazosin in the GnRH neurones and of the uptake of (-)-noradrenaline in the SK-N-SH neurones (Table 11.3.1). At pH 8.1, uptake of prazosin was 54% and uptake of (-)-noradrenaline was 68% of the appropriate control value at pH 7.40 (Table 11.3.1).

11.3.3 Effects of calcium channel blockade

The phenylethylamine verapamil is a substrate for Transport-P (see Section 11.6). As verapamil is a blocker of calcium channels, it seemed possible that entry of prazosin into the GnRH cells may be dependent on calcium channels.

Lanthanum and cadmium block all calcium channels (Hille, 1992). Lanthanum chloride (up to 10^{-4} M) and cadmium chloride (up to 10^{-4} M) had no consistent effect on the uptake of prazosin (at 10^{-6} M) in the GnRH neurones (Figure 11.3.6). In these experiments, desipramine 10^{-5} M inhibited the uptake of prazosin (at 10^{-6} M) in the GnRH neurones, confirming that active uptake was taking place.

11.3.4 Comment

Cooling the GnRH cells inhibited the incorporation of [^{3}H]prazosin (at 2.1×10^{-10} M) and abolished the paradoxical increase in accumulation of [^{3}H]prazosin (Figure 11.1.1). This suggested that the increase in accumulation of [^{3}H]prazosin may be due to an energy-requiring, active uptake process. The experiments which are described in this Section attempted to define the source of this energy.

Neurones accumulate amine transmitters by two active transport processes: carrier molecules in the plasma membranes of pre-synaptic nerve terminals recapture released neurotransmitters from the extracellular synaptic space into the cytoplasm. A second set of carriers in the membranes of neurosecretory vesicles then transport the recaptured amines from the cytoplasm for storage in the vesicles. In addition to transport into pre-synaptic nerve terminals, neurotransmitters may also be accumulated in some non-neuronal cells, such as myocytes and glia (see Section 7.5).

The plasma membrane transporters derive their energy from the electrochemical gradient of sodium ions which is generated by the Na^+/K^+-ATPase ('sodium pump'; Pedersen & Carafoli, 1987; Nelson, 1991). Thus, uptake of noradrenaline is absolutely dependent on the presence of sodium in the extracellular space (Iversen & Kravitz, 1966; Pacholczyk et al, 1991). This was confirmed in the SK-N-SH neurones (Figure 11.3.1). In contrast, in GnRH neurones studied in an identical manner, exclusion of sodium caused only a minor reduction of prazosin uptake; in the absence of sodium, desipramine-blockable uptake was evident, representing 70% of the uptake of prazosin at 60 minutes (Figure 11.3.1). Thus, uptake of prazosin in the GnRH neurones, unlike the known plasma membrane neurotransmitter transporters, is not absolutely dependent on the presence of sodium. Vanadate inhibits P-ATPase pumps, including the Na^+/K^+-ATPase (Cantley et al, 1978; Pedersen & Carafoli, 1987). Vanadate diminished the uptake of noradrenaline in SK-N-SH neurones but had no effect on the uptake of prazosin in GnRH neurones which were studied identically (Figure 11.3.2). This confirmed that prazosin uptake in GnRH neurones is not dependent on energy from the electrochemical gradient of sodium or a P-ATPase.

The vesicular neurotransmitter transporters derive their energy from the electrochemical gradient of protons which is generated by V-ATPase (Bashford et al, 1975a&b; Pedersen & Carafoli, 1987; Nelson, 1991). BafilomycinA1 is an inhibitor of V-ATPase proton pumps which are responsible for generating intracellular acidity (Bowman et al, 1988). BafilomycinA1 10^{-6} M increased the pH of lysosomes of cultured cells from 5.1 to 6.3 (Yoshimori et al, 1991). BafilomycinA1 completely inhibited prazosin uptake in GnRH cells (Figure 11.3.3), suggesting that uptake requires energy from an electrochemical proton gradient generated by a V-ATPase ion pump. Thus, enhanced prazosin accumulation at higher concentrations of the drug may be into an acidified intracellular compartment, such as neurosecretory vesicles. Inhibition of the uptake of prazosin by increases in extracellular pH (Table 11.3.1) is consistent with this conclusion. Further dependence for prazosin uptake on a proton gradient was obtained from experiments that used monensin and chloroquine. Monensin is a monovalent carboxylic ionophore which forms lipid-soluble complexes with cations. It traverses the lipid phase of cellular membranes, resulting in movement of sodium ions into cells, in exchange for protons (Pressman & Fahim, 1982; Ledger & Tanzer, 1984). At a concentration of $6x10^{-6}$ M, monensin increased the pH of intracellular acidified vesicles in fibroblasts from 5.0 to 6.2 (Maxfield, 1982). Chloroquine is an organic base which

diffuses into intracellular acidified particles; at a concentration of 10^{-4} M, chloroquine increased the pH of intracellular acidified vesicles in macrophages from approximately 4.7 to 6.4 (Ohkuma & Poole, 1978). These compounds, which increase intracellular pH by different mechanisms, inhibited the uptake of prazosin in the GnRH cells, as did increasing extracellular pH. This was consistent with the view that accumulation of prazosin in GnRH cells is likely to be into acidified intracellular vesicles. In contrast, chloroquine had no comparable inhibitory effect on the accumulaion of prazosin in COS-7 cells (Figure 11.3.5). This confirmed that accumulation of prazosin in GnRH cells is unlikely to be into acidified vesicles such as mitochondria or lysosomes which are present in all eukaryotic cells, but is more likely to be into some specialised neuronal compartment, such as neurosecretory vesicles or internalised clathrin-coated pits.

Lysosomotropic drugs are cationic amphiphilic compounds which exist as bases at physiological pH (pKa approximately 8; De Duve et al, 1974). They enter cells by diffusion in unprotonated form. They become protonated in the acidic environment of lysosomes, where they remain trapped due to their inability to diffuse through the lipid phase of the membrane (De Duve et al, 1974). The pKa of prazosin is 6.8 (Alabaster et al, 1987), which makes it an unlikely candidate for a lysosomotropic drug. Lysosomes are present in all eukaryotic cells but the organic base chloroquine did not reduce the association of prazosin with COS-7 kidney cells (Figure 11.3.5). This confirmed that prazosin is unlikely to accumulate as a result of lysosomotropic properties. Further, the lysosomotropic effect is accelerated by increasing extracellular pH, due to an increase in the proportion of the compound that is unprotonated and therefore able to diffuse into cells (De Duve et al, 1974; Marini et al, 1992). However, uptake of prazosin was in fact inhibited by increasing extracellular pH (Table 11.3.1). It therefore seems unlikely that uptake of prazosin is due to a lysosomotropic effect.

Thus, peptidergic neurones accumulate prazosin by an unusual, antidepressant-sensitive process which derives its energy from the electrochemical gradient of protons which is generated by V-ATPase.

Figure 11.3.1:

Effects of desipramine and exclusion of sodium on the uptake of amines in SK-N-SH noradrenergic neurones and GT1-1 GnRH peptidergic neurones.

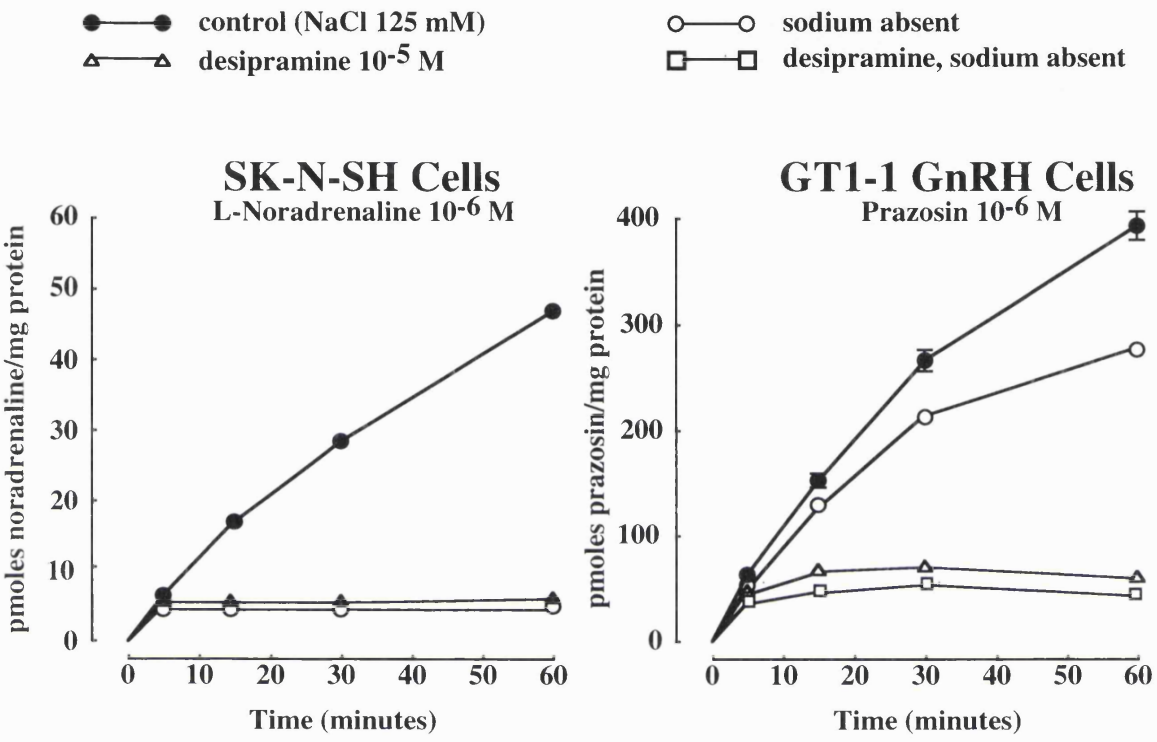


Figure 11.3.2:

Effects of sodium orthovanadate on the uptake of amines in SK-N-SH noradrenergic neurones and GT1-1 GnRH peptidergic neurones.

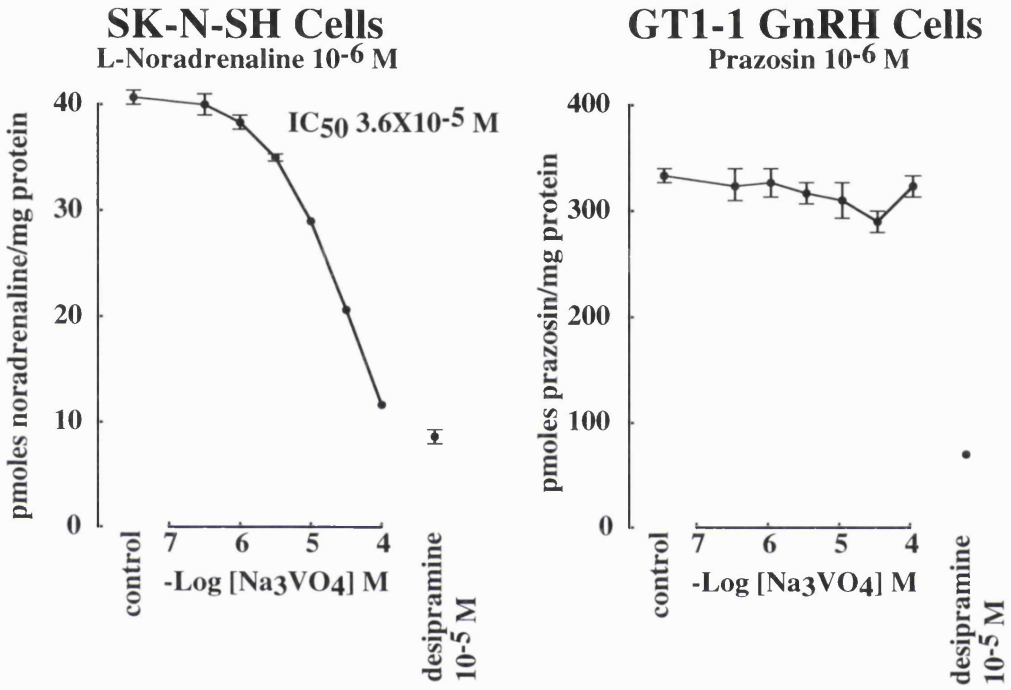


Figure 11.3.3:

Effects of bafilomycinA1 on the uptake of amines in SK-N-SH noradrenergic neurones and GT1-1 GnRH peptidergic neurones.

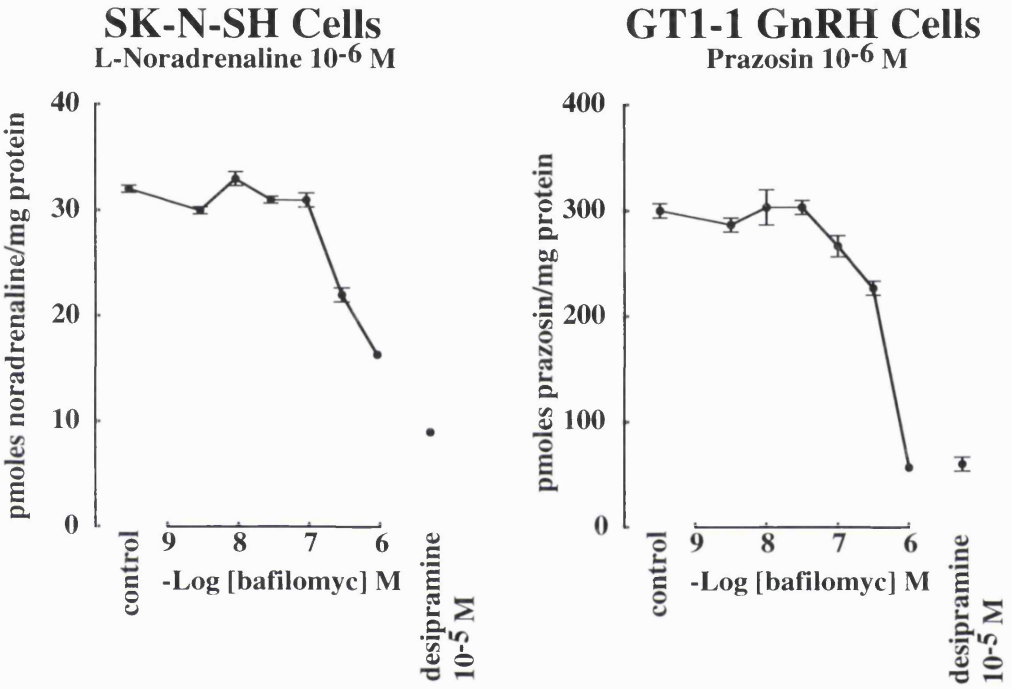


Figure 11.3.4:

Effects of monensin on the uptake of amines in SK-N-SH noradrenergic neurones and in GT1-1 GnRH peptidergic neurones.

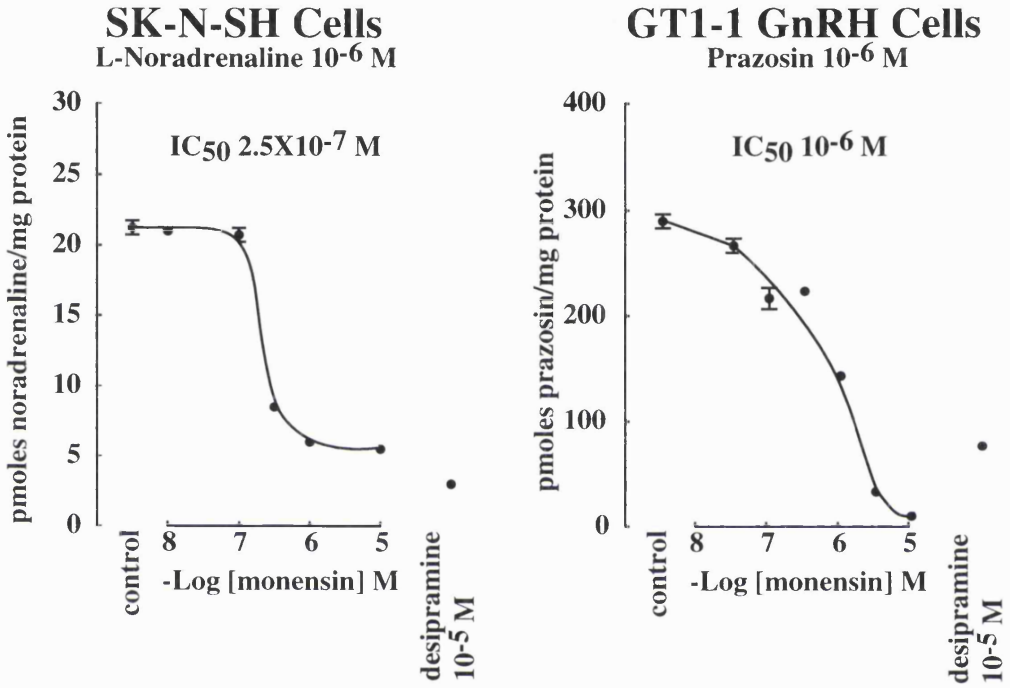


Figure 11.3.5:

Effects of chloroquine on the uptake of amines in SK-N-SH noradrenergic neurones, GT1-1 GnRH peptidergic neurones and COS-7 kidney cells

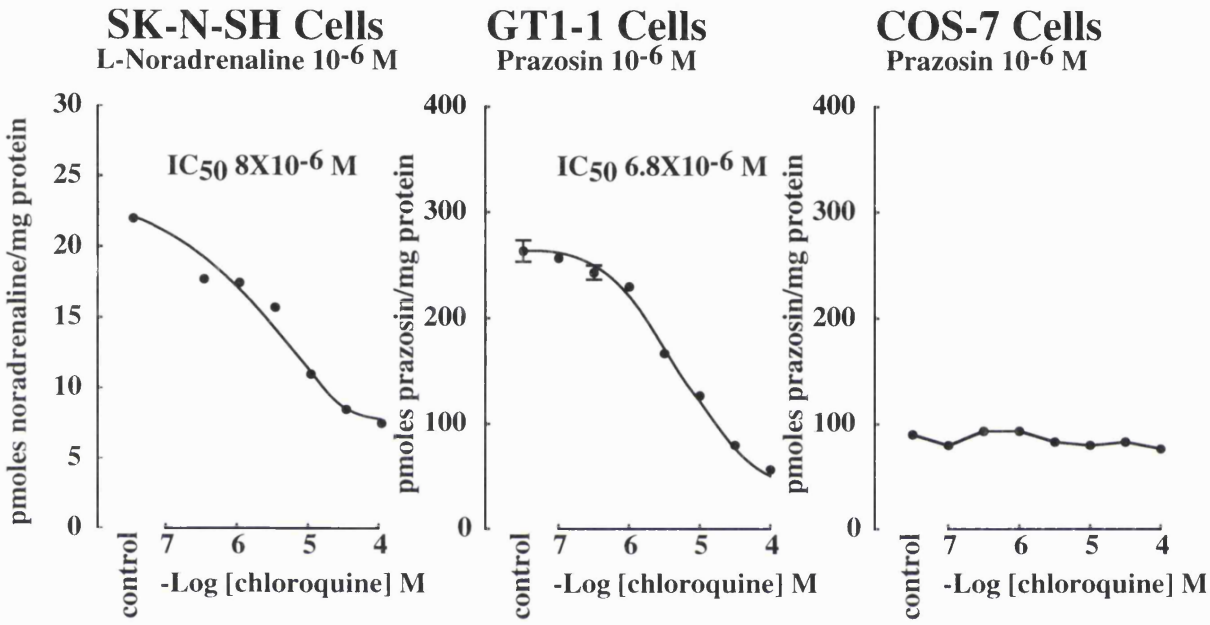


Figure 11.3.6:

Effects of lanthanum and cadmium on the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH neurones.

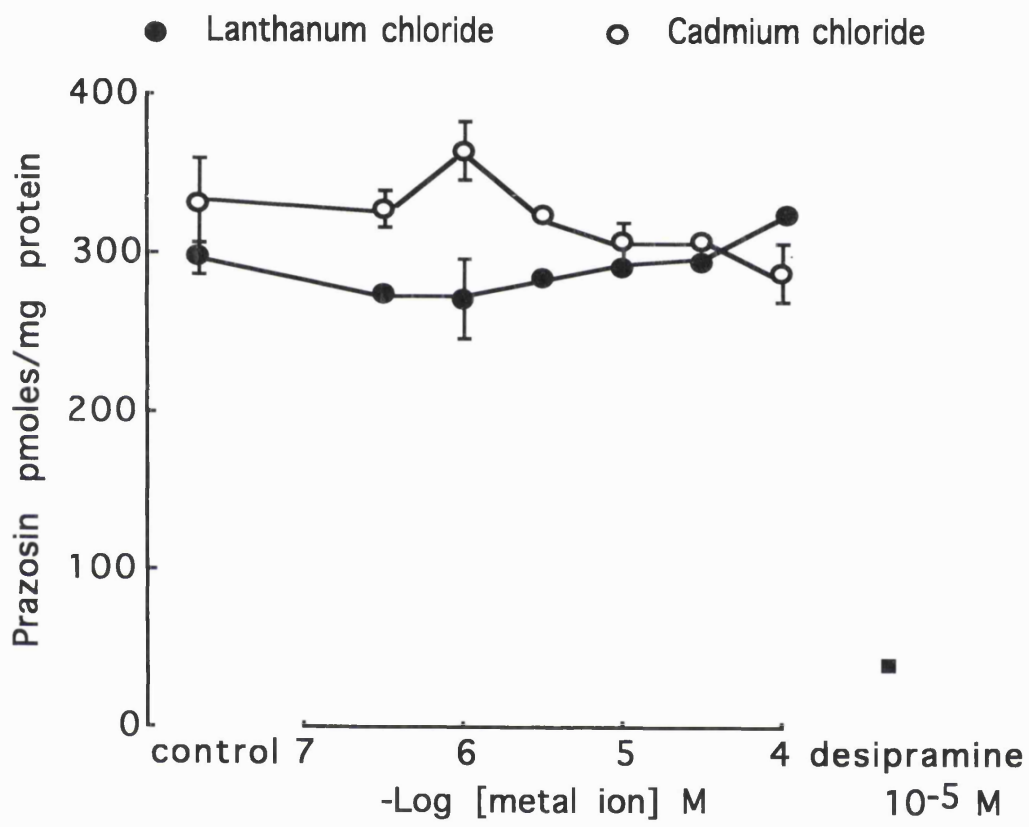


Table 11.3.1:

Effects of extracellular pH on the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH neurones and (-)-noradrenaline in SK-N-SH noradrenergic neurones.

pH	Prazosin (pmoles/mg) in GT1-1 cells	% control	Noradrenaline (pmoles/mg) in SK-N-SH cells	% control
7.40	292.8 ± 10.1	100%	33.8 ± 0.2	100%
7.50	306.5 ± 14.9	104%	34.4 ± 0.1	101%
7.60	278.6 ± 12.6	95%	32.5 ±0.4	96%
7.70	285.0 ± 11.2	97%	32.2 ± 0.1	95%
7.80	249.4 ± 11.3	85%	30.4 ± 0.5	90%
7.90	210.4 ± 5.9	71%	27.2 ± 0.0	80%
8.00	193.4 ± 10.9	66%	23.8 ± 0.2	70%
8.10	159.8 ± 15.4	54%	23.1 ± 0.1	68%

11.4 OTHER FUNCTIONAL PROPERTIES OF TRANSPORT-P

11.4.1 The prazosin equilibrium curve

Figure 11.4.1 shows linear plots of the uptake of prazosin in GT1-1 GnRH neurones at equilibrium (60 minutes). The lower panel describes the effect of unlabelled prazosin on the uptake of [^{3}H]prazosin (at $1.8x10^{-10}$ M), in the presence or absence of desipramine (at 10^{-4} M). Unlabelled prazosin concentrations ranged from 10^{-7} M to $4.3x10^{-6}$ M. The upper panel describes specific uptake of prazosin (labelled and unlabelled) by correcting for the fall in specific activity consequent upon isotope dilution. Non-specific uptake was defined as uptake in the presence of desipramine and specific uptake was obtained by subtracting non-specific from total uptake.

In the lower panel of Figure 11.4.1, it can be seen that increasing the concentration of unlabelled prazosin from 10^{-7} M to $4x10^{-7}$ M has no effect on the uptake of [^{3}H]prazosin; the paradoxical increase in accumulation of [^{3}H]prazosin appears at $6x10^{-7}$ M unlabelled prazosin. Uptake of [^{3}H]prazosin increases up to $2x10^{-6}$ M unlabelled prazosin, saturates and begins to decline at greater concentrations of unlabelled prazosin, due to competition between the radiolabelled ligand and the unlabelled ligand at these saturating concentrations.

In the upper panel of Figure 11.4.1, it can be seen that the accumulation of prazosin (labelled and unlabelled) in GnRH neurones is described by a sigmoidal curve which does not conform to the model of Michaelis & Menten (1913). In contrast, all the known uptake processes in pre-synaptic nerve terminals and in glial cells follow the Michaelis-Menten model, in which the increase in uptake is described by a parabola (Section 7.5).

The data from the experiment which is shown in Figure 11.4.1 are represented as a Hill plot in Figure 11.4.2A. In the vertical axis, "Y" is the fractional uptake of prazosin, ie, uptake/maximal uptake. Maximal uptake was obtained from a Lineweaver-Burk plot, using only the data for the greatest prazosin concentrations, as shown in Figure 11.4.2B; maximal uptake in this plot was obtained by extrapolating the linear trend line to infinite prazosin concentration (Blangy et al, 1968). The Hill coefficient is the slope of the curve at half-maximal uptake (0 on the vertical axis of Figure 11.4.2A). The Hill coefficient in this graph was 2.08. This is discussed further in Section 12.3.

The conclusion from these studies is that uptake of prazosin is cooperative; cooperativity is defined as increasing affinity for the ligand, as ligand binding proceeds (Baldwin & Chothia, 1979). The activation of Transport-P by a concentration of prazosin which is above a certain threshold suggests that the Transport-P carrier may be an allosteric membrane protein to which prazosin binds in a cooperative manner. This is discussed in detail in Section 12.3.

11.4.2 Effects of blockers of known transporters

From preceding Sections, it is clear that Transport-P shares some functional properties with other amine uptake processes: Transport-P resembles pre-synaptic vesicular transporters in its dependence on the electrochemical gradient of protons which is generated by V-ATPase (Section 11.3.2). Transport-P also resembles pre-synaptic plasma membrane transporters in that it is blocked by antidepressants (Section 11.2). In this Section, the functional properties of Transport-P were explored further by examining the effects of pharmacological manipulations which are known to block the known uptake processes for neurotransmitters.

11.4.2.1 Effects of blockers of pre-synaptic plasma membrane transporters

The plasma membranes of pre-synaptic nerve terminals possess transporter molecules which accumulate the amines noradrenaline, dopamine, serotonin or histamine. The excitatory and inhibitory amino acids (glutamate, aspartate and GABA) are also re-accumulated by the pre-synaptic nerve terminals whence they are released. These compounds were tested for their ability to inhibit competitively the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH peptidergic neurones, and they were all inactive as inhibitors of Transport-P (Table 11.4.1). Therefore, uptake of prazosin in the peptidergic neurones is unlikely to be via one of these known transporters.

Cocaine, which blocks the pre-synaptic re-uptake of noradrenaline, dopamine and serotonin, was effective in blocking Transport-P (Figure 11.4.3). Guanethidine and *meta*-iodobenzylguanidine (MIBG) are synthetic compounds which are accumulated by the pre-synaptic plasma membrane noradrenaline transporter; these compounds were inactive in blocking the accumulation of prazosin (at 10^{-6} M) in GT1-1 GnRH neurones (Table 11.4.1).

Thus, the functional properties of Transport-P are distinguishable from known plasma membrane neurotransmitter transporters (Table 11.4.2).

11.4.2.2 Effects of blockers of vesicular transporters

Pre-synaptic vesicular monoamine transporters accumulate the amines serotonin, dopamine, noradrenaline and histamine (Slotkin et al, 1979; Peter et al, 1994; Merickel & Edwards, 1995) but these amines did not compete with prazosin in GnRH neurones (Table 11.4.1). Reserpine blocks pre-synaptic vesicular monoamine transporters (see Section 7.5.5). In SK-N-SH cells, reserpine inhibited the uptake of (-)-noradrenaline (at 10^{-6} M) in a concentration-dependent manner (IC_{50} $1.3x10^{-8}$ M; Figure 11.4.4). This is similar to the potency of reserpine in membranes of CHO cells which had been transfected with the chromaffin cell vesicular amine transporter (IC_{50} $2.8x10^{-8}$ M; Liu et al, 1992c). In contrast, in GnRH neurones which were studied in an identical manner, reserpine had no consistent effect on the uptake of prazosin (Figure 11.4.4; prazosin uptake at 60 minutes in GnRH neurones: control 305.1 ± 10.7; reserpine 10^{-7} M 313.3 ± 8.5 pmoles/mg protein). Cocaine has no effect on the vesicular monoamine transporters (Liu et al, 1992c) but it was effective in blocking Transport-P (Figure 11.4.3). Thus, accumulation of prazosin in peptidergic neurones is not via a known vesicular amine transporter.

Uptake of prazosin was unaffected by acetylcholine or vesamicol (Table 11.4.1) which bind to the pre-synaptic vesicular transporter in cholinergic neurones (Liu & Edwards, 1997), so uptake of prazosin in peptidergic neurones is unlikely to be via the vesicular acetylcholine transporter.

GABA and glutamate are accumulated into acidified vesicles via specific transport proteins (Section 7.5.14). However, these two neurotransmitters did not inhibit the uptake of prazosin in the GT1-1 cells (Table 11.4.1). Therefore, uptake of prazosin in peptidergic neurones is not via one of these transporters.

These findings indicated that accumulation of prazosin in peptidergic neurones is not via a known vesicular neurotransmitter transporter.

11.4.2.3 Effects of blockers of $Uptake_2$

Prazosin is a substrate for $Uptake_2$ in non-neuronal cells (Grohmann & Trendelenburg, 1984). However, $Uptake_2$ is insensitive to desipramine and is independent of protons but is

blocked by steroid hormones (Iversen & Salt, 1970; Salt, 1972; Schomig et al, 1992; Russ et al, 1996). Uptake of prazosin in GnRH neurones was unaffected by hydrocortisone, corticosterone, β-oestradiol, progesterone or testosterone in concentrations of 10^{-5} M (Table 11.4.1). Two separate experiments were carried out, in which the steroid hormones were added in 10-fold excess over prazosin (steroids at 10^{-5} M; prazosin at 10^{-6} M) and in 5000 fold excess over prazosin (steroids at 10^{-5} M; prazosin at $2x10^{-9}$ M).

$Uptake_2$ accumulates a wide range of compounds, including phenoxybenzamine, serotonin, histamine and isoprenaline (Iversen et al, 1972; Grohmann & Trendelenburg, 1984); these compounds did not interact with Transport-P (Table 11.4.1). This indicated that accumulation of prazosin in GnRH neurones is not via $Uptake_2$.

11.4.2.4 Comment

The functional properties of Transport-P are clearly distinguishable from known neurotransmitter transporters (Table 11.4.2), indicating that uptake of prazosin is likely to be via an unidentified transport protein.

Figure 11.4.1:

Linear plots of the uptake of prazosin in GT1-1 GnRH neurones. The lower panel describes uptake of [^{3}H]prazosin, in the presence or absence of desipramine. The upper panel describes specific uptake of prazosin (labelled and unlabelled) by correcting for the fall in specific activity consequent upon isotope dilution. Non-specific accumulation was defined as uptake in the presence of desipramine and specific uptake was obtained by subtracting non-specific from total uptake. Accumulation of prazosin in GnRH neurones is described by a sigmoidal curve which is cooperative and is likely to be mediated by an allosteric carrier.

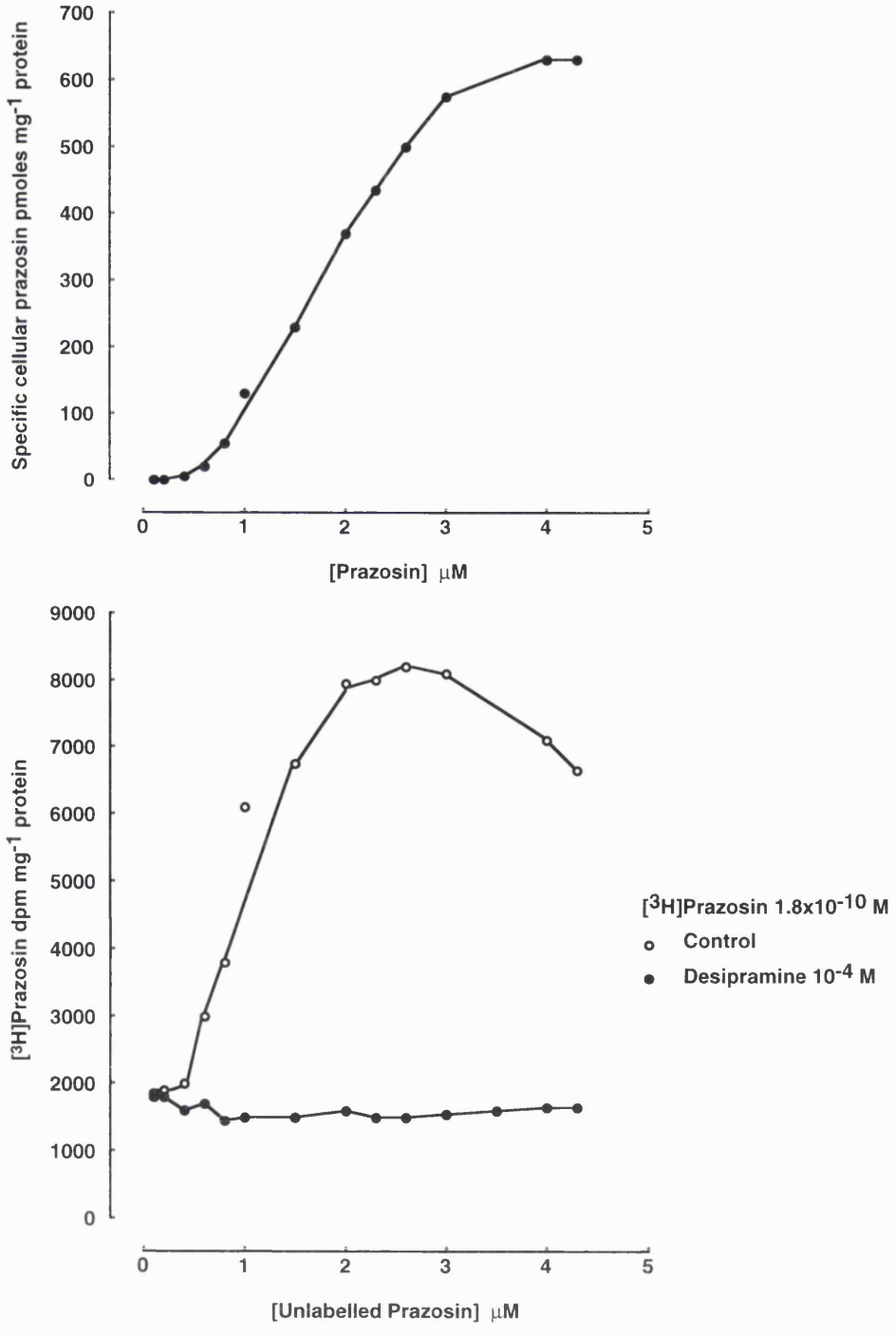
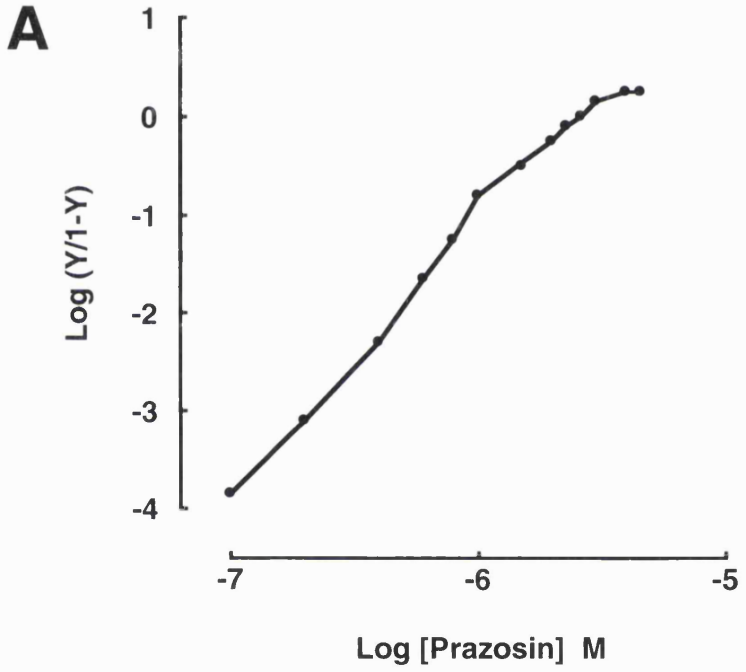


Figure 11.4.2:

Panel A shows a Hill plot of the uptake of prazosin in GT1-1 GnRH neurones. The data are from the experiment which is shown in Figure 11.4.1. In the vertical axis, "Y" is the fractional uptake of prazosin, ie, uptake/maximal uptake. Maximal uptake was obtained from a Lineweaver-Burk plot, using only the data for the greatest prazosin concentrations, shown in Panel B; maximal uptake in this plot was obtained by extrapolating the linear trend line to infinite prazosin concentration. In panel A, the Hill coefficient is the slope of the curve at half-maximal uptake (0 on the vertical axis). The Hill coefficient in this graph was 2.08.



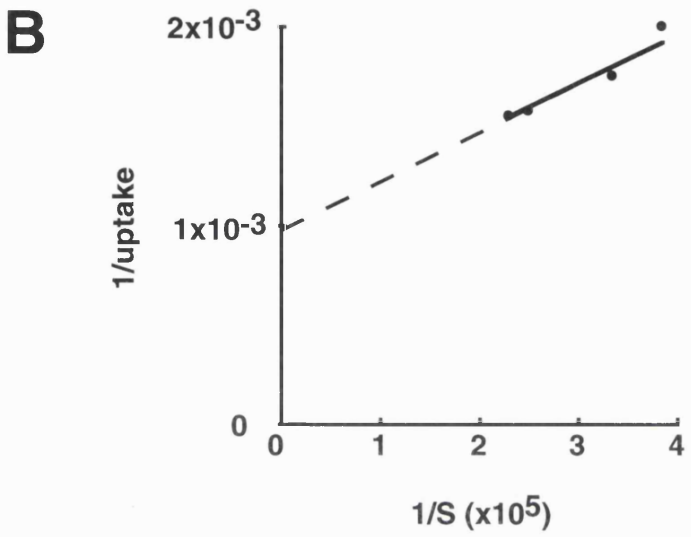
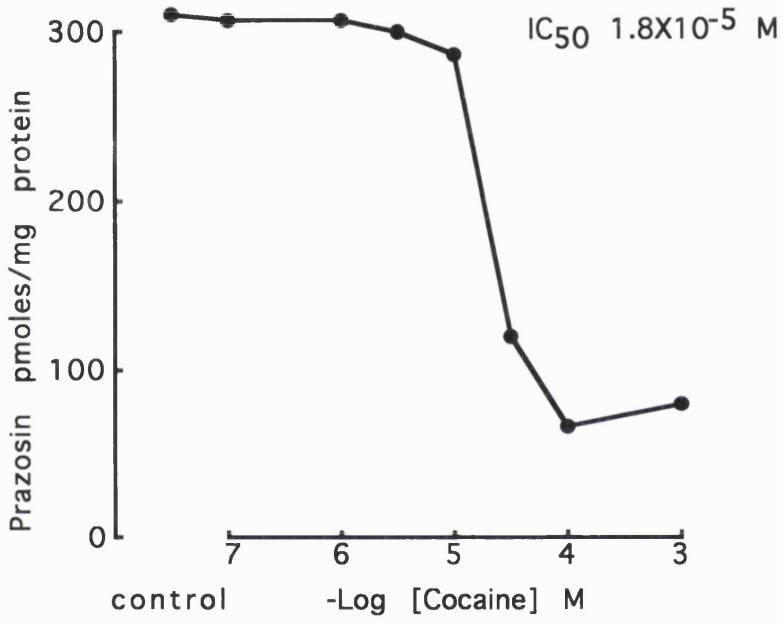
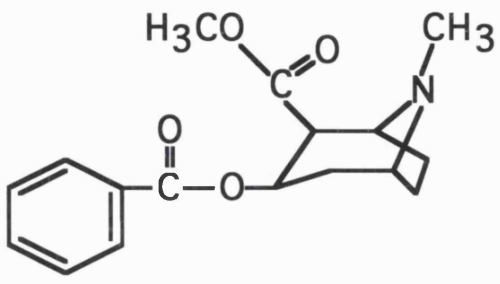


Figure 11.4.3:

Effect of cocaine, an inhibitor of plasma membrane amine transporters, on the uptake of prazosin in GT1-1 GnRH peptidergic neurones.





Cocaine

Figure 11.4.4:

Effects of reserpine, an inhibitor of vesicular amine transporters, on the uptake of amines in SK-N-SH noradrenergic neurones and in GT1-1 GnRH peptidergic neurones.

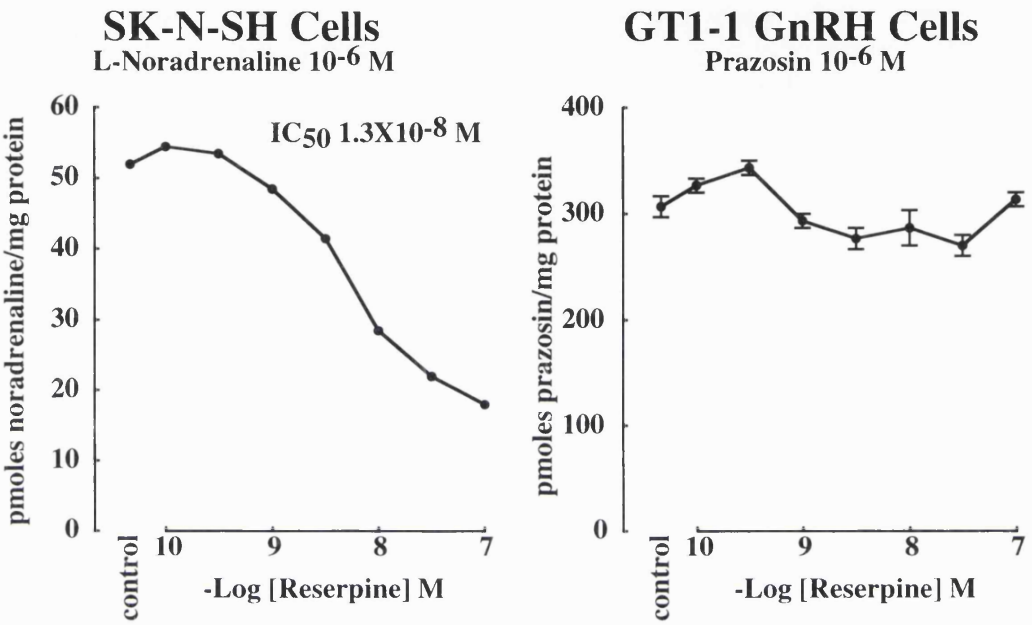


Table 11.4.1:

Effects of amines and neurotransmitters on the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH peptidergic neurones.

Compound	Efficacy %
(-)-Noradrenaline 10^{-3} M	28
Dopamine 10^{-3} M	41
(-)-Isoprenaline 10^{-4} M	18
Serotonin 10^{-4} M	0
L-Tryptophan 10^{-4} M	3
Histamine 10^{-4} M	4
L-Histidine 10^{-4} M	8
Acetylcholine 10^{-4} M	5
Choline 10^{-4} M	9
DL-Vesamicol 10^{-5} M	29
L-Glutamate 10^{-4} M	0
L-Aspartate 10^{-4} M	0
Glycine 10^{-4} M	0
GABA 10^{-4} M	0
ATP 10^{-4} M	20
Adenosine 10^{-3} M	17
Cocaine 10^{-4} M	100
Guanethidine 10^{-4} M	8
meta-iodobenzylguanidine (MIBG) 10^{-3} M	25
Phenoxybenzamine 10^{-4} M	22
Hydrocortisone 10^{-5} M	1
Corticosterone 10^{-5} M	0
β-Oestradiol 10^{-5} M	6
Progesterone 10^{-5} M	0
Testosterone 10^{-5} M	2

Table 11.4.2:

Comparison of some of the functional properties of Transport-P to other uptake processes in the brain.

	Transport-P	*Plasma membrane*	*Vesicular*	*Uptake$_2$*
Location	Post-synaptic	Pre-synaptic	Pre-synaptic	Non-neuronal
Amines activate	Yes	No	No	No
Energy source	V-ATPase	P-ATPase	V-ATPase	?
Ion dependence	H^+	Na^+	H^+	None
Antidepressants	Block	Block	No effect	No effect
Cocaine	Blocks	Blocks	No effect	No effect
Guanethidine	No effect	Blocks		
MIBG	No effect	Blocks		
Reserpine	No effect	No effect	Blocks	No effect
Steroids	No effect	No effect	No effect	Block

11.5 STRUCTURAL PROPERTIES OF LIGANDS FOR TRANSPORT-P

The work which is described in this Section aimed to define some of the structural properties of compounds which interact with Transport-P. The methods which were used are described in detail in Section 10.5. Briefly, compounds were tested for their ability to inhibit the uptake of [^{3}H]prazosin $2x10^{-9}$ M with unlabelled prazosin 10^{-6} M; this concentration of prazosin activates Transport-P. Non-specific uptake was defined as uptake in the presence of desipramine 10^{-4} M and specific (desipramine-sensitive) uptake was obtained by subtracting non-specific from total uptake. Typically, desipramine 10^{-4} M inhibited the accumulation of prazosin 10^{-6} M by 80% (Figure 11.5.1). Efficacy was defined as % inhibition of the uptake of prazosin 10^{-6} M when the test compound was used in a concentration of 10^{-4} M. Efficacy was expressed as % of the effect of a maximal inhibitory concentration of desipramine (10^{-4} M). Half-maximal inhibitory concentrations (IC_{50} values) were calculated from the concentration-response curves. IC_{50} values were calculated only for compounds which achieved a maximal inhibitory response, defined as 90% of the inhibitory effect of desipramine 10^{-4} M. When a compound did not achieve the maximal inhibitory response, IC_{50} values were not calculated and the data were expressed only as efficacy (% inhibition relative to desipramine 10^{-4} M). A representative member of each group of compounds was tested for its ability to inhibit the uptake of prazosin competitively. This was done by examining the effects of different concentrations of the test compound in the presence of different concentrations of unlabelled prazosin, while the concentration of [^{3}H]prazosin was kept constant. Figure 11.5.2 shows that in four separate experiments, increasing the concentration of unlabelled prazosin in the range 10^{-6} to $3x10^{-6}$ M caused the paradoxical increase in accumulation of [^{3}H]prazosin. Phenylethylamine, methylamphetamine, 3,4-dichloromethylamphetamine and 2,5-dimethoxyphenylethylamine represent different series of compounds which are active at Transport-P (see below). The inhibitory effects of these compounds could be reversed by increasing the concentration of unlabelled prazosin (Figure 11.5.2), indicating that they are competitive inhibitors of Transport-P.

11.5.1 Structural properties of phenylethylamines which inhibit Transport-P

The α_1 adrenoceptor agonist methoxamine is a phenylethylamine derivative which has

prominent effects on hypothalamic neuroendocrine function (Al-Damluji, 1993). The structural similarity between methoxamine and prazosin prompted an examination of the effects of phenylethylamine derivatives. The study examined phenylethylamine analogues for their ability to antagonise competitively the uptake of prazosin (at 10^{-6} M) in immortalised peptidergic neurones (GT1-1 GnRH cells) which possess Transport-P. In the presence of unlabelled prazosin 10^{-6} M, [^{3}H]prazosin is completely displaced from α_1 adrenoceptors (Figures 11.1.8 and 11.1.15). Nevertheless, as prazosin and methoxamine are ligands for α_1 adrenoceptors, the findings were compared to the structural properties of phenylethylamine derivatives which are active at α_1 adrenoceptors and at other amine uptake processes.

11.5.1.1 The amine group

Absence of an alkyl amine abolished the ability of phenylethylamines to inhibit the uptake of prazosin 10^{-6} M. Thus, phenylethanolamine was fully active in inhibiting the uptake of prazosin (efficacy 99%; IC_{50} $54x10^{-6}$ M) whereas its analogue phenylethylalcohol was inactive (efficacy 4%; Figure 11.5.3). Table 11.5.1 lists a series of phenylalkyl compounds which lack an alkyl amine, all of which were essentially inactive in inhibiting the accumulation of prazosin 10^{-6} M.

Presence of a carboxyl group on the α carbon abolished the ability of phenylethylamines to inhibit the uptake of prazosin; phenylethylamine (efficacy 100%; IC_{50} $16x10^{-6}$ M) and tyramine (efficacy 100%; IC_{50} $800x10^{-6}$ M) were fully active whereas their respective carboxylated analogues phenylalanine (efficacy 2%) and tyrosine (efficacy 0%) were inactive (Figure 11.5.3 and Table 11.5.1). Table 11.5.1 lists other carboxylated compounds which were inactive in inhibiting the accumulation of prazosin.

Presence of an aminomethyl group slightly reduced potency (Figure 11.5.4). Thus, amphetamine (IC_{50} $6x10^{-6}$ M) was 1.5 fold more potent than methylamphetamine (IC_{50} $15x10^{-6}$ M), and norephedrine (IC_{50} $37x10^{-6}$ M) was 0.2 fold more potent than ephedrine

(IC_{50} 43x10^{-6} M). However, these secondary amines retained full efficacy in inhibiting the uptake of prazosin, as did tertiary amines (Table 11.5.1). In contrast, quaternary amines and guanidines were inactive. Thus, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a tertiary amine, was fully active (efficacy 100%; IC_{50} 16x10^{-6} M) in contrast to its quaternary amine analogue 1-methyl-4-phenylpyridinium (MPP^+; efficacy 14%; Figure 11.5.3). Similarly, tyramine (efficacy 100%; IC_{50} 800x10^{-6} M) was fully active in contrast to N-guanyltyramine (efficacy 18%; Figure 11.5.3). Table 11.5.1 lists further quaternary and guanyl-amines which were inactive in inhibiting the uptake of prazosin 10^{-6} M.

11.5.1.2 The alkyl side chain, α-methyl and β-hydroxyl groups

Aniline was essentially inactive in inhibiting the uptake of prazosin 10^{-6} M but lengthening the alkyl side chain progressively increased potency (Figure 11.5.5).

Presence of a methyl group on the α carbon enhanced potency at Transport-P (Figure 11.5.4). This effect was observed in the following series of compounds: amphetamine (IC_{50} 6x10^{-6} M) was 1.7 fold more potent than phenylethylamine (IC_{50} 16x10^{-6} M); norephedrine (IC_{50} 37x10^{-6} M) was 0.5 fold more potent than phenylethanolamine (IC_{50} 54x10^{-6} M).

Presence of a hydroxyl group on the β carbon reduced potency at Transport-P (Figure 11.5.4). This effect was observed in the following series of compounds: phenylethylamine (IC_{50} 16x10^{-6} M) was 2.4 fold more potent than phenylethanolamine (IC_{50} 54x10^{-6} M); amphetamine (IC_{50} 6x10^{-6} M) was 5.2 fold more potent than norephedrine (IC_{50} 37x10^{-6} M); methylamphetamine (IC_{50} 15x10^{-6} M) was 1.9 fold more potent than ephedrine (IC_{50} 43x10^{-6} M). Further, tyramine was more potent than octopamine (efficacy 100% vs 21%) and dopamine was more potent than noradrenaline (efficacy 41% vs 28%; Figure 11.5.6). The combined effect of an α methyl and absence of a β hydroxyl increased potency 8 fold (amphetamine IC_{50} 6x10^{-6} M vs phenylethanolamine IC_{50} 54x10^{-6} M; Figure 11.5.4).

R-(-)-Amphetamine was equipotent to **S**-(+)-amphetamine (IC_{50} $6.7x10^{-6}$ M and $6x10^{-6}$ M, respectively). 1**R**,2**S**-(-)-Ephedrine was equipotent with 1**S**,2**R**-(+)-ephedrine (IC_{50} $3.4x10^{-5}$ M and $4.3x10^{-5}$ M, respectively). 1**R**,2**S**-(-)-Norephedrine was equipotent with 1**S**,2**R**-(+)-norephedrine (IC_{50} $4.0x10^{-5}$ M and $3.7x10^{-5}$ M, respectively).

11.5.1.3 The phenyl group and phenolic substitutions

Absence of the phenyl group abolished the ability of phenylalkylamines to inhibit the uptake of prazosin (Figure 11.5.5). Thus, methylamine, ethylamine, propylamine and butylamine (efficacy 14%, 10%, 18% and 20%, respectively) were essentially inactive in comparison to their respective phenylalkyl analogues (phenylmethylamine efficacy 100%, IC_{50} $37x10^{-6}$ M; phenylethylamine efficacy 100%, IC_{50} $16x10^{-6}$ M; phenylpropylamine efficacy 100%, IC_{50} $12x10^{-6}$ M; phenylbutylamine efficacy 100%, IC_{50} $6x10^{-6}$ M).

Presence of a single phenolic hydroxyl group in the *para* position strongly reduced potency at Transport-P (Figure 11.5.6). Thus, phenylethylamine (IC_{50} $16x10^{-6}$ M) was 49 fold more potent than tyramine (IC_{50} $800x10^{-6}$ M) in inhibiting the uptake of prazosin. Presence of a second phenolic hydroxyl group in the *meta* position further reduced potency at Transport-P (dopamine efficacy 41%). The effect of the phenolic *para* hydroxyl group was also seen in the following series of compounds: phenylethanolamine and octopamine; norephedrine and α-methyloctopamine (Figure 11.5.6).

Phenolic chlorine atoms increased potency at Transport-P (Figure 11.5.7). Thus, 2,4-dichlorophenylethylamine (IC_{50} $4x10^{-6}$ M) was 3 fold more potent than phenylethylamine (IC_{50} $16x10^{-6}$ M); 3,4-dichlorophenylethanolamine (IC_{50} $4x10^{-6}$ M) was 12.5 fold more potent than phenylethanolamine (IC_{50} $54x10^{-6}$ M); 3,4-dichloromethylamphetamine (IC_{50} $3x10^{-6}$ M) was 3.7 fold more potent than methylamphetamine (IC_{50} $14x10^{-6}$ M). Substitution of chlorine atoms with hydroxyl groups in the same positions reduced potency (3,4-dichlorophenylethanolamine efficacy 100% vs noradrenaline efficacy 28%).

Analogues of phenylethylamine which possessed one phenolic methoxyl group were equipotent with the parent compound, regardless of whether the methoxyl group was in the *ortho, meta* or *para* position (Figure 11.5.8). Further, methoxyphenamine was equipotent with methylamphetamine (Figure 11.5.8). Of the three compounds which possessed a dimethoxyphenyl group, 2,5-dimethoxyphenylethylamine was equipotent with phenylethylamine but 3,4-dimethoxyphenylethylamine (IC_{50} $69x10^{-6}$ M) was 3.3 fold less potent than phenylethylamine (IC_{50} $16x10^{-6}$ M) and methoxamine (IC_{50} $68x10^{-6}$ M) was 0.7 fold less potent than norephedrine (IC_{50} $40x10^{-6}$ M; Figure 11.5.8).

11.5.1.4 Comment on phenylethylamines as ligands for Transport-P and comparison to ligands of other amine transporters and α adrenergic receptors

This study defined the structural properties of phenylethylamines which interact with Transport-P. The essential structure is a hydrophobic phenyl ring and an amine which is separated from the phenyl ring by a carbon chain. These properties are different from those of phenylethylamines which interact with other amine transporters and receptors.

There is an absolute requirement for an amine in the side chain; absence of the amine or neutralization by a nearby carboxyl group on the α-carbon abolished activity (Figure 11.5.3 and Table 11.5.1). An aminomethyl group slightly reduced potency at Transport-P (Figure 11.5.4). Despite the lower potency of these secondary amines, it is clear that secondary and tertiary amines are fully active at Transport-P (Table 11.5.1). However, quaternary and guanyl amines are inactive (Figure 11.5.3 and Table 11.5.1). At pH 7.4, the amine exists in a protonated form (Maxwell et al, 1970; Lentzen & Philippu, 1981) and this presumably enables interaction with a negatively charged group in the Transport-P site, allowing entry into the cells. An aminomethyl group causes steric hindrance which may reduce potency. It is possible that the permanent positive charge in quaternary and guanyl amines may prohibit interaction with a strongly hydrophobic residue in the Transport-P site. Aminomethyl groups also reduced the affinity of phenylethylamines for the pre-synaptic plasma membrane dopamine and noradrenaline $Uptake_1$ sites but they enhanced affinity for noradrenaline $Uptake_2$ and for α_1 and α_2 adrenoceptors (Table 11.5.2; Burgen & Iversen, 1965; Grohman

& Trendelenburg, 1984; Horn, 1973a; Ruffolo et al, 1988; Nichols & Ruffolo, 1991). Aminomethyl groups had no effect on affinity for the vesicular uptake process in rat brain or in bovine adrenal medulla (Table 11.5.2; Slotkin & Kirshner, 1971; Slotkin et al, 1979; Peter et al, 1994).

Potency was reduced by a β-hydroxyl group and increased by an α-methyl group (Figure 11.5.4 and 11.5.6). These substitutions alter the molecular conformation of the alkylamine side chain; presence of a β-hydroxyl group exerts an electrostatic pull on the positively charged amine, resulting in preponderence of a conformational form in which the amine is folded towards the β-hydroxyl group (Pullman et al, 1972; Ison et al, 1973). Conversely, presence of an α-methyl group causes steric hindrance which reduces the likelihood of such folding (Ison et al, 1973). Phenolic hydroxyl groups exert no significant electrostatic effect on the rotational conformation of the amine (Pullman et al, 1972; Ison et al, 1973). The enhancement of potency by an α-methyl group and reduction by a β-hydroxyl group suggest that folding of the side chain does not favour interaction with the Transport-P site, which presumably favours a conformation in which the side chain is fully extended away from the phenyl group. In support of this suggestion, shortening the side chain progressively reduced potency whereas lengthening the side chain increased potency at Transport-P (Figure 11.5.5). An alternative explanation for the effects of the β-hydroxyl and α-methyl groups is that they may alter the hydrophobicity of the compounds (see below).

β-Hydroxyl and α-methyl groups influence the potencies of phenylethylamines at Transport-P in a similar manner to their effects on the affinities of these compounds for the pre-synaptic plasma membrane transporters for dopamine and noradrenaline and the vesicular transporters in rat brain and adrenal medulla (Table 11.5.2; Burgen & Iversen, 1965; Horn, 1973a; Slotkin et al, 1975; 1979; Pacholczyk et al, 1991; Giros et al, 1994). Studies which used rigid analogues confirmed that the side chain of phenylethylamines is in a fully extended conformation when these compounds interact with the pre-synaptic plasma membrane dopamine and noradrenaline $Uptake_1$ sites (Horn & Snyder, 1972; Miller et al, 1973; Horn, 1974). In contrast, a β-hydroxyl group is essential for agonist activity at α_1

and α_2 adrenoceptors (Table 11.5.2; Ruffolo et al, 1988; Nichols & Ruffolo, 1991). However, an α-methyl group enhances affinity of phenylethylamines for α_2 adrenoceptors but reduces affinity for α_1 adrenoceptors (Table 11.5.2; Ruffolo et al, 1988; Nichols & Ruffolo, 1991). These receptors presumably require different conformations of the side chain for maximal agonist binding (DeMarinis et al, 1981). As in the case of α_1 adrenoceptors, affinity for $Uptake_2$ is enhanced by a β-hydroxyl group and reduced by an α-methyl group (Table 11.5.2; Burgen & Iversen, 1965; Grohmann & Trendelenburg, 1984).

Amphetamine possesses a single chiral centre around the α-carbon whereas ephedrine and norephedrine possess two chiral centres around the α- and β-carbons. The equipotent effects of these three sets of enantiomers suggest that Transport-P may not distinguish between stereochemical arrangements of a methyl group at the α-carbon or a hydroxyl group at the β-carbon of phenylethylamines. The pre-synaptic plasma membrane dopamine transporter in rat brain recognises asymmetry of a methyl group on the α-carbon but does not distinguish asymmetry of a hydroxyl group on the β-carbon (Iversen et al, 1971; Ferris et al, 1972; Harris & Baldessarini, 1973; Thornburg & Moore, 1973; Koe, 1976; Meiergerd & Schenk, 1994; Giros et al, 1994). In contrast, α_1 adrenoceptors, the pre-synaptic plasma membrane noradrenaline transporter and $Uptake_2$ distinguish asymmetry of a hydroxyl group at the β-carbon but not a methyl group at the α-carbon (Table 11.5.2; Iversen et al, 1971; Ferris et al, 1972; Grohman & Trendelenburg, 1984; Bryan & O'Donnell, 1984; Ruffolo et al, 1988). α_2 Adrenoceptors and pre-synaptic vesicular monoamine transporters distinguish asymmetry of both a hydroxyl group at the β-carbon and a methyl group at the α-carbon (Table 11.5.2; Slotkin et al, 1979; Ferris & Tang, 1979; Ruffolo et al, 1988; Peter et al, 1994). Hydroxyl and methyl groups are small entities; it is possible that larger substitutions at the α- or β-carbons may be recognised stereospecifically by Transport-P.

In the present series, a phenyl group was essential for activity at Transport-P; alkyl amines of up to four carbons were inactive in comparison to their phenylalkyl analogues (Figure 11.5.5). Phenolic hydroxyl groups reduced potency (Figure 11.5.6) suggesting that the phenyl group should be hydrophobic for optimum activity at Transport-P. This suggestion is strengthened by the finding that hydrophobic chlorine atoms in the phenyl ring increased potency (Figure 11.5.7). Substitution of chlorine atoms with hydroxyl groups in the same positions reduced potency, suggesting that the enhancing effect of chlorine is unlikely to be due to an electronegative effect, but is more likely due to the hydrophobic nature of chlorine. These surprising findings are in striking contrast to the structural properties of phenylethylamines which bind α_1 and α_2 adrenoceptors, where phenolic hydroxyl groups strongly increased affinity (Table 11.5.2; Ruffolo et al, 1988; Nichols & Ruffolo, 1991). Phenolic hydroxyl groups also increased the affinity of phenylethylamines for pre-synaptic plasma membrane dopamine and noradrenaline $Uptake_1$ transporters, and for vesicular transporters in rat brain and adrenal medulla, although their effects on these transporters were less prominent than on α_1 and α_2 adrenoceptors (Table 11.5.2; Burgen & Iversen, 1965; Horn, 1973a; Slotkin et al, 1975; 1979; Peter et al, 1994). These findings explain the previous observation that very small amounts of noradrenaline accumulated in hypothalamic neurones (fmoles noradrenaline/mg protein *vs* pmoles prazosin/mg protein; Al-Damluji et al, 1993); potency at Transport-P is reduced by the phenolic and β-hydroxyl groups of noradrenaline (Figure 11.5.6).

A single phenolic methoxyl group had no effect on potency at Transport-P (Figure 11.5.8). The lack of effect of these neutral groups is consistent with the suggestion that the phenyl ring of these compounds should be hydrophobic for optimum activity at Transport-P. In clear contrast, a single phenolic methoxyl group reduced the affinity of phenylethylamines for the pre-synaptic plasma membrane dopamine and noradrenaline $Uptake_1$ sites (Table 11.5.2; Burgen & Iversen, 1965; Horn, 1973a). Phenolic methoxyl groups enhanced the affinity of phenylethylamines for noradrenaline $Uptake_2$ (Burgen & Iversen, 1965; Grohman & Trendelenburg, 1984) and for α_1 adrenoceptors (DeMarinis et al, 1981), but they had no effect on the affinity of these compounds for α_2 adrenoceptors (Table 11.5.2; Ruffolo et al,

1988).

Phenylethylamine and its derivatives inhibited the uptake of prazosin competitively (Figure 11.5.2), suggesting that these compounds and prazosin act on the same Transport-P carrier molecule in GnRH neurones. It therefore seems likely that the structural properties which were described in this study may define some of the requirements for interaction of phenylethylamines with the Transport-P carrier molecule in peptidergic neurones. An alternative explanation is that these amines may have inhibited the uptake of prazosin by dint of their lipophilic nature, which may have enabled them to diffuse across cell membranes, resulting in neutralisation of the acidified intracellular compartment in which prazosin is accumulated. However, this seems an unlikely explanation as uptake of prazosin was unaffected by some highly lipophilic amines, including reserpine, phenoxybenzamine and vesamicol (Figure 11.4.2; Table 11.4.1). At present, the most likely explanation for the findings is that these compounds compete with prazosin for binding to a carrier molecule in peptidergic neurones.

The work which is described in this Section identified the structures of phenylethylamine derivatives which interact with Transport-P. These Transport-P ligands can be clearly distinguished from ligands for other amine transporters and receptors (Table 11.5.2).

11.5.2 The basicity of ligands for Transport-P

The work on phenylethylamines which is decribed in the previous Sections had indicated that in order to be effective ligands for Transport-P, compounds of this class must possess a hydrophobic phenyl group, a side chain and an amine. Presence of a negatively charged carboxyl group adjacent to the amine abolished activity at Transport-P. This indicated that a positively charged amine was an essential requirement for activity at Transport-P. Surprisingly, compounds which possess a permanent positive charge on the nitrogen (eg, quaternary and guanyl amines) were inactive at Transport-P (Section 11.5.1.1). Further, aniline was inactive at Transport-P (Section 11.5.1.2). The amine in aniline is neutral at physiological pH because the nitrogen lone-pair electrons is delocalised by orbital overlap with the π electron system of the phenyl ring (McMurry, 1992). It was unknown whether the lack of activity of aniline at Transport-P was due to this weak basicity or to the fact that the amine is in close proximity to the phenyl ring, regardless of basicity.

This problem was addressed by examining the effect of an additional hydrophobic phenyl group which is located at a distance from the amine which is appropriate for activity at Transport-P. The distance chosen was three carbon atoms, which is the distance between the amine and the phenyl ring in phenylpropylamine, which is fully active at Transport-P (Figure 11.5.9). Compounds such as 2-benzylaniline in which a hydrophobic phenyl group is located three carbon atoms from the anilino-amine were inactive at Transport-P (Figure 11.5.9). Other compounds in which a phenoxyl group is located in the *ortho, meta* or *para* position were also inactive (Figure 11.5.9). This indicated that the lack of effect of aniline is not due simply to the proximity of the amine to the benzene ring; the lack of effect of aniline is likely to be due to the neutral charge of its amine at physiological pH.

To test this hypothesis further, the following compounds were examined:

1. Amides: The nitrogen is neutral in amides, due to delocalisation of the lone-pair electrons with the carbonyl group (McMurry, 1992). Phenylethylamide (phenylacetamide) was inactive at Transport-P, whereas its basic analogue phenylethylamine had full activity (Figure 11.5.10).
2. Pyridine, which is a very weak base (pK_a 5.2) was much less active at Transport-P than its basic analogue piperidine (pK_a 11.1; Figure 11.5.10).
3. 1-Aminonaphthalene and 1-aminofluorene are weak bases because as in aniline, the nitrogen lone-pair electrons is delocalised by orbital overlap with the π electron system of the phenyl ring. 1-Aminonaphthalene and 1-aminofluorene were inactive whereas their basic analogues 1-naphthalenemethylamine and 9-aminofluorene had full activity at Transport-P (Figure 11.5.10).

In conclusion, compounds which are active at Transport-P must have an amine which is neither permanently neutral nor permanently positively charged. The amine group must be able to acquire and to shed its charge, presumably while it traverses the Transport-P carrier. The significance of these findings is discussed further in Sections 12.3 and 12.4.

11.5.3 The carbon skeleton

The work which is described in this Section was part of a collaboration with Professor CR Ganellin FRS. In order to test the specificity of the uptake process, Professor Ganellin supplied three commercially available compounds, dipentylamine, dihexylamine and didecylamine. Surprisingly, dipentylamine and dihexylamine were active at Transport-P but

didecylamine was inactive. This was particularly surprising because didecylamine is much more hydrophobic than dipentylamine and dihexylamine. Further, amitriptyline and trimipramine have full activity at Transport-P (Section 11.2); these two antidepressants possess 20 carbon atoms, like didecylamine. These initial findings suggested that Transport-P may be able to accumulate small hydrophobic amines regardless of structure, but that large hydrophobic amines can only be accumulated if they possess phenyl groups. This hypothesis was tested by studying in detail the effects of a series of aliphatic amines, in comparison with their aromatic counterparts, including antidepressant compounds (Table 11.5.3). The findings were as follows:

1. Transport-P cannot accumulate aliphatic amines consisting of 1-4 carbon atoms, confirming previous observations (Figure 11.5.5). Thus, methylamine, ethylamine, propylamine and butylamine were all inactive (Table 11.5.3).

2. Transport-P is capable of accumulating aliphatic amines consisting of 5-13 carbon atoms. Thus, compounds in the progressive series pentylamine to tridecylamine were all fully active at Transport-P, defined as >90% inhibition of the specific uptake of prazosin 10^{-6} M, when the test compound was used in a concentration of 10^{-4} M (Table 11.5.3).

3. The affinity of the C10-C12 aliphatic amines for Transport-P is not much less than the affinity of their phenolic counterparts. Thus, the IC_{50} values for the 10-carbon compounds dipentylamine, phenylbutylamine and methylamphetamine were $7.9x10^{-6}$ M, $3.1x10^{-6}$ M and $1.0x10^{-6}$ M, respectively, and the IC_{50} values for the 12-carbon compounds dihexylamine, MPTP and benzylpiperidine were also in a similar range (Table 11.5.3). This suggests that in this size of compounds, Transport-P is capable of accumulating hydrophobic amines regardless of structure.

4. Aliphatic amines consisting of a single chain of 14 or more carbon atoms cannot be accumulated effectively by Transport-P. Thus, in the series tetradecylamine, pentadecylamine, hexadecylamine and octadecylamine (C14-C18), there was progressive reduction in efficacy at Transport-P (Table 11.5.3). Further, the 19-carbon compound N-methyloctadecylamine was inactive at Transport-P.

5. Although aliphatic amines consisting of a single chain of 15-18 carbon atoms were inactive at Transport-P, equivalent aliphatic compounds consisting of the same number of carbon atoms arranged in two chains were active at Transport-P. Thus, hexadecylamine was inactive whereas dioctylamine was active; octadecylamine was inactive whereas trihexylamine was active (Table 11.5.3). These surprising findings suggest that reducing the molecular size of the aliphatic amine increases its activity at Transport-P. Thus, Transport-P is capable of accumulating hydrophobic amines only if they are within a certain molecular size.

6. Didecylamine was inactive at Transport-P (Table 11.5.3). As dioctylamine was active at Transport-P, the data suggest that Transport-P cannot accumulate aliphatic amines which consist of two carbon chains if the chain lengths exceed 9 carbon atoms.

7. Whereas the aliphatic amines octadecylamine (C18), N-methyloctadecylamine (C19) and didecylamine (C20) were inactive at Transport-P, their aromatic counterparts desipramine (C18), nortriptyline (C19), imipramine (C19), amitriptyline (C20) and trimipramine (C20) were fully active (Table 11.5.3). Aromatic compounds are condensed structures; the findings are therefore consistent with the conclusion that Transport-P can only accumulate compounds which are smaller than a certain size.

8. Desipramine (C18) was much more potent than the equivalent aliphatic compound trihexylamine (C18), despite the greater lipophilicity of trihexylamine (Table 11.5.3). This is consistent with the conclusion that in the C18-C20 range of compounds, the compact phenolic structure is more effective at Transport-P than the branched aliphatic arrangement.

9. Among aromatic compounds, increasing size increased affinity for Transport-P (Table 11.5.3). Thus, the antidepressants desipramine (C18), imipramine (C19) and trimipramine (C20) were much more potent than phenylbutylamine (C10), methylamphetamine (C10) and benzylpiperidine (C12). This may be due to the greater lipophilicity of the larger compounds, although this point requires further investigation.

10. Among aliphatic compounds, increasing size reduced the affinity for Transport-P (Table 11.5.3). Thus, dipentylamine and dihexylamine were much more potent than trihexylamine. This may be due to the requirement for a relatively compact structure. As trihexylamine is

much more lipophilic than dipentylamine and dihexylamine, the findings suggest that molecular size is the critical determinant of activity at Transport-P.

11. Pentylamine (C5) approximates to LogP of 1.5 and this may be the minimum lipophilicity of ligands which can be accumulated by Transport-P.

11.5.4 The importance of the furan group in activating Transport-P

In the collaboration with Professor Ganellin, novel compounds have been synthesised in Professor Ganellin's laboratory and these compounds are then tested by the author. The aim is to identify the structural properties of the prazosin molecule which are responsible for the phenomena under observation. UCL-2381 is an analogue of prazosin in which the furan has been reduced to tetrahydrofuran. This compound was far weaker than prazosin in activating Transport-P, indicating that an aromatic group is required for maximal activation of Transport-P (Figure 11.5.11). UCL-2362 is another prazosin analogue in which the furan group has been substituted with benzene. This compound was far weaker than prazosin in activating Transport-P, indicating that furan is better than benzene (Figure 11.5.11). This conclusion has been supported by further evidence from a series of analogues; the data are not described in this Thesis, in order to maintain the confidentiality of the intellectual property at this stage.

11.5.5 Conclusion:

A large series of compounds has been examined for:

A. The ability to activate Transport-P; the furan is a critical determinant of this function.

B. The ability to inhibit competitively the uptake of prazosin via Transport-P. Compounds which have the greatest affinity consist of:

   1. A basic amine which is neither permanently neutral nor permanently charged;
   2. A condensed cyclic structure of 18-20 carbons;
   3. Phenolic hydroxyls reduce affinity;
   4. Phenolic halogens enhance affinity.

The significance of these findings is discussed further in Sections 12.3 and 12.4.

Figure 11.5.1:

Effects of phenylethylamine and two of its derivatives (3,4-dichloromethylamphetamine and methoxamine) on the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH peptidergic neurones. The effect of desipramine is also shown for comparison. The vertical axis shows the total amount of prazosin accumulated in the cells by correcting for specific activity, ie, [^{3}H]prazosin + unlabelled prazosin/mg protein.

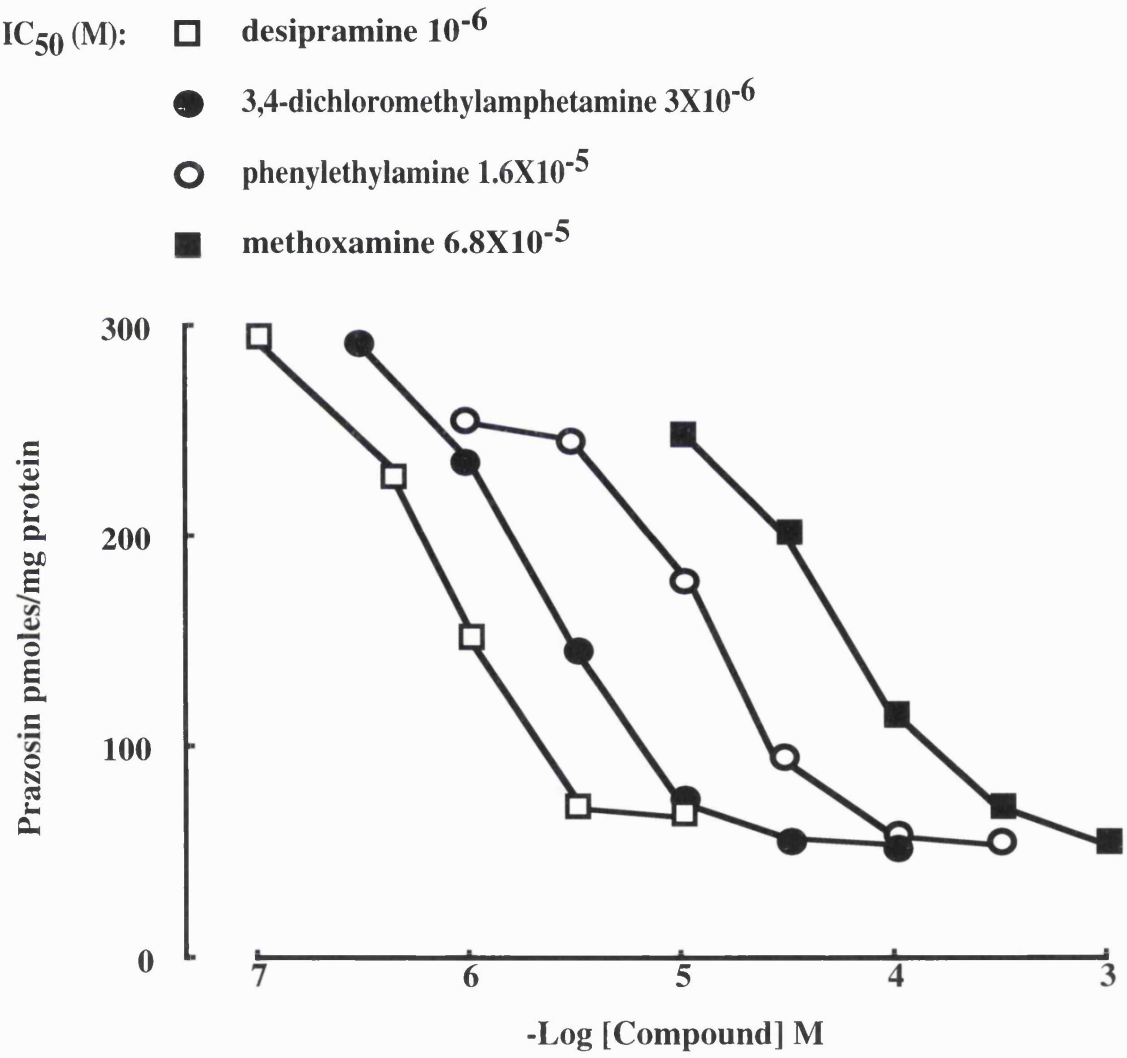


Figure 11.5.2:

Reversal of the inhibitory effect of phenylethylamine derivatives by increasing concentrations of unlabelled prazosin; indicating that the effects of the phenylethylamines are competitive. The points do not fall on straight lines because they form sigmoidal concentration-response curves in these log-linear plots (see Figure 11.5.1). Note that the vertical axis shows only the amount of [^{3}H]prazosin/mg protein accumulated in the cells; this demonstrates that increasing concentrations of unlabelled prazosin cause the paradoxical increase in accumulation of [^{3}H]prazosin in the peptidergic neurones.

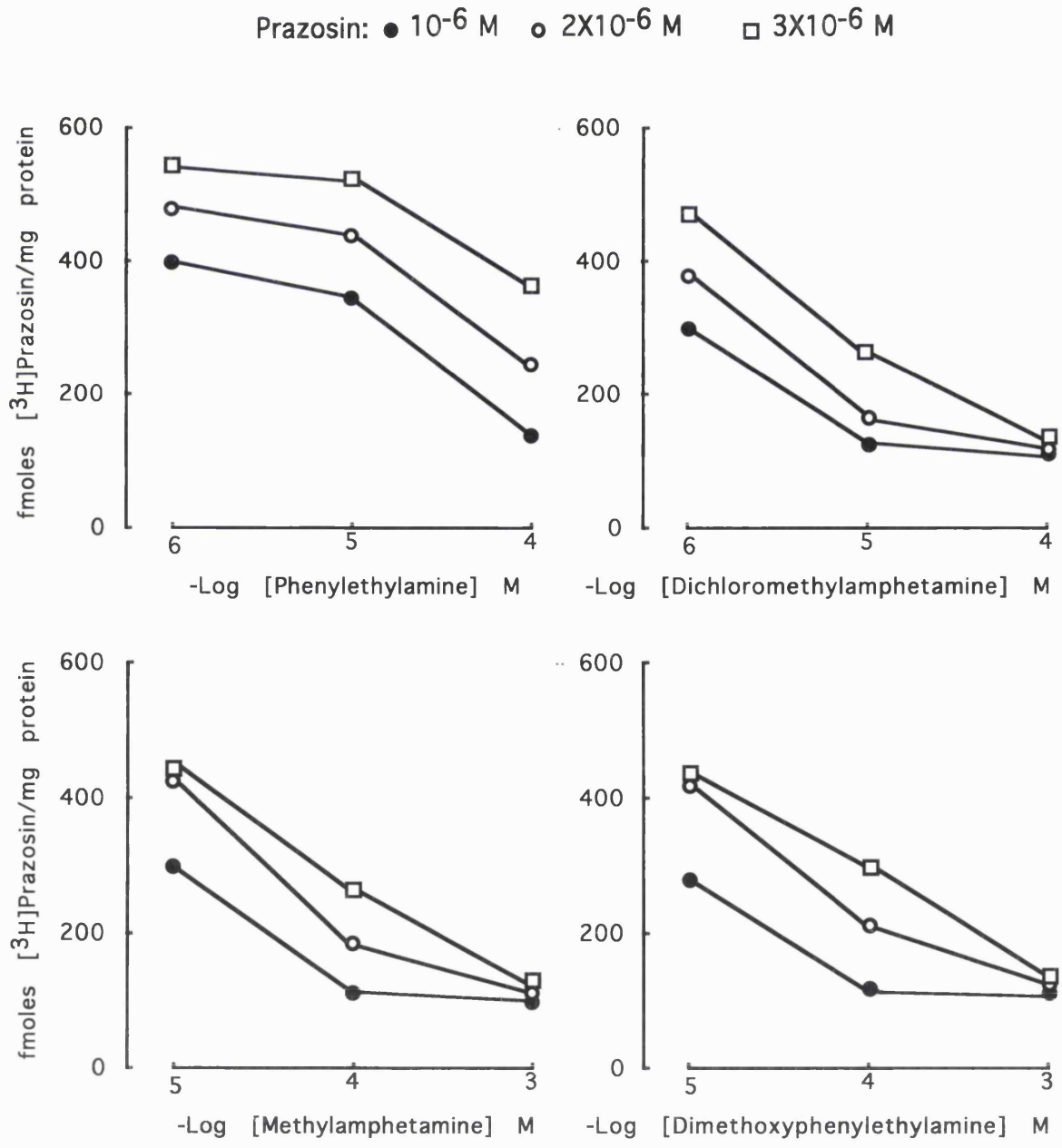
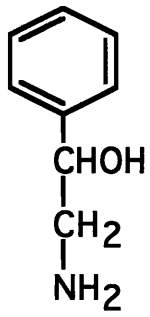
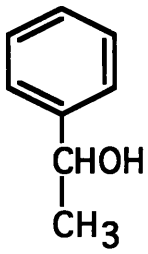


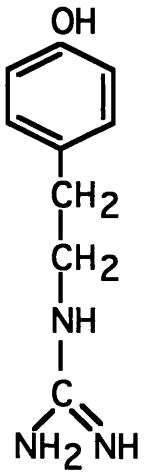
Figure 11.5.3:

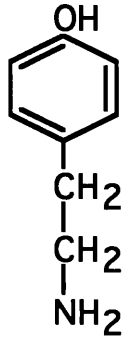
Effects of substitutions of the alkyl amine on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin (at 10^{-6} M) in GnRH peptidergic neurones.

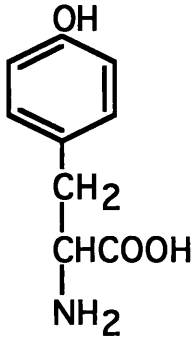




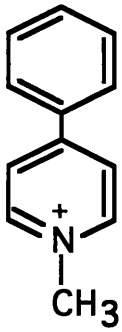
	Phenylethanolamine	Phenylethylalcohol
IC_{50} (μM)	54	—
Efficacy	99%	4% at 10^{-3} M

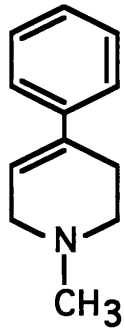






	N-Guanyl-Tyramine	Tyramine	L-Tyrosine
IC_{50} (μM)	—	800	—
Efficacy	18% at 10^{-3} M	100%	0% at 10^{-3} M

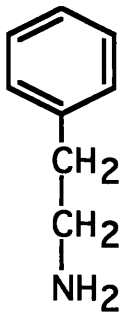


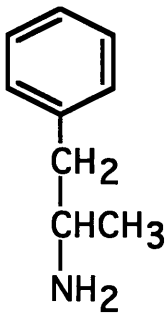


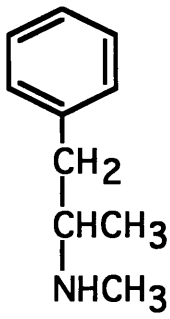
	MPP^+	MPTP
IC_{50} (μM)	—	16
Efficacy	14% at 10^{-3} M	100%

Figure 11.5.4:

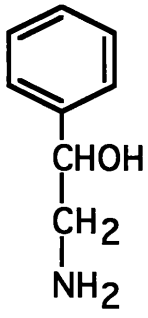
Effects of aminomethyl, α-methyl and β-hydroxyl groups on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin (at 10^{-6} M) in GnRH peptidergic neurones.

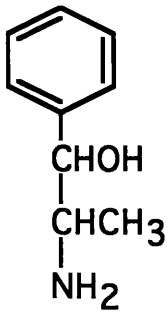


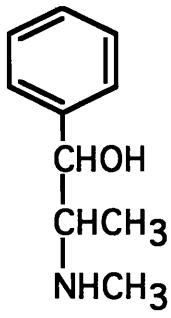




	Phenylethylamine	D-Amphetamine	Methylamphetamine
IC_{50} (μM)	16	6	15
Efficacy	100%	100%	94%



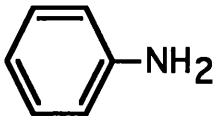




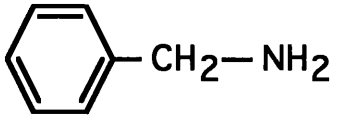
	Phenylethanolamine	D-Norephedrine	D-Ephedrine
IC_{50} (μM)	54	37	43
Efficacy	99%	96%	93%

Figure 11.5.5:

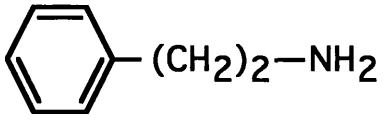
Effects of the phenyl group and length of the alkyl side chain on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin (at 10^{-6} M) in immortalised peptidergic neurones.



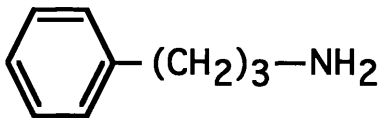
	Aniline
IC_{50} (μM)	—
Efficacy	19% at 10^{-3} M



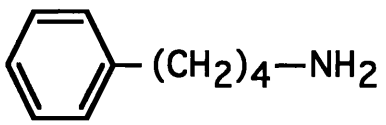
	Phenylmethylamine	CH_3NH_2 Methylamine
IC_{50} (μM)	37	—
Efficacy	100%	14% at 10^{-4} M



	Phenylethylamine	$CH_3CH_2NH_2$ Ethylamine
IC_{50} (μM)	16	—
Efficacy	100%	10% at 10^{-4} M



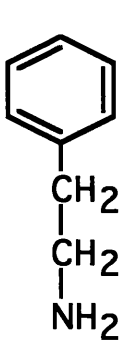
	Phenylpropylamine	$CH_3(CH_2)_2NH_2$ Propylamine
IC_{50} (μM)	12	—
Efficacy	100%	18% at 10^{-4} M

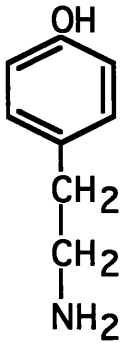


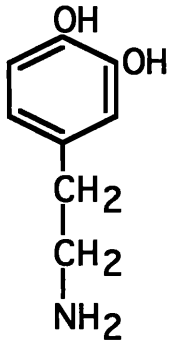
	Phenylbutylamine	$CH_3(CH_2)_3NH_2$ Butylamine
IC_{50} (μM)	6	—
Efficacy	100%	20% at 10^{-4} M

Figure 11.5.6:

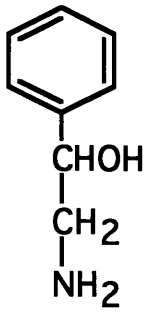
Effects of phenolic hydroxyls and β-hydroxyls on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in GnRH neurones.

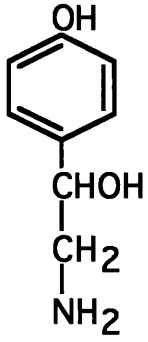


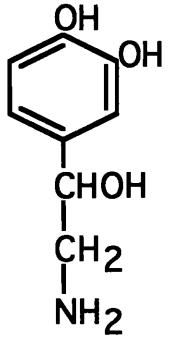




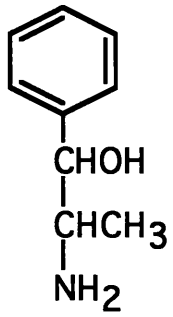
	Phenylethylamine	Tyramine	Dopamine
IC_{50} (μM)	16	800	—
Efficacy	100%	100%	41% at 10^{-3} M

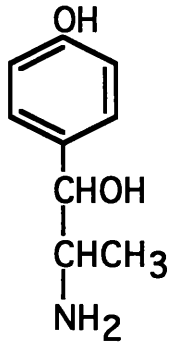


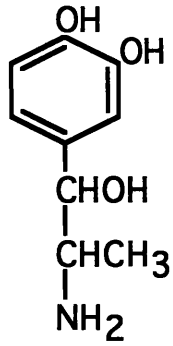




	Phenylethanolamine	Octopamine	L-Noradrenaline
IC_{50} (μM)	54	—	—
Efficacy	99%	21% at 10^{-3} M	28% at 10^{-3} M



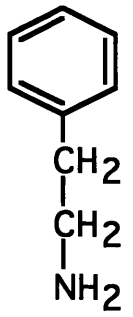




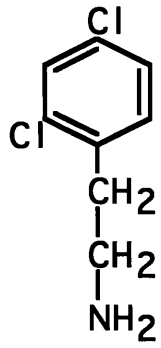
	L-Norephedrine	α-methyl-octop.	α-methyl-noradren.
IC_{50} (μM)	40	—	—
Efficacy	95%	0% at 10^{-3} M	20% at 10^{-3} M

Figure 11.5.7:

Effects of phenolic chlorine substitutions on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalised GnRH neurones.

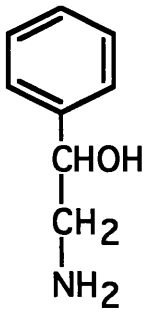


Phenylethylamine

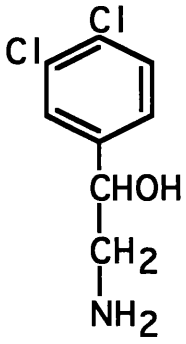


2,4-Dichloro-PEA

IC_{50} (μM)	16	4
Efficacy	100%	100%

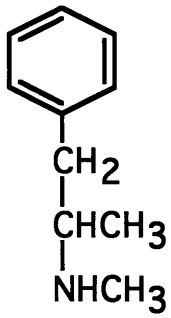


Phenylethanolamine

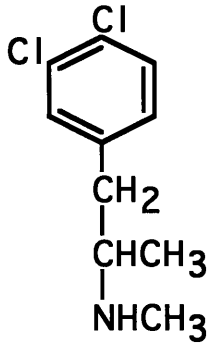


3,4-Dichloro-PEolA

IC_{50} (μM)	54	4
Efficacy	99%	100%



Methylamphetamine

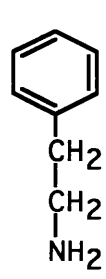


3,4-Dichloromethylamphet.

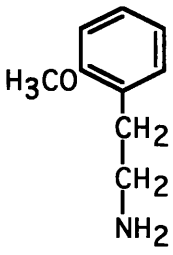
IC_{50} (μM)	14	3
Efficacy	94%	100%

Figure 11.5.8:

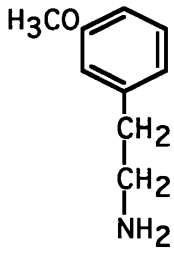
Effects of phenolic methoxyl groups on the potency and efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin 10^{-6} M in immortalised GnRH neurones.



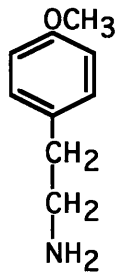
Phenylethylamine



2-Methoxy-PEA

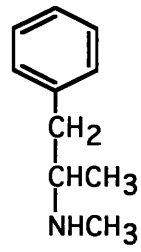


3-Methoxy-PEA

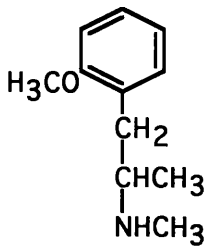


4-Methoxy-PEA

IC_{50} (μM)	16	19	19	16
Efficacy	100%	91%	98%	100%

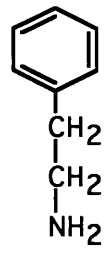


Methylamphetamine

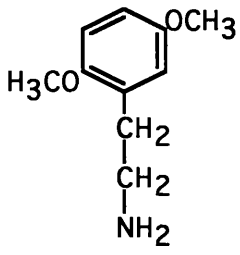


Methoxyphenamine

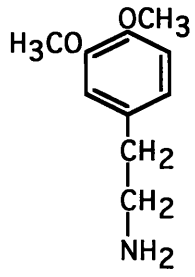
IC_{50} (μM)	14	17
Efficacy	94%	90%



Phenylethylamine

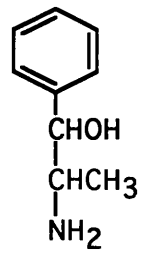


2,5-Dimethoxy-PEA

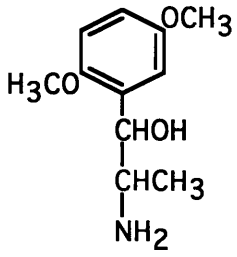


3,4-Dimethoxy-PEA

IC_{50} (μM)	16	18	69
Efficacy	100%	96%	93%



L-Norephedrine



Methoxamine

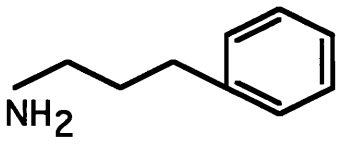
IC_{50} (μM)	40	68
Efficacy	95%	100%

Figure 11.5.9:

Basicity of the amine: effects of aniline and its derivatives on the uptake of prazosin 10^{-6} M in GT1-1 GnRH peptidergic neurones.

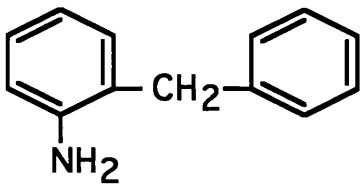
Aniline

Efficacy 0%



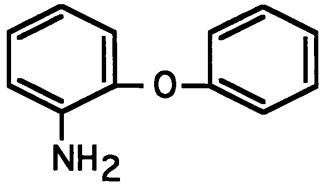
3-Phenyl-1-propylamine

97%



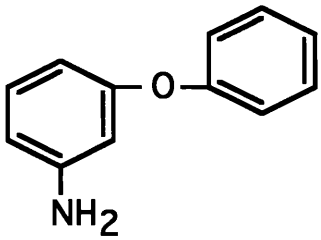
2-Benzylaniline

Efficacy 0%



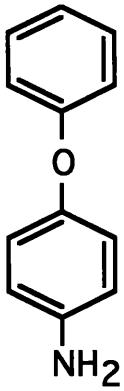
2-Phenoxyaniline

Efficacy 0%



3-Phenoxyaniline

0%

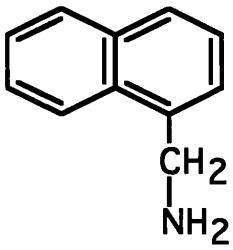


4-Phenoxyaniline

0%

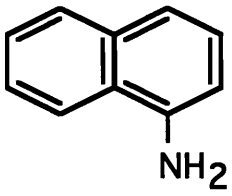
Figure 11.5.10:

Basicity of the amine: effects of neutral amines and basic analogues on the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH peptidergic neurones.



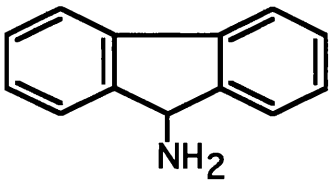
1-Naphthalenemethylamine

Efficacy **98%**



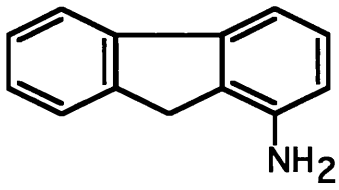
1-Aminonaphthalene

40%



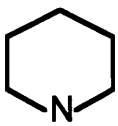
9-Aminofluorene

Efficacy **91%**



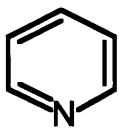
1-Aminofluorene

1%



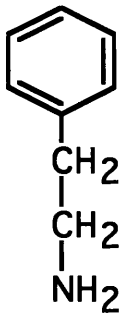
Piperidine

Efficacy **72%**



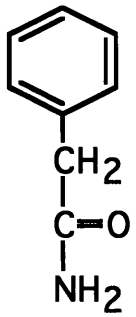
Pyridine

34%



Phenylethylamine

Efficacy **100%**

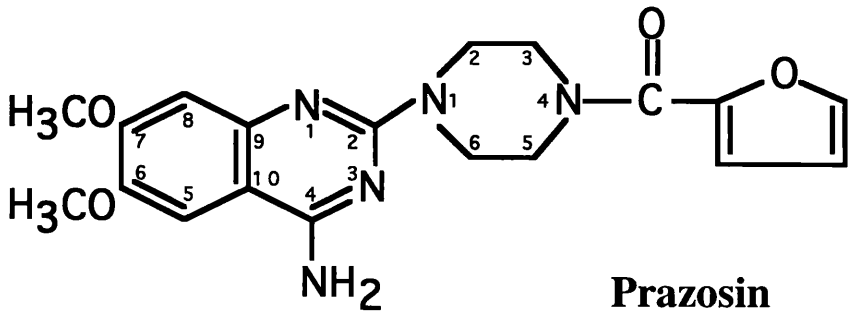


Phenylacetamide (Phenylethylamide)

19%

Figure 11.5.11:

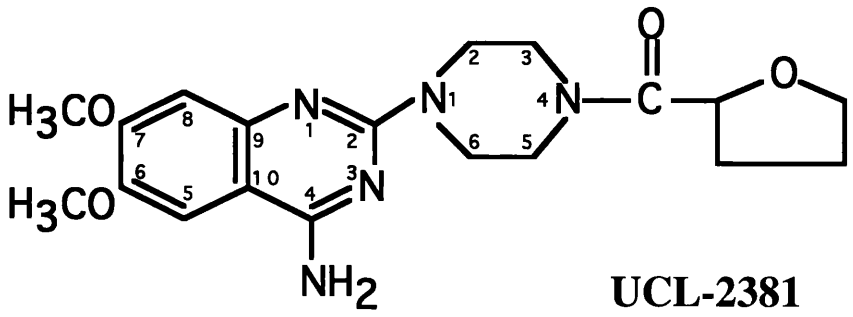
UCL-2381 is an analogue of prazosin in which the furan has been reduced to tetrahydrofuran. UCL-2362 is another analogue in which the furan group has been substituted with benzene. The data show that an aromatic group is more effective in activating Transport-P and that furan is better than benzene. UCL-2381 and UCL-2362 are part of a series of compounds which are being synthesised specifically for this project by Professor C.R. Ganellin and his colleagues in the Department of Chemistry at University College London.



Prazosin

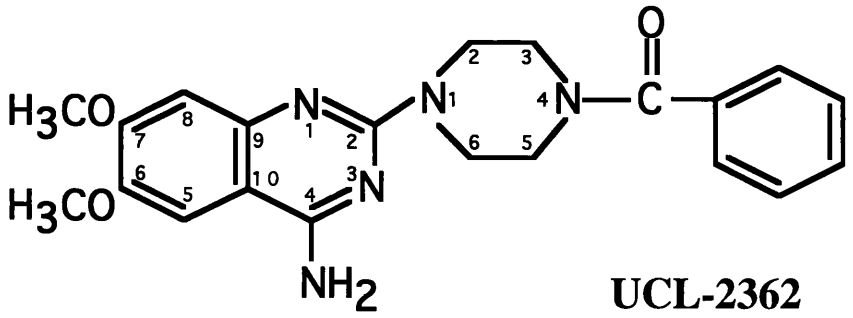
Activation

132 ± 16%
at 10^{-6} M



UCL-2381

None at 10^{-6} M
None at 10^{-5} M
42% at 10^{-4} M



UCL-2362

None at 10^{-6} M
57% at 10^{-5} M
59% at 10^{-4} M

Table 11.5.1:

Effects of selected substitutions on the efficacy of phenylethylamine derivatives at inhibition of the uptake of prazosin (at 10^{-6} M) in immortalised peptidergic neurones.

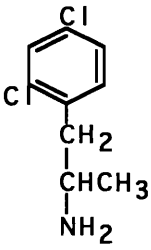
Compound	Efficacy %
Absence of alkyl amine	
2,5-dimethoxyacetophenone 10^{-3} M	0
3,4-dimethoxyacetophenone 10^{-3} M	5
2,5-dimethoxybenzaldehyde 10^{-3} M	0
1,4-dimethoxybenzene 10^{-3} M	0
3,4-dimethoxyphenylacetic acid 10^{-3} M	3
Presence of α-carboxyl group	
L-Phenylalanine 10^{-3} M	2
L-Tyrosine 10^{-3} M	0
L-DOPA 10^{-3} M	0
L-Serine 10^{-4} M	0
Secondary, tertiary, quaternary and guanyl amines	
HEAT (2-[β-(4-hydroxyphenyl)ethylaminomethyl]tetralone; secondary) 10^{-3} M	100
Fluoxetine (secondary) 10^{-4} M	100
Verapamil (tertiary) 10^{-4} M	100
MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; tertiary) 10^{-4} M	100
MPP^+ (1-methyl-4-phenylpyridinium; quaternary) 10^{-3} M	14
Bretylium (quaternary) 10^{-4} M	7
MIBG (*meta* -iodobenzylguanidine) 10^{-3} M	25
N-guanyltyramine 10^{-3} M	18
Guanethidine 10^{-4} M	8
Phenolic hydroxyl and methoxyl groups	
(-)-Metaraminol (1-[3-hydroxyphenyl]-2-amino-1-propanol) 10^{-3} M	13
(-)-Adrenaline (1-[3,4-dihydroxyphenyl]-2-methylamino-ethanol) 10^{-4} M	14
(-)-Phenylephrine (1-[3-hydroxyphenyl]-2-methylamino-ethanol) 10^{-3} M	37
(±)-Normetanephrine (1-[3-methoxy-4-hydroxyphenyl]-ethanolamine) 10^{-3} M	14
(-)-Isoprenaline (1-[3,4-dihydroxyphenyl]-2-isopropylamino-ethanol) 10^{-4} M	18

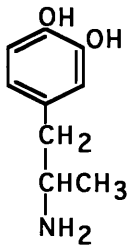
Table 11.5.2:

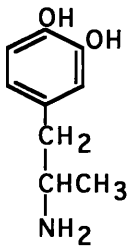
Comparison of the structural properties of phenylethylamines which are Transport-P ligands to ligands of α adrenoceptors and other amine transporters in the brain.

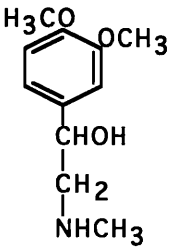
Substitution	*Transport-P*	*Noradren Uptake$_1$*	*Vesicular*	*Noradren Uptake$_2$*	*Dopamine*	*Adrenergic Receptors* α_1	α_2
Phenolic OH^-	Inhibit	Enhance	Enhance	Inhibit	Enhance	Enhance	Enhance
Phenolic OCH_3	No effect	Inhibit	Inhibit	Enhance	Inhibit	Enhance	No effect
β–OH^-	Inhibit	Inhibit	Inhibit	Enhance	Inhibit	Enhance	Enhance
configuration	Equal	**R**-(-)	**R**-(-)	**R**-(-)	Equal	**R**-(-)	**R**-(-)
α-methyl	Enhance	Enhance	Enhance	Inhibit	Enhance	Inhibit	Enhance
configuration	Equal	Equal	**S**-(+)		**S**-(+)	Equal	**S**-(+)
Amino methyl	Inhibit	Inhibit	No effect	Enhance	Inhibit	Enhance	Enhance

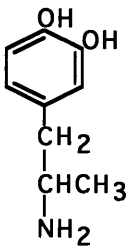
Expected optimum substitutions from available data

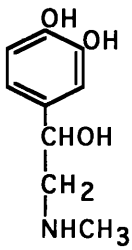












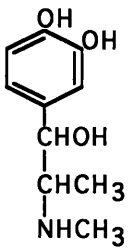


Table 11.5.3:

Effects of aliphatic and aromatic amines on the uptake of prazosin (at 10^{-6} M) in GT1-1 GnRH peptidergic neurones. Full activity was defined as efficacy greater than 90%.

Carbons	Formula	Compound	Efficacy	IC50 (M)
1	C1H5N	Methylamine	33%	
2	C2H7N	Ethylamine	29%	
3	C3H9N	Propylamine	66%	
4	C4H11N	Butylamine	68%	
5	C5H13N	Pentylamine	93%	
6	C6H15N	Hexylamine	96%	
7	C7H17N	Heptylamine	100%	
8	C8H19N	Octylamine	100%	
9	C9H21N	Nonylamine	100%	
10	C10H23N	Decylamine	99%	
	C10H23N	Dipentylamine	97%	7.90E-06
	C10H15N	4-Phenylbutylamine	92%	3.10E-06
	C10H15N	Methylamphetamine	94%	1.00E-06
11	C11H25N	Undecylamine	100%	
12	C12H27N	Dodecylamine	100%	
	C12H27N	Dihexylamine	99%	3.40E-06
	C12H15N	MPTP	97%	1.10E-06
	C12H17N	4-Benzylpiperidine	97%	4.50E-06
13	C13H29N	Tridecylamine	100%	
14	C14H31N	Tetradecylamine	89%	
15	C15H33N	Pentadecylamine	88%	
16	C16H35N	Hexadecylamine	30%	
	C16H35N	Dioctylamine	100%	
18	C18H39N	Octadecylamine	3%	
	C18H39N	Trihexylamine	90%	3.80E-05
	C18H22N2	Desipramine	100%	5.30E-07
19	C19H41N	N-methyloctadecylamine	19%	
	C19H21N	Nortriptyline	100%	
	C19H24N2	Imipramine	99%	1.80E-07
20	C20H43N	Didecylamine	26%	
	C20H23N	Amitriptyline	100%	
	C20H26N2	Trimipramine	98%	2.70E-07

11.6 VERAPAMIL AS A SUBSTRATE FOR TRANSPORT-P

The conclusions of the previous Section were based on the potencies of phenylethylamine derivatives at Transport-P, as indicated by inhibition of the uptake of prazosin. Inhibition of uptake does not necessarily indicate that these compounds are themselves internalised by the uptake process; that would require direct measurement of the accumulation of radioactively labelled compounds in the cells. The accumulation of [^{3}H]verapamil was therefore studied, as this compound is a phenylethylamine derivative which possesses the structural properties which were defined as being important for interaction with Transport-P (Figure 11.6.1).

Immortalised GT1-1 GnRH peptidergic neurones accumulated [^{3}H]verapamil by a desipramine-sensitive mechanism (Figure 11.6.2). The inhibitory effect of desipramine was dose-dependent (IC_{50} $4.9x10^{-6}$ M; Figure 11.6.3). Thus, uptake of verapamil, like the uptake of prazosin, is sensitive to antidepressants. Further, verapamil inhibited the uptake of prazosin (at 10^{-6} M) in a dose-dependent manner (IC_{50} $2.8x10^{-6}$ M; Figure 11.6.4). The inhibitory effect of verapamil was competitive with prazosin (Figure 11.6.5). Taken together, these findings indicated that prazosin and verapamil are accumulated by the same uptake process in the peptidergic neurones.

Although verapamil accumulated via Transport-P, it did not activate the uptake process; [^{3}H]verapamil was displaced by unlabelled verapamil but there was no increase in the accumulation of [^{3}H]verapamil at concentrations of unlabelled verapamil up to 10^{-6} M (Figure 11.6.6). In this regard, the action of verapamil was similar to the actions of the antidepressants (Section 11.2). Thus, Transport-P ligands can be divided into two groups:

Group A compounds activate Transport-P and are internalised by Transport-P in peptidergic neurones. Prazosin is the prototype of this group.

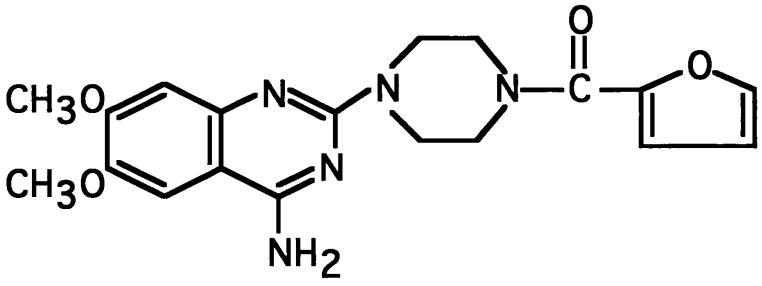
Group B compounds are internalised by Transport-P but they do not activate the uptake process. This group includes the antidepressants and phenylethylamine derivatives such as verapamil.

The significance of these findings will be addressed further in the General Discussion (Section 12.3).

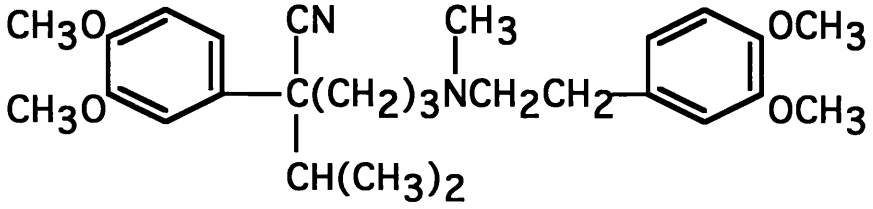
In conclusion, the findings on verapamil indicate that Transport-P accumulates the phenylethylamine derivatives which inhibit the uptake of prazosin. The structural properties which were identified by competition studies (Section 11.5) may indeed define some of the requirements for interaction of phenylethylamines with the Transport-P carrier in peptidergic neurones.

Figure 11.6.1:

The chemical structures of prazosin and verapamil.



Prazosin



Verapamil

Figure 11.6.2:

Time course of the accumulation of [^{3}H]verapamil in GT1-1 immortalised GnRH neurones.

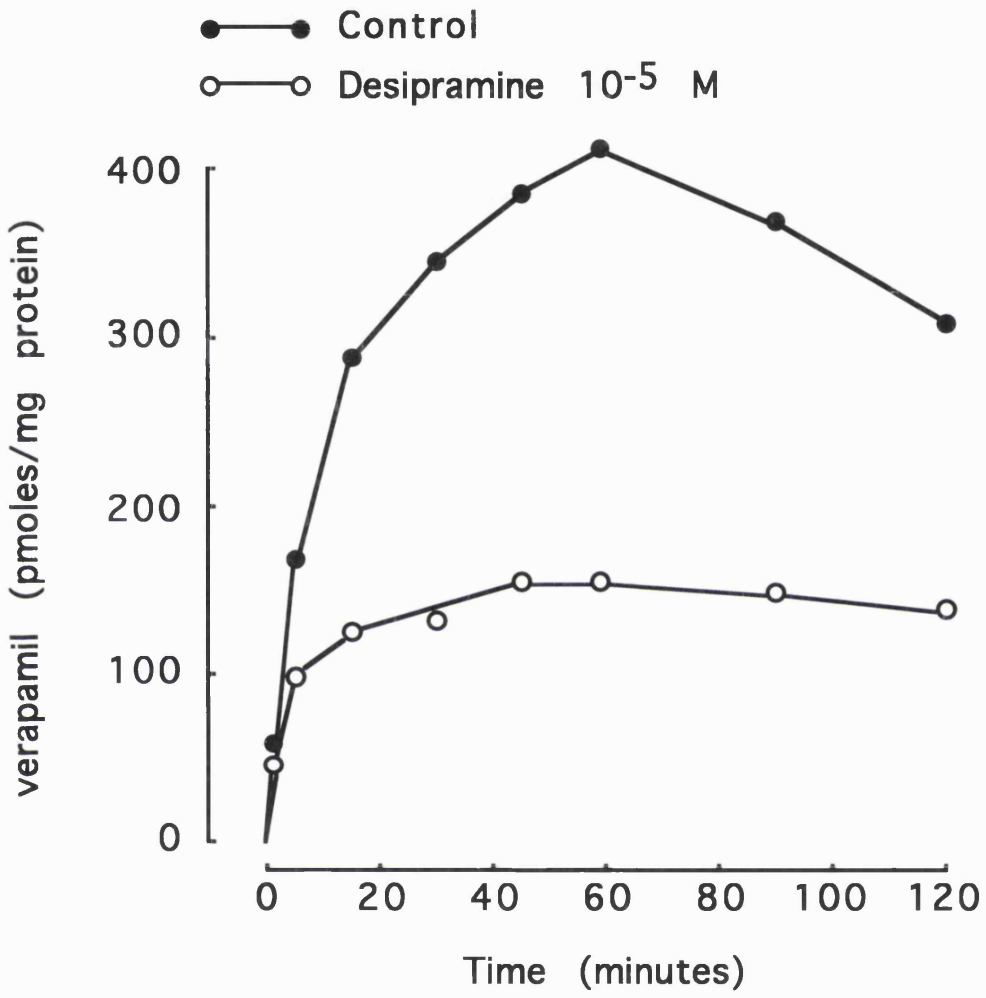


Figure 11.6.3:

Effect of desipramine on the accumulation of [^{3}H]verapamil in GT1-1 immortalised GnRH neurones.

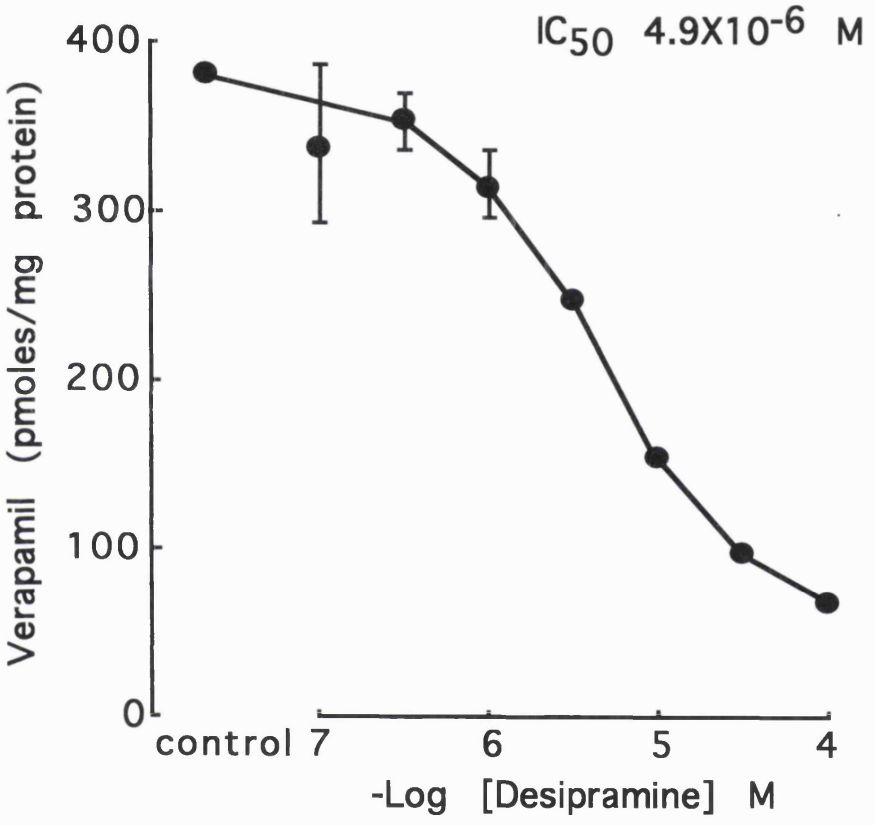


Figure 11.6.4:

Effect of verapamil on the accumulation of prazosin (at 10^{-6} M) in GT1-1 immortalised GnRH neurones.

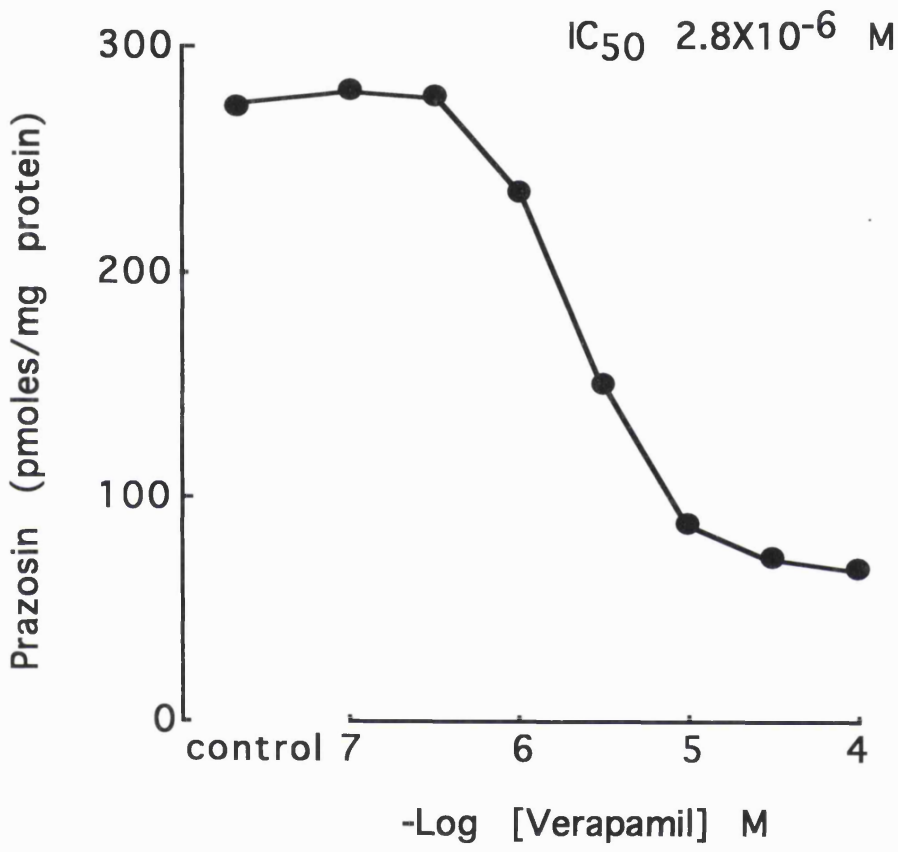


Figure 11.6.5:

Reversal of the inhibitory effect of verapamil in GnRH neurones by increasing concentrations of unlabelled prazosin; indicating that the effect of verapamil is competitive. The points do not fall on straight lines because they form sigmoidal concentration-response curves (see Figure 11.6.4).

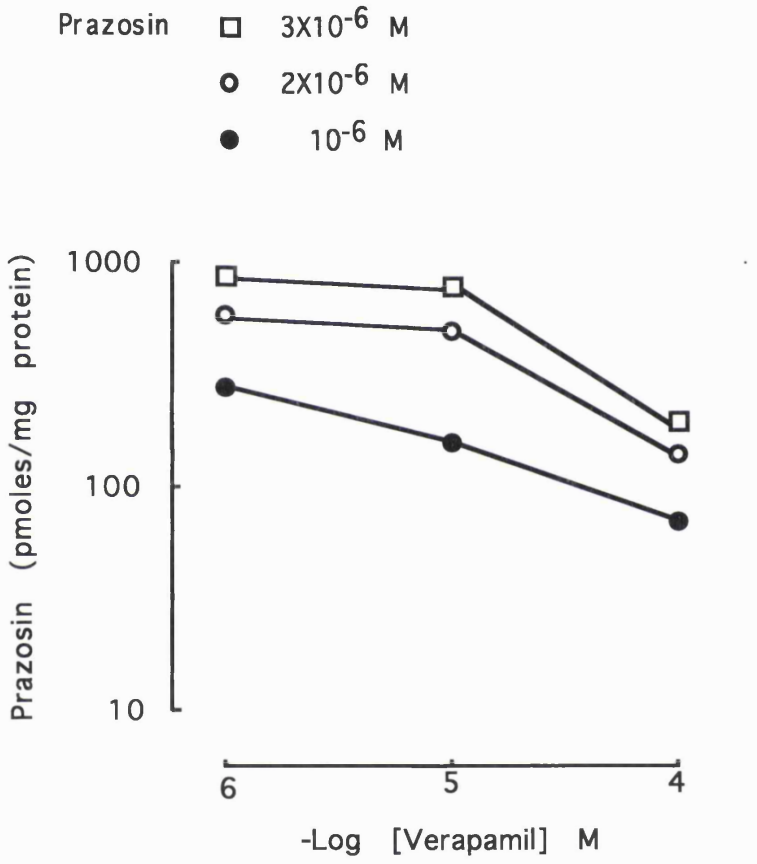
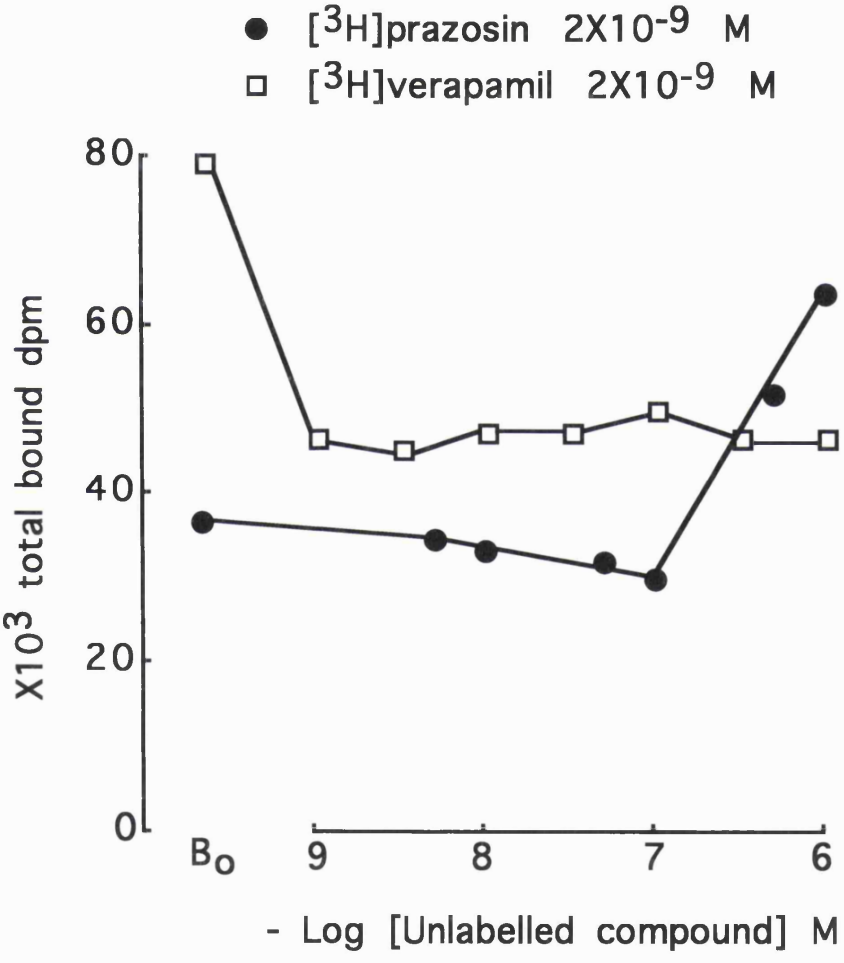


Figure 11.6.6:

Effect of unlabelled verapamil on the accumulation of [^{3}H]verapamil; comparison to the effect of unlabelled prazosin on the accumulation of [^{3}H]prazosin in GT1-1 immortalised GnRH neurones. [^{3}H]verapamil is displaced by unlabelled verapamil and there is no paradoxical increase up to 10^{-6} M unlabelled verapamil.



11.7 VISUAL DETECTION OF TRANSPORT-P

The work which is described in this Section aimed to test the hypothesis that the peptidergic neurones of the hypothalamus accumulate prazosin by an antidepressant-sensitive mechanism. This was done by developing a method for visual detection of the uptake process using a fluorescent analogue of prazosin.

11.7.1 Spectrophotofluorimetry

Prazosin has intrinsic fluorescence but its emission spectrum is predominantly ultraviolet (excitation and emission maxima 340 and 400 nm, respectively; Figure 11.7.1). The spectral properties of prazosin are dependent on the pH of the buffer in which prazosin is dissolved; at acid pH, the emission spectrum acquires a shoulder at 380 nm and there is a pH-insensitive isosbastic point at 420 nm. A plot of the ratio of fluorescence at 380 nm:420 nm enables measurement of the pH of the medium in which prazosin is dissolved (Figure 11.7.1). The pKa of prazosin measured by this method was 7.2 which is similar to the pKa of 6.8 which was reported by previous investigators (Alabaster et al, 1987).

BODIPY FL prazosin is an analogue of prazosin in which the furan ring has been substituted with the fluorescent group BODIPY FL (Figure 11.7.2). The emission spectrum of BODIPY FL prazosin was within the visible range (excitation and emission maxima 500 and 512 nm, respectively; Figure 11.7.3). Changes in pH in the range 4.0 to 9.1 influenced the intensity of fluorescence but they did not affect the wavelength of the emission maximum of BODIPY FL prazosin (Figure 11.7.3). Intensity of fluorescence was greatest at pH 7.4.

With an excitation wavelength of 450 nm and an emission window of 480-600 nm, autofluorescence was minimal in GnRH cells grown on glass coverslips (Figure 11.7.3). After incubation in the presence of BODIPY FL prazosin (1.77×10^{-7} M) for 60 minutes and washing, fluorescence of BODIPY FL prazosin was clearly evident, indicating that the cells had accumulated the compound (Figure 11.7.3). The emission maximum of BODIPY FL prazosin in GnRH cells was at 517-518 nm (Figure 11.7.3). When the cell-coated cover slips were removed from the cuvette, no fluorescence was detected in the buffer, confirming that the measured fluorescence was in the cell-coated coverslips (not shown).

Desipramine 10^{-5} M had a negligible effect on the emission spectrum of BODIPY FL prazosin in solution; the wavelength of the emission peak was unaffected but the amplitude

was slightly increased (Figure 11.7.4). Desipramine 10^{-5} M inhibited the accumulation of BODIPY FL prazoisn ($1.77x10^{-7}$ M) in GnRH cells (Figure 11.7.4). In the presence of desipramine 10^{-5} M, the emission maximum of BODIPY FL prazosin in GnRH cells was at 516-517 nm. The inhibitory effect of desipramine was dose-dependent (IC_{50} $8x10^{-9}$ M; Figure 11.7.5). In a limited experiment, specific uptake of BODIPY FL prazosin in GnRH cells (defined as total uptake - uptake in the presence of desipramine 10^{-5} M) began to saturate at concentrations greater than $1.77x10^{-7}$ M (Figure 11.7.5).

Unlabelled prazosin 10^{-5} M had a negligible effect on the emission spectrum of BODIPY FL prazosin in solution; the wavelength of the emission peak was unaffected but the amplitude was slightly increased (Figure 11.7.6). Unlabelled prazosin 10^{-6} M reduced the accumulation of BODIPY FL prazosin $1.77x10^{-7}$ M in GnRH cells (Figure 11.7.6). Desipramine 10^{-7} M almost completely blocked the accumulation of BODIPY FL prazosin, in the presence and absence of unlabelled prazosin (Table 11.7.1). In the presence of unlabelled prazosin 10^{-6} M, the emission spectrum of BODIPY FL prazosin in GnRH cells was shifted to 522 nm (Figure 11.7.6 and Table 11.7.1). At concentrations up to 10^{-5} M, unlabelled prazosin did not cause an increase in the accumulation of BODIPY FL prazosin $1.77x10^{-7}$ M (data not shown).

11.7.2 Fluorescence microscopy

Autofluorescence was minimal in GnRH cells and in hypothalamic cells from fetal rats, with a 470-490 nm excitation filter, 520-560 nm emission filter and a 510 nm dichroic mirror (Figure 11.7.7). GT1-1 GnRH neurones incubated at 37^0C in the presence of BODIPY FL prazosin ($1.77x10^{-7}$ M) acquired an intense green fluorescence, indicating that they had accumulated the compound (Figure 11.7.7). The acquisition of intense fluorescence was abolished by desipramine 10^{-5} M (Figure 11.7.8). In the presence of desipramine, only a faint fluorescence was evident in these cells (Figure 11.7.8). This presumably represents a combination of non-specific binding and binding of BODIPY FL prazosin to α_1 adrenergic receptors, as unlabelled prazosin further reduced the faint fluorescence.

In the presence of unlabelled prazosin 10^{-6} M, BODIPY FL prazosin accumulated in GnRH cells in a granular pattern (Figure 11.7.8). Accumulation of BODIPY FL prazosin in GnRH cells was inhibited by chloroquine 10^{-4} M and by reducing the incubation temperature from 37^0C to 0^0C; under these conditions, the appearance of the cells resembled their appearance in the presence of desipramine (Figure 11.7.8).

Hypothalamic cells from fetal rats also accumulated BODIPY FL prazosin and most of the intensely labelled cells appeared to be neurones (Figures 11.7.9 and 11.7.10). Accumulation of BODIPY FL prazosin in fetal hypothalamic cells was inhibited by desipramine 10^{-5} M (Figure 11.7.9). Glial cells were identifiable in these cultures but they were not intensely labelled with the fluorescent compound. Their faint fluorescence resembled the appearance of neuronal cells incubated in the presence of desipramine (Figures 11.7.9 and 11.7.10).

11.7.3 Comment

The previous studies with [^{3}H]prazosin provided evidence for the existence of a new uptake process for amines in post-synaptic (peptidergic) neurones. The work which is described in this Section extended the previous findings by providing visual evidence for amine uptake in peptidergic neurones.

Prazosin is fluorescent but most of its emission peak was invisible to the human eye (Figure 11.7.1). In studies on the structural properties of ligands for Transport-P, the furan group of prazosin was found to be unnecessary for uptake but it was required for the paradoxical increase in accumulation of [^{3}H]prazosin (not shown). Thus, analogues of prazosin which lacked the furan group accumulated in GnRH neurones by a desipramine-sensitive process, but they did not display the paradoxical increase in accumulation of [^{3}H]prazosin. This study utilised an analogue of prazosin in which the furan ring had been substituted with a fluorescent group (BODIPY FL) whose emission peak is in the green part of the visible spectrum (Figure 11.7.3).

Under the present experimental conditions, autofluorescence was minimal in GnRH cells examined with both the spectrophotofluorimeter and the fluorescence microscope (Figures

11.7.3 and 11.7.7). The spectrophotofluorimetric studies demonstrated that these cells accumulated BODIPY FL prazosin by a saturable, desipramine-sensitive process (Figures 11.7.4 and 11.7.5). Unlabelled prazosin 10^{-6} M partially inhibited the accumulation of BODIPY FL prazosin (Figure 11.7.6). As previously described for prazosin (Sections 11.1 and 11.3), accumulation of BODIPY FL prazosin was inhibited in the cold and by the organic base chloroquine. This indicated that prazosin and its analogue are internalised by the same antidepressant-sensitive, proton-dependent uptake process (Transport-P). The absence of a paradoxical increase in accumulation of BODIPY FL prazosin in the presence of unlabelled prazosin (up to 10^{-5} M) is consistent with the previous finding that this increase requires the presence of a furan ring, which is lacking in BODIPY FL prazosin (Figure 11.7.2).

Fluorescence microscopy confirmed that GnRH neurones accumulate BODIPY FL prazosin by a desipramine-sensitive process; in the presence of desipramine, the intense labelling of GnRH cells was abolished, and only faint labelling was seen (Figure 11.7.8). Unlabelled prazosin further reduced the faint labelling. This faint labelling presumably represents a combination of binding of BODIPY FL prazosin to α_1 adrenoceptors and non-specific binding of this lipophilic compound. Hypothalamic cells from fetal rats also accumulated BODIPY FL prazosin by a desipramine-sensitive process (Figure 11.7.9). Most of the intensely labelled cells appeared to be neurones, whereas glial cells were not intensely labelled (Figures 11.7.9 and 11.7.10). These findings indicate that in the hypothalamus, Transport-P is located predominantly in neurones, rather than in glial cells.

Previous studies had demonstrated that accumulation of prazosin in GnRH cells was dependent on an electrochemical gradient of protons which is generated by V-ATPase (Section 11.3). It therefore seemed likely that prazosin was internalised in some acidified intracellular particles, such as neurosecretory vesicles or internalised clathrin-coated pits. In the present study, unlabelled prazosin 10^{-6} M partially inhibited the accumulation of BODIPY FL prazosin (Figure 11.7.6). This manoeuvre, which was intended to displace BODIPY FL prazosin from surface α_1 adrenoceptors, revealed that the intracellular distribution of BODIPY FL prazosin was in a granular pattern, presumably representing accumulation in intracellular vesicles (Figure 11.7.8).

Although the emission spectrum of BODIPY FL prazosin was similar in solution and in GnRH cells, there was a slight red shift in the emission maximum in GnRH cells. This shift is unlikely to be due to the pH of the intracellular compartment in which BODIPY FL prazosin is stored, as the wavelength of the emission maximum of this compound was insensitive to pH changes in the range 4.0 to 9.1 (Figure 11.7.3). The shift was accentuated by unlabelled prazosin and persisted in the presence of desipramine, indicating that it is unlikely to be due to association of the fluorescent compound with α_1 adrenoceptors or Transport-P. The nature of this shift is unclear, but may be due to non-specific binding of this compound to various cellular components. The fluorescence microscopy method was insensitive to this slight change in the wavelength of the emission maximum, since the emission filter included wavelengths up to 560 nm.

In conclusion, the present results provide visual evidence for the existence of Transport-P in peptidergic neurones. This approach should be useful for detailed studies of the anatomical distribution of this uptake process in the brain.

Figure 11.7.1:

Effect of pH on the excitation and emission spectra of prazosin. Prazosin has intrinsic fluorescence but its emission spectrum is predominantly ultraviolet. The spectral properties of prazosin are dependent on the pH of the buffer in which prazosin is dissolved; at acid pH, the emission spectrum acquires a shoulder at 380 nm and there is a pH-insensitive isosbastic point at 420 nm. A plot of the ratio of fluorescence at 380 nm:420 nm enables measurement of the pH of the medium in which prazosin is dissolved. The pKa of prazosin measured by this method was 7.2 which is similar to the pKa of 6.8 which was reported by previous investigators (Alabaster et al, 1987).

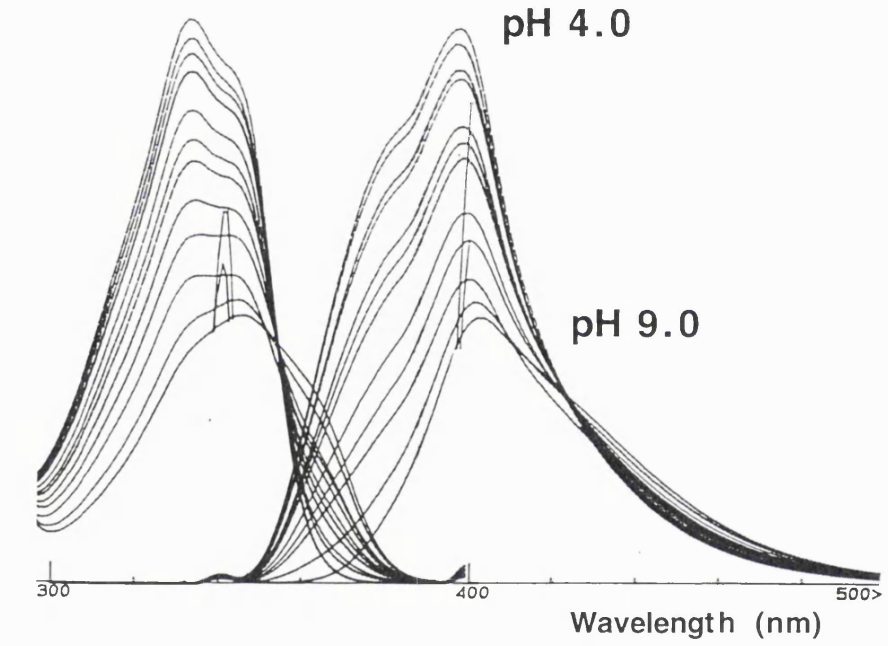
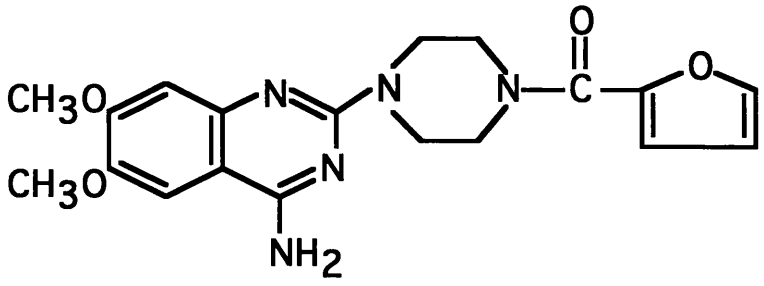
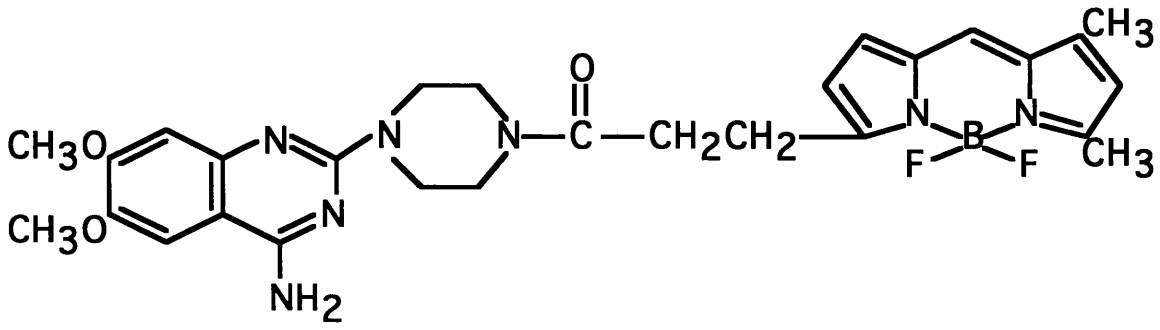


Figure 11.7.2:

Chemical structures of prazosin and BODIPY FL prazosin.



Prazosin

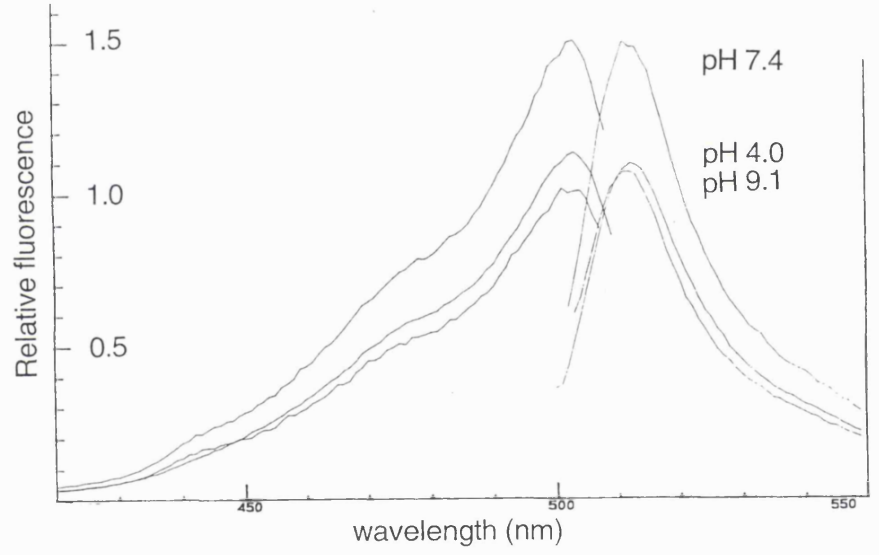


BODIPY FL Prazosin

Figure 11.7.3:

Upper panel: Excitation (left) and emission (right) spectra of BODIPY FL prazosin in solution at different pH values. The wavelength of the emission maximum (512 nm) is in the green part of the visible spectrum and is unaffected by changes in pH in the range 4.0 to 9.1. Fluorescence intensity is greatest at pH 7.4.

Lower panel: GT1-1 GnRH neurones were grown on glass coverslips coated with poly-D-lysine and laminin. Autofluorescence was minimal before exposure to BODIPY FL prazosin ("control GnRH cells"). The cells were then incubated in the presence of BODIPY FL prazosin 1.77×10^{-7} M for 60 minutes at 37^0C, washed, placed in a cuvette and excited with a wavelength of 450 nm. The emission spectrum characteristic of BODIPY FL prazosin is seen in the cells, indicating that they had accumulated the compound.



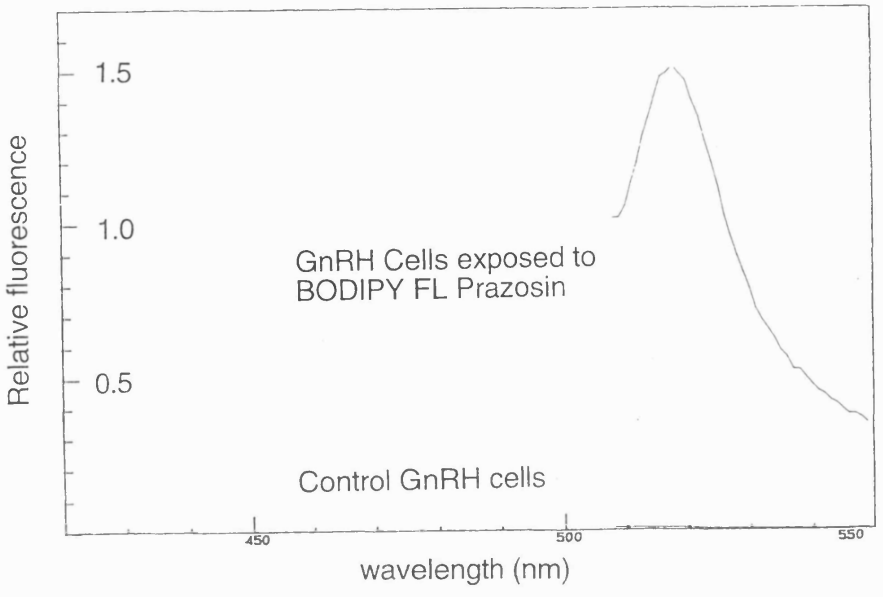


Figure 11.7.4:

Effect of desipramine on the uptake of BODIPY FL prazosin in GT1-1 GnRH neurones. Upper panel: Emission spectrum of BODIPY FL prazosin in solution, with and without desipramine 10^{-5} M. Desipramine had a negligible effect on the emission spectrum; the wavelength of the emission maximum was unchanged but there was a slight increase in fluorescence intensity in the presence of desipramine. Excitation wavelength was 450 nm. Lower panel: GnRH cells grown on glass coverslips were incubated for 60 minutes at 37^{0}C in the presence of BODIPY FL prazosin 1.77×10^{-7} M, with or without desipramine 10^{-5} M. The cells were then washed, placed in a cuvette and excited with a wavelength of 450 nm. BODIPY FL prazosin accumulated in the GnRH cells, as indicated by the characteristic emission spectrum. Accumulation of the compound was blocked by desipramine, indicating that uptake of the fluorescent analogue has similar properties to uptake of prazosin in GnRH cells. Three replicates were performed for each experiment.

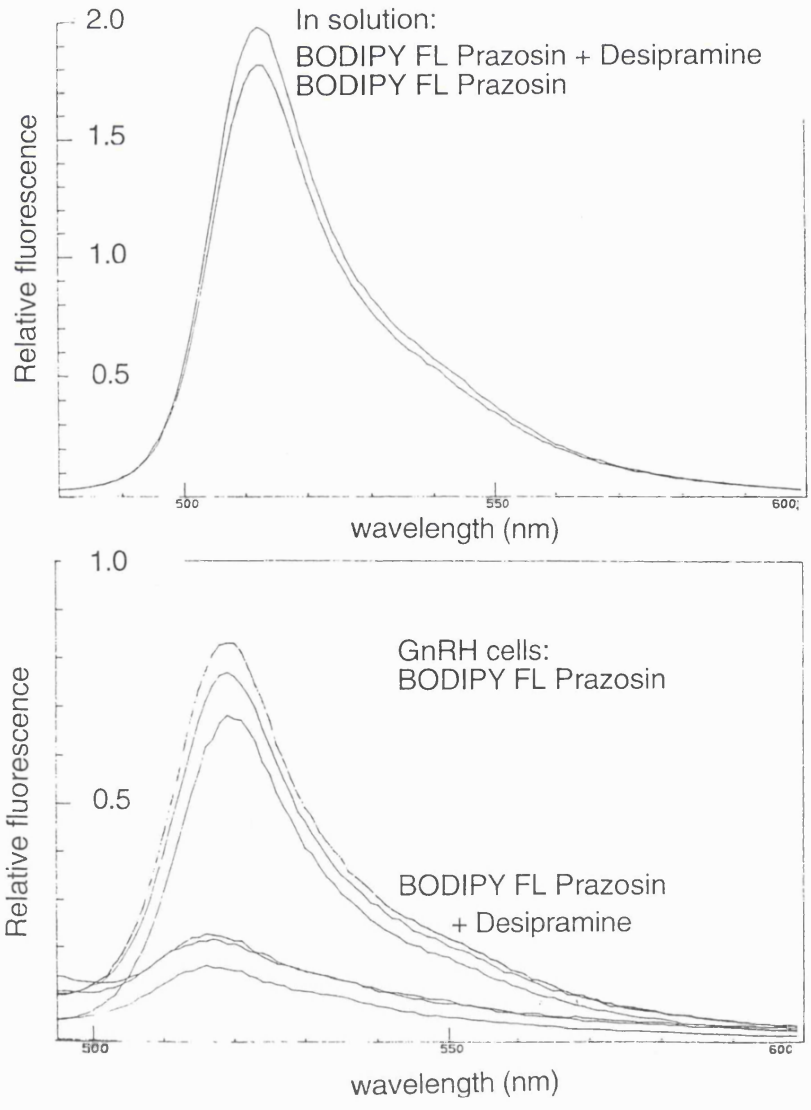


Figure 11.7.5:

A. Effects of increasing concentrations of desipramine on the uptake of BODIPY FL prazosin 1.77×10^{-7} M in GnRH neurones. Each point represents the mean of three replicates. Standard error bars were smaller than the sizes of the symbols.

B. Uptake of increasing concentrations of BODIPY FL prazosin in GnRH neurones. Specific uptake was defined as total uptake - uptake in the presence of desipramine 10^{-5} M. Specific uptake begins to saturate at concentrations greater than 1.77×10^{-7} M. Data are means of three replicates; the vertical lines show standard errors of the mean. The curves were fitted manually.

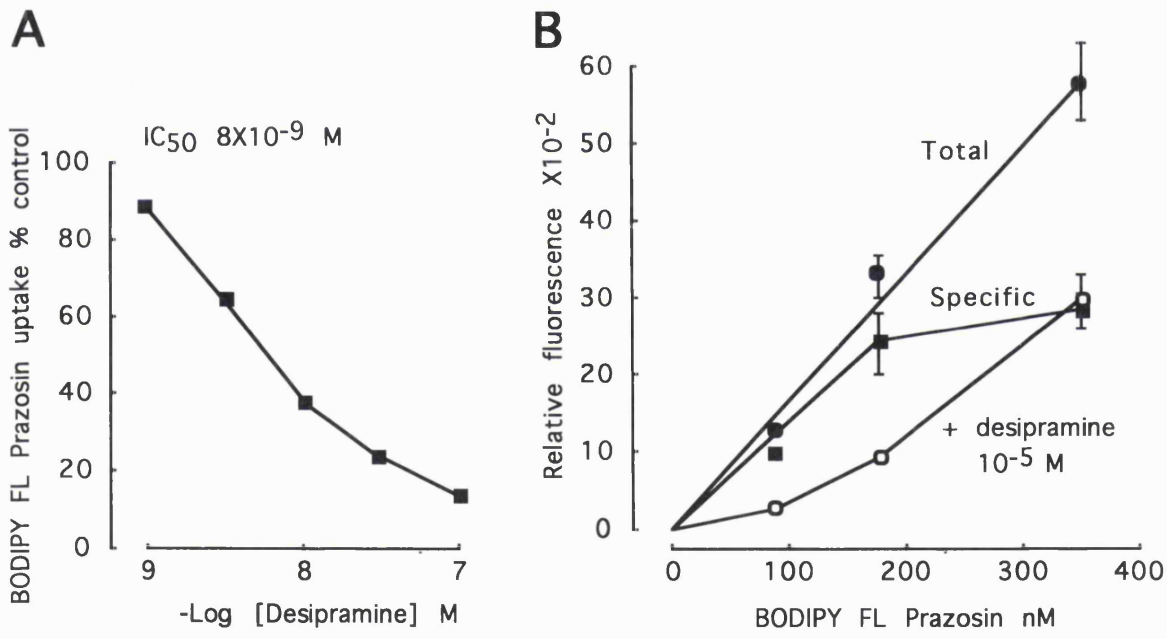


Figure 11.7.6:

Effect of unlabelled prazosin on the uptake of BODIPY FL prazosin in GnRH neurones. **A**: Emission spectrum of BODIPY FL prazosin in solution, with and without unlabelled prazosin 10^{-5} M. Prazosin had a negligible effect on the emission spectrum; the wavelength of the emission peak was unchanged but there was a slight increase in intensity of fluorescence in the presence of unlabelled prazosin. Excitation wavelength was 450 nm. **B**: GnRH cells grown on coverslips were incubated for 60 min at 37^0C in the presence of BODIPY FL prazosin $1.77x10^{-7}$ M, with or without unlabelled prazosin 10^{-6} M. The cells were then washed, placed in a cuvette and excited with a wavelength of 450 nm. The cells accumulated BODIPY FL prazosin, as indicated by the characteristic emission spectrum. Uptake of the compound was partially blocked by unlabelled prazosin. Desipramine almost completely blocked the uptake of BODIPY FL prazosin, in the presence or absence of unlabelled prazosin (Table 11.7.1). Three replicates were performed for each experiment.

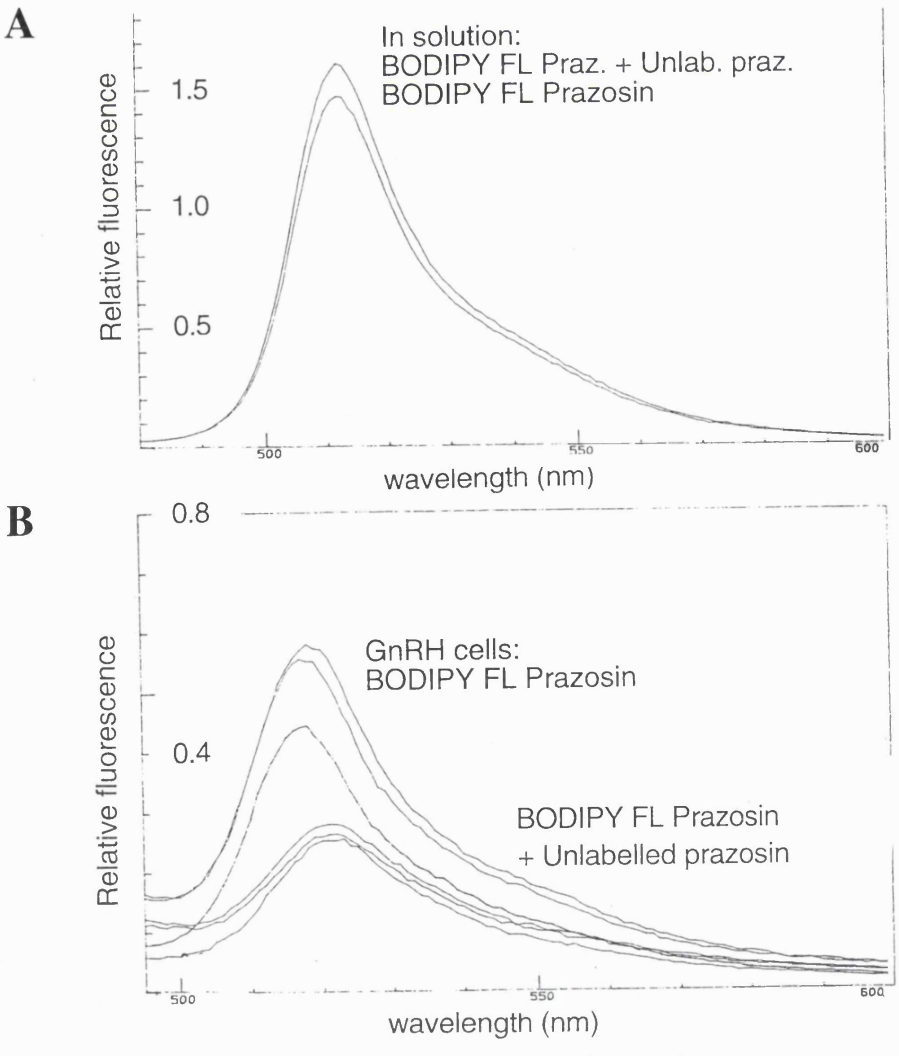


Figure 11.7.7:

Accumulation of BODIPY FL prazosin in immortalised GT1-1 GnRH neurones, detected by fluorescence microscopy. The cells were grown in serum-free medium on glass microscope slides coated with poly-D-lysine and laminin. Forty eight hours later, the cells were incubated for one hour in the presence of buffer (control, A) or the fluorescent prazosin analogue BODIPY FL prazosin 1.77×10^{-7} M (B and C). The cells were then washed and fixed. Fluorescence microscopy demonstrates that autofluorescence is minimal under these conditions (A). The cells accumulated BODIPY FL prazosin, as indicated by the intense green appearance which is characteristic of this compound (B and C). The scale bar represents 14 μm in A and B and 7 μm in C.

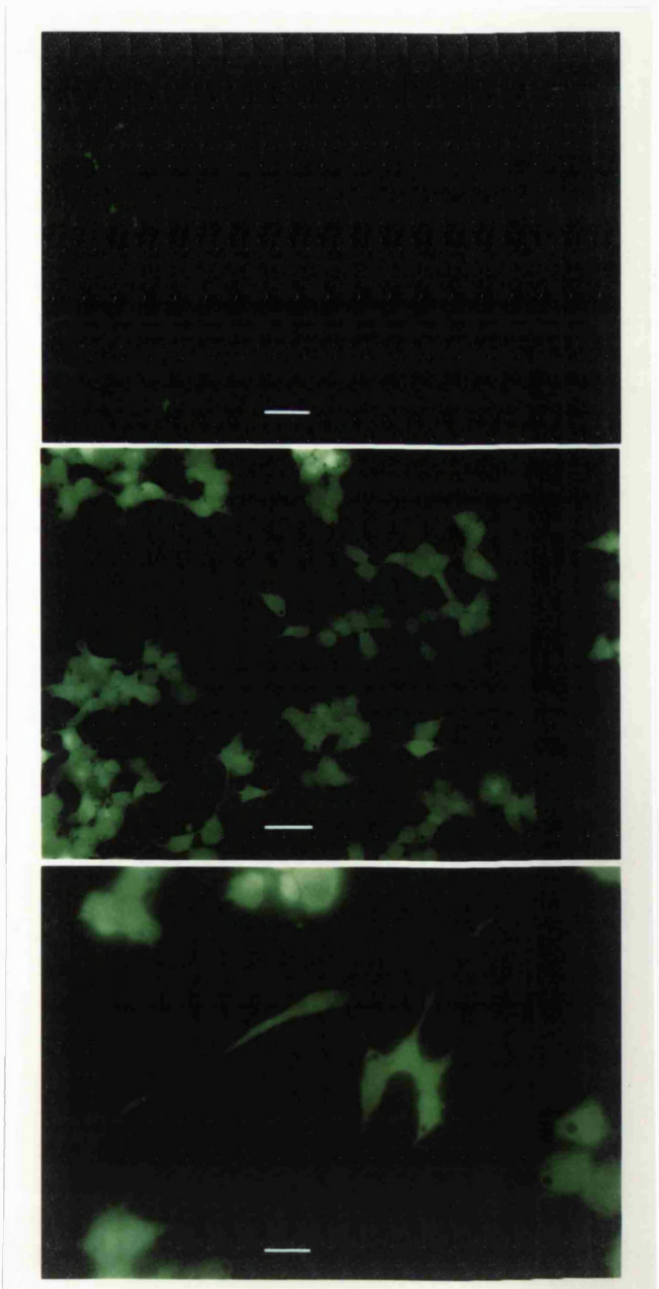


Figure 11.7.8:

Effects of desipramine and unlabelled prazosin on the uptake of BODIPY FL prazosin in GnRH neurones. Cells were grown in serum-free medium on glass slides coated with poly-D-lysine and laminin. Forty eight hours later, the cells were incubated for one hour in the presence of BODIPY FL prazosin 177 nM. The cells accumulated the amine (control, upper panel) by a desipramine-inhibitable mechanism (middle panel; desipramine 10^{-5} M). In the lower panels, the cells were incubated with both BODIPY FL prazosin 177 nM and unlabelled prazosin 10^{-6} M. This manoeuvre was intended to block binding of BODIPY FL prazosin to α_1 adrenoceptors; this reveals that accumulation of BODIPY FL prazosin is in a granular distribution, which is consistent with localisation in intracellular vesicles.

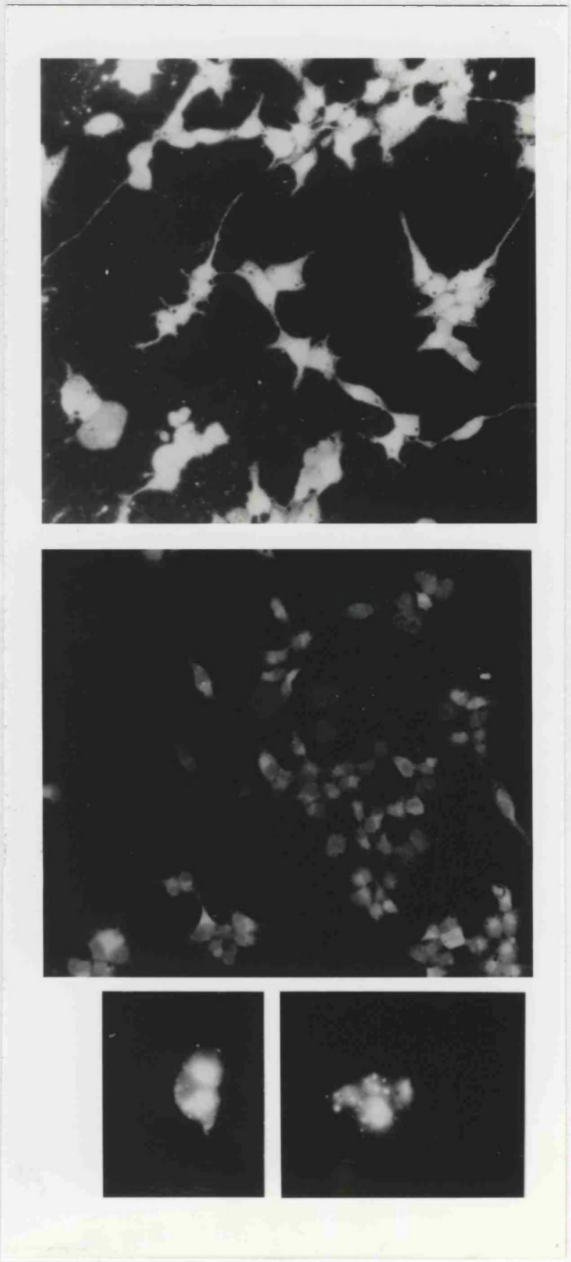
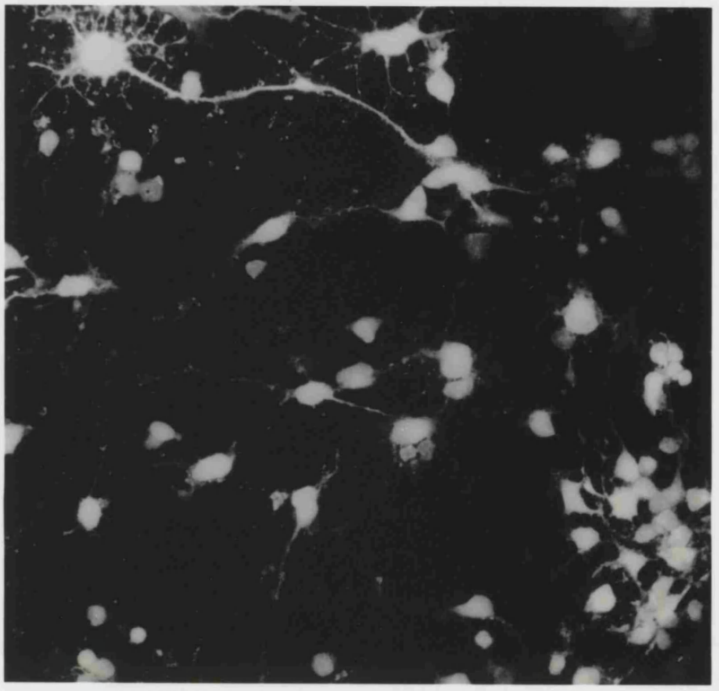


Figure 11.7.9:

Visual evidence for Transport-P. Hypothalamic cells from fetal day 18 rats were grown in medium containing 10% FBS on glass slides coated with poly-D-lysine and laminin. Forty eight hours later, the cells were incubated for one hour in the presence of BODIPY FL prazosin 1.77×10^{-7} M, with or without desipramine 10^{-5} M. The cells accumulated the fluorescent amine (control, A) by a desipramine-inhibitable mechanism (B). The intensely labelled cells in A have processes with varicosities which are typical of axons and dendrites. Thus, in the hypothalamus, Transport-P is predominantly in neurones rather than in glia.

A



B

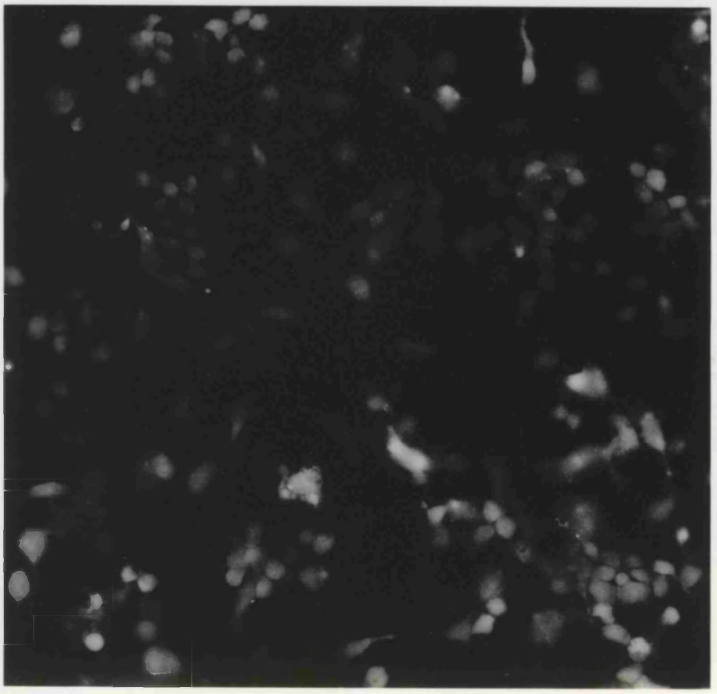


Figure 11.7.10:

Visual evidence for amine uptake in hypothalamic neurones. Hypothalamic cells from fetal day 18 rats were grown as described in Figure 11.7.9, and incubated for one hour in the presence of BODIPY FL prazosin 1.77×10^{-7} M. There is an intensely labelled neurone in the upper left corner. On the right, there is an oligodendrocyte and an astrocyte which are not intensely labelled; their appearance is similar to cells which are incubated in the presence of desipramine (see Figure 11.7.9). There are also many round cells (microglia) which are not intensely labelled. The findings indicate that in the hypothalamus, Transport-P is located predominantly in neurones rather than in glial cells.

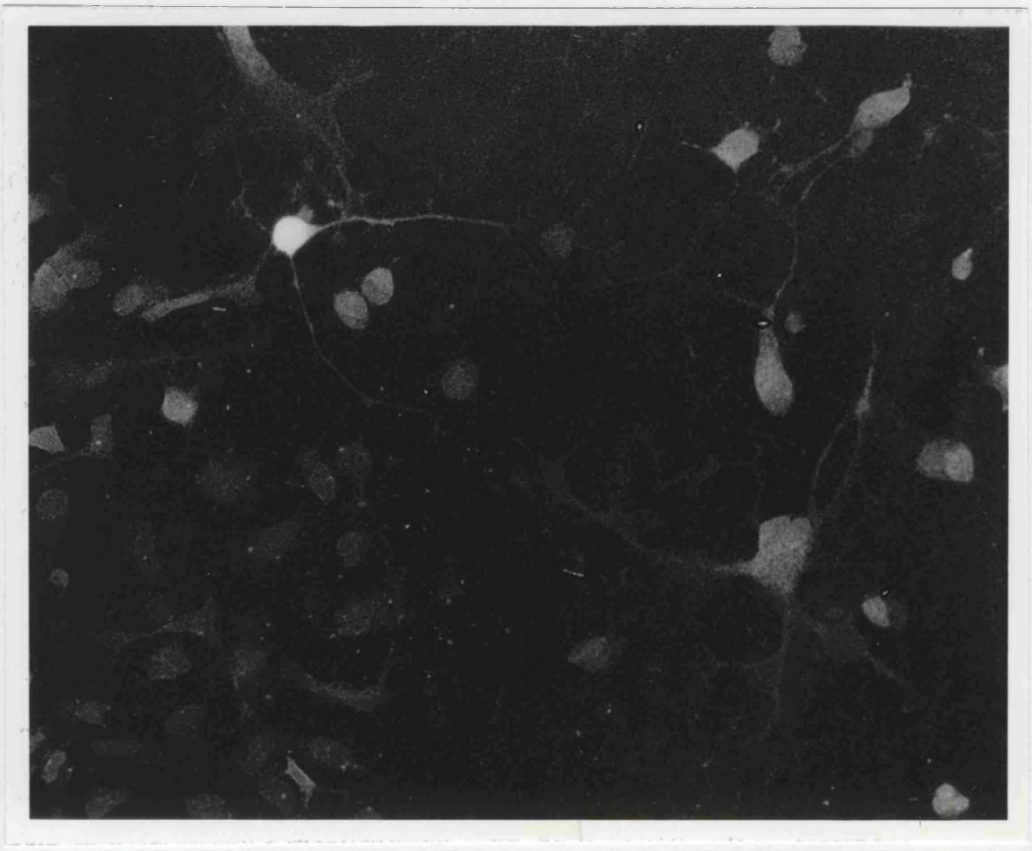


Table 11.7.1:

Effects of desipramine and unlabelled prazosin on the accumulation and the wavelength of the emission maximum of BODIPY FL prazosin in GT1-1 immortalised GnRH neurones. In these experiments, the excitation wavelength was 450 nm and the fluorescence of BODIPY FL prazosin was measured at the emission maximum, relative to rhodamine as standard. Desipramine and unlabelled prazosin had negligible effects on the fluorescence of BODIPY FL prazosin in solution (see Figures 11.7.4 and 11.7.6).

Compound	*Relative fluorescence*	*Emission maximum*
BODIPY Fl Prazosin 1.77×10^{-7} M in solution		512 nm
In GnRH cells:		
BODIPY Fl Prazosin 1.77×10^{-7} M	0.34 ± 0.01	518 nm
+ Desipramine 10^{-7} M	0.06 ± 0.01	517 nm
+ Prazosin 10^{-6} M	0.11 ± 0.01	522 nm
+ Desipramine + prazosin	0.05 ± 0.02	518 nm

11.8 RELEASE OF AMINES FOLLOWING UPTAKE

The work which is described in this Section was aimed to test the hypothesis that following uptake by Transport-P, the amines are internalised in an acidified cellular compartment. This was done by examining the effects of manipulations of cellular acidity on the release of amines from the peptidergic neurones.

11.8.1 Release of prazosin

Following uptake by Transport-P, there was a decline in the total cellular content of prazosin which was accelerated by desipramine 10^{-5} M (Figure 11.8.1A). Non-specific uptake was defined as the residual amount of cellular prazosin in the presence of desipramine 10^{-5} M at 120 minutes (Figure 11.8.1A). In the presence of desipramine, the decline of the specific cellular content of prazosin was exponential and could be described by a straight line on a log-linear plot (Figure 11.8.1B; R^2 value for the curve fit 0.9908; control: release constant 7.3×10^{-5} sec^{-1}, half life 69 minutes; desipramine 10^{-5} M: release constant 4.7×10^{-4} sec^{-1}, half life 11 minutes; ANOVA: $P = 0.01$). The effect of desipramine was concentration-dependent (IC_{50} 3.0×10^{-6} M). As desipramine inhibits the uptake of prazosin in these cells (Section 11.2), the effect of desipramine on the cellular content of prazosin was interpreted as being due to inhibition of re-uptake of released prazosin from the incubation medium. This hypothesis was tested further by examining the effect of unlabelled prazosin in the release buffer on the cellular content of [^{3}H]prazosin. One of the functional properties of Transport-P is that at concentrations of unlabelled prazosin greater than 10^{-7} M, there is a paradoxical increase in accumulation of [^{3}H]prazosin which can be blocked by desipramine (Al-Damluji et al, 1993; Figure 11.8.2A). In order to test the hypothesis that released prazosin can be re-accumulated from the medium via Transport-P, the effect of unlabelled prazosin 10^{-6} M was examined, with and without the antidepressant desipramine in the release buffer. All the cells were pre-loaded with [^{3}H]prazosin and unlabelled prazosin 10^{-6} M; release was then studied in the absence of drugs (control) or in the presence of unlabelled prazosin 10^{-6} M, desipramine 10^{-5} M or the combination of unlabelled prazosin and desipramine. In the presence of unlabelled prazosin in the release buffer, the cellular content of [^{3}H]prazosin was increased ($P<0.01$ at 60 minutes; Figure 11.8.2B); this increase was

abolished by desipramine (cellular content of [^{3}H]prazosin at 60 minutes: control: 15,096 ± 983 dpm; unlabelled prazosin 10^{-6} M: 25,521 ± 493 dpm; desipramine 10^{-5} M: 4,966 ± 56 dpm; unlabelled prazosin + desipramine: 13,381 ± 675 dpm). Thus, the cells can accumulate the small amounts of [^{3}H]prazosin which are present in the release buffer by a mechanism whose functional properties are identical to the uptake process which has been described in these cells (Transport-P). The rate of release of [^{3}H]prazosin was very similar in cells which were studied in the presence of desipramine + unlabelled prazosin and in the cells which were studied in the presence of desipramine alone in the release buffer (release constant: control 9.9×10^{-5} sec^{-1}; unlabelled prazosin 3.5×10^{-5} sec^{-1}; desipramine 2.4×10^{-4} sec^{-1}; unlabelled prazosin + desipramine 1.4×10^{-4} sec^{-1}).

In order to avoid interference from re-uptake of released prazosin, further experiments were carried out in the presence of desipramine 10^{-5} M. This concentration of desipramine maximally inhibits the uptake of prazosin in GnRH cells (Figure 11.2.6).

In 16 independent experiments in the presence of desipramine 10^{-5} M at 37^0C, the coefficient of variation (CV) of the release constant was 17% and the CV of the half life was 18%.

In the presence of desipramine 10^{-5} M, release of prazosin was acutely sensitive to the temperature of the incubation medium (Figure 11.8.3). This is clearly evident when the data in Figure 11.8.3 are presented as a linear plot of temperature *vs* release constant (Figure 11.8.4A). There was essentially no prazosin release at 0^0C (release constant 3.3×10^{-6} sec^{-1}; half life 1510 minutes). There was a small increase in release at temperatures up to 25^0C (release constant 4.2×10^{-5} sec^{-1}; half life 120 minutes). However, at temperatures greater than 25^0C, there was a sharp acceleration in release which reached a maximum at 33^0C (release constant 2.5×10^{-4} sec^{-1}; half life 20 minutes). There was no further increase in release at 37^0C (Figure 11.8.4A). The approximate value of Q_{10} calculated from the linear plot was 2.6 (Figure 11.8.4A). The data in Figure 11.8.3 are also presented as an Arrhenius plot of temperature K^{-1} against the natural log of the release constant (Figure 11.8.4B). The

data fitted a straight line ($R^2 = 0.9625$) whose gradient was -10^4 K. The gradient is also given in the expression -Ea/R (Cornish-Bowden, 1995). From this, a value of 83.1 $kJmol^{-1}$ was obtained for the activation energy for the release of prazosin from GnRH cells. Q_{10} is also given by the expression $e^{Ea/75000}$ (Price & Stevens, 1989) from which a value of 3.0 was obtained. This is similar to the approximate Q_{10} value which was obtained from the linear plot.

The organic base chloroquine accelerated prazosin release in the presence of desipramine (Figure 11.8.5A; control: release constant $2.5x10^{-4}$ sec^{-1}; half life: 20 minutes; chloroquine 10^{-4} M: release constant $7.3x10^{-4}$ sec^{-1}; half life 7 minutes; ANOVA: $P<0.01$). The effect of chloroquine was concentration-dependent (Figure 1.8.5B; IC_{50} $4.6x10^{-5}$ M).

The ionophore monensin accelerated the release of prazosin in the presence of desipramine at 37^0C (Figure 11.8.6A; control: release constant $1.9x10^{-4}$ sec^{-1}; half life: 26 minutes; monensin 10^{-5} M: release constant $5.7x10^{-4}$ sec^{-1}; half life 9 minutes; ANOVA: $P<0.01$). The effect of monensin was concentration-dependent (Figure 11.8.6B; IC_{50} $3.8x10^{-6}$ M).

In the presence of desipramine 10^{-5} M at 37^0C, release of prazosin was accelerated by the V-ATPase inhibitor bafilomycinA1 (Figure 11.8.7; control: release constant $2.1x10^{-4}$ sec^{-1}; half life: 24 minutes; bafilomycinA1 10^{-6} M: release constant $4.5x10^{-4}$ sec^{-1}; half life 11 minutes; ANOVA: $P = 0.01$).

Increasing extracellular pH accelerated prazosin release at 37^0C in the presence of desipramine 10^{-5} M (Figure 11.8.8A; pH 7.4: release constant $1.9x10^{-4}$ sec^{-1}; half life: 27 minutes; pH 7.7: release constant $3.0x10^{-4}$ sec^{-1}; half life: 17 min; pH 8.3: release constant $4.4x10^{-4}$ sec^{-1}; half life: 11 minutes; ANOVA: pH 7.4 *vs* pH 7.7: $P<0.05$; pH 7.4 *vs* pH 8.3: $P<0.001$). In contrast, the release of prazosin was slowed in acidic extracellular pH (Figure 11.8.8B; ANOVA pH 7.4 *vs* pH 6.4: $P<0.05$).

11.8.2 Release of BODIPY FL prazosin

GT1-1 GnRH cells cultured in the presence of FBS had a predominantly round appearance with few processes (Figure 11.8.9), in contrast to the neuronal appearance of these cells when grown in serum-free medium (Figures 11.7.7 and 11.7.8). Autofluorescence of GnRH cells (ie, fluorescence in the absence of BODIPY FL prazosin) was minimal, confirming previous observations (Section 11.7). GnRH cells incubated at 37^0C in the presence of the fluorescent analogue BODIPY FL prazosin accumulated the fluorescent compound and became intensely fluorescent. Acquisition of fluorescence was blocked by the antidepressant desipramine 10^{-5} M, confirming that BODIPY FL prazosin accumulates via Transport-P, as previously described (Section 11.7). BODIPY FL prazosin is accumulated via Transport-P and it also binds to α_1 adrenoceptors which are located on the cell surface. In the presence of unlabelled prazosin (which blocks the binding of BODIPY FL prazosin to the α_1 adrenoceptors), the nucleus is relatively free of fluorescent staining and the distribution of the fluorescence is in a punctate pattern, as can be expected from accumulation in intracellular vesicles (Figures 11.8.9A and 11.8.9B). Experiments on the release of BODIPY FL prazosin were carried out in the presence of desipramine 10^{-5} M in order to avoid interference from re-uptake of the released compound. GnRH cells which had accumulated BODIPY FL prazosin at 37^0C, washed and incubated in the presence of desipramine 10^{-5} M for 15 minutes at 37^0C remained fluorescent, indicating that some of the fluorescent compound had been retained in the cells (Figure 11.8.9C). When release of BODIPY FL prazosin was studied for 15 minutes at 0^0C, the cells retained much more of the fluorescent analogue, as indicated by a more intensely fluorescent appearance of the cells (Figure 11.8.9D). In contrast, when release of BODIPY FL prazosin was studied for 15 minutes at 37^0C in the presence of chloroquine 10^{-4} M or monensin 10^{-5} M, cellular fluorescence was greatly diminished (Figures 11.8.9E and 11.8.9F), indicating that release of the fluorescent compound had been accelerated.

11.8.3 Comment

Previous work had demonstrated that peptidergic neurones accumulate the amines prazosin and BODIPY FL prazosin via Transport-P. The present study demonstrates that these cells are also capable of releasing the accumulated amines. In addition to accumulation by

Transport-P, GnRH neurones possess α_{1B} adrenoceptors which also bind [^{3}H]prazosin (Section 11.1). However, the present study examined the release of [^{3}H]prazosin which had been accumulated in the presence of unlabelled prazosin 10^{-6} M; this concentration of unlabelled prazosin completely blocks the binding of [^{3}H]prazosin to α_{1B} adrenoceptors (Figure 11.1.15). The decline of cellular radioactivity therefore represents release of prazosin accumulated by Transport-P, rather than dissociation of [^{3}H]prazosin from α_{1B} adrenoceptors in GnRH neurones.

Many transporters are capable of reversing the direction of transport if the concentrations of their substrates are reversed; this applies to the pre-synaptic transporters for noradrenaline, dopamine and serotonin and for the Uptake$_2$ carrier in non-neuronal cells (Paton, 1973; Raiteri et al, 1977 & 1979; Grohmann, 1988). These transporter-mediated release processes are inhibited by appropriate blocking agents such as desipramine, cocaine, nomifensine or O-methyl-isoprenaline which inhibit the uptake of these amines via the transporters (Paton, 1973; Raiteri et al, 1979; Grohmann, 1988). However, release of prazosin and BODIPY FL prazosin was not blocked by desipramine 10^{-5} M. As this concentration of desipramine completely blocks Transport-P (Figure 11.2.6), the findings indicate that release of prazosin occurs by a mechanism other than Transport-P.

In fact, desipramine accelerated the decline of the cellular content of prazosin (Figure 11.8.1), presumably by inhibiting re-uptake of released prazosin from the incubation medium. This interpretation is supported by the finding that the presence of unlabelled prazosin in the release buffer increased the cellular content of [^{3}H]prazosin by an antidepressant-sensitive process (Figure 11.8.2B); these findings are identical to the functional properties of Transport-P which is activated by concentrations of prazosin greater than 10^{-7} M. Thus, the cells can accumulate the small amounts of [^{3}H]prazosin which are present in the release buffer via Transport-P. It therefore appears that in the control experiment in Figure 11.8.1A, the decline in the cellular content of prazosin is not truly mono-exponential, as it represents a composite of the release of cellular prazosin and re-uptake of released prazosin. However, when re-uptake is inhibited by desipramine, the decline in the cellular content of prazosin is probably truly mono-exponential.

An alternative interpretation of the effect of desipramine is that it may exert its effect at an intracellular site such as the vesicles which accumulate prazosin. Exit of prazosin from the cells may be by a two-stage process: exit across the vesicular membrane followed by exit across the plasma membrane. It is possible that desipramine may block re-uptake of prazosin from the cytoplasm into the vesicles; accumulation of prazosin in the cytoplasm would then accelerate its exit from the cells. The present work has described some of the functional properties of the release of amines from peptidergic neurones but has not identified the rate limiting step which is likely to be the site of action of the experimental manipulations.

Release of amines from GnRH cells was acutely sensitive to temperature. Release was minimal below 30^0C and accelerated sharply at higher temperatures. The kinetics of temperature dependence conformed to the Arrhenius equation in which there is an exponential increase in reaction rate with temperature (Figure 11.8.4; Price & Stevens, 1989). This is typical of cellular processes such as enzymatic reactions (Cornish-Bowden, 1995). In contrast, simple diffusion along a concentration gradient increases linearly with temperature, as described in the Fick-Einstein and the Stokes-Einstein equations (Stein, 1986). Release of prazosin from GnRH neurones is clearly not simply by a process of passive diffusion down a concentration gradient. In common with other biological systems, release of prazosin from GnRH neurones requires an activation energy. The values which were obtained for the activation energy (Ea) and for the temperature quotient (Q_{10}) were within the range of values which are expected in a biological system (Price & Stevens, 1989). An alternative explanation for the temperature dependence of the release process is that cellular phospholipid membranes may undergo a "melting" process which may influence the release of amines; such a possibility cannot be excluded by the available data and will have to be addressed in future studies.

Transport-P derives its energy from the electrochemical proton gradient (Section 11.3), so the present study examined the effects of manipulations of intracellular acidity on the release of prazosin and BODIPY FL prazosin. Chloroquine is an organic base which diffuses into intracellular acidified particles; at a concentration of 10^{-4} M, chloroquine increased the pH of intracellular acidified vesicles in macrophages from approximately 4.7 to 6.4 (Ohkuma & Poole, 1978). Monensin is a monovalent carboxylic ionophore which forms lipid-soluble complexes with cations. It traverses the lipid phase of cellular membranes, resulting in

movement of sodium ions into cells, in exchange for protons (Pressman & Fahim, 1982; Ledger & Tanzer, 1984). At a concentration of $6x10^{-6}$ M, monensin increased the pH of intracellular acidified vesicles in fibroblasts from 5.0 to 6.2 (Maxfield, 1982). BafilomycinA1 is an inhibitor of V-ATPase proton pumps which are responsible for generating intracellular acidity (Bowman et al, 1988). BafilomycinA1 10^{-6} M increased the pH of lysosomes of cultured cells from 5.1 to 6.3 (Yoshimori et al, 1991). These compounds increase intracellular pH by different mechanisms. Uptake of prazosin and BODIPY FL prazosin via Transport-P is blocked by all these pharmacological manipulations and by increasing extracellular pH (Section 11.3 and 11.7). In contrast, release of prazosin and BODIPY FL prazosin was not blocked by any of these pharmacological manipulations or by increasing extracellular pH. These findings further distinguish the release process from Transport-P.

In fact, release of prazosin was accelerated by these pharmacological manipulations and by increasing extracellular pH. These experiments were carried out in the presence of desipramine 10^{-5} M which completely inhibits uptake of amines via Transport-P (Figure 11.2.6). Therefore, the accelerated decline of cellular radioactivity which was caused by these manipulations is not due to inhibition of re-uptake of released amines via Transport-P. The present observation that release of prazosin was accelerated by manipulations which neutralise intracellular acidity is consistent with the conclusion that Transport-P accumulates amines in acidified intracellular vesicles (Section 11.3). Clearly, intracellular acidity is required for retention of amines which are accumulated via Transport-P in peptidergic neurones.

Lysosomotropic drugs are cationic amphiphilic compounds which exist as bases at physiological pH (pKa approximately 8; De Duve et al, 1974). They enter cells by diffusion in unprotonated form. They become protonated in the acidic environment of lysosomes, where they remain trapped due to their inability to diffuse through the lipid phase of the membrane (De Duve et al, 1974). The pKa of prazosin is 6.8 (Alabaster et al, 1987), which makes it an unlikely candidate for a lysosomotropic drug. Lysosomes are present in all eukaryotic cells but the organic base chloroquine did not reduce the association of prazosin with COS-7 kidney cells, indicating that prazosin is unlikely to accumulate as a result of lysosomotropic properties (Figure 11.3.5). Further, the lysosomotropic effect is accelerated

by increasing extracellular pH, due to an increase in the proportion of the compound that is unprotonated and therefore able to diffuse into cells (De Duve et al, 1974). However, uptake of prazosin was in fact inhibited by increasing extracellular pH (Table 11.3.1). It therefore seems unlikely that uptake of prazosin is due to a lysosomotropic effect. Conversely, manipulations of intracellular pH may exert an effect on the equilibrium between neutral and charged forms of basic compounds, and this may influence the retention of these compounds within cells. For a compound whose pKa is 6.8, an increase in the pH of intracellular vesicles from 5.0 to 6.5 would reduce the proportion of the ionised species of the compound from 98.4% to 66.6% (Newton & Kluza, 1978). The neutral species of the compound would then be more likely to escape across cellular membranes.

In conclusion, following uptake by Transport-P in peptidergic neurones, amines are accumulated in acidified intracellular stores; their retention in peptidergic neurones requires maintenance of intracellular acidity. The amines can then be released by a temperature-dependent process which is resistant to antidepressants.

Figure 11.8.1:

Effect of desipramine on the release of prazosin from GnRH cells following accumulation by Transport-P. The data are presented as linear plots of the total cellular content of prazosin in "A" and as log-linear plots of the specific cellular content of prazosin in "B". Non-specific uptake was defined as the residual amount of cellular prazosin in the presence of desipramine 10^{-5} M at 120 minutes. Specific content was obtained by subtracting non-specific content from total content. In the presence of desipramine, release of prazosin follows an exponential time course which is described by a straight line in the log-linear plot. The "goodness" of these curve fits was assessed by R^2 values (Section 10.4). The R^2 values in panel B were: control 0.968; desipramine 0.9908. The study was carried out at 37^0C. In this and in subsequent Figures, units were omitted when data were log-transformed.

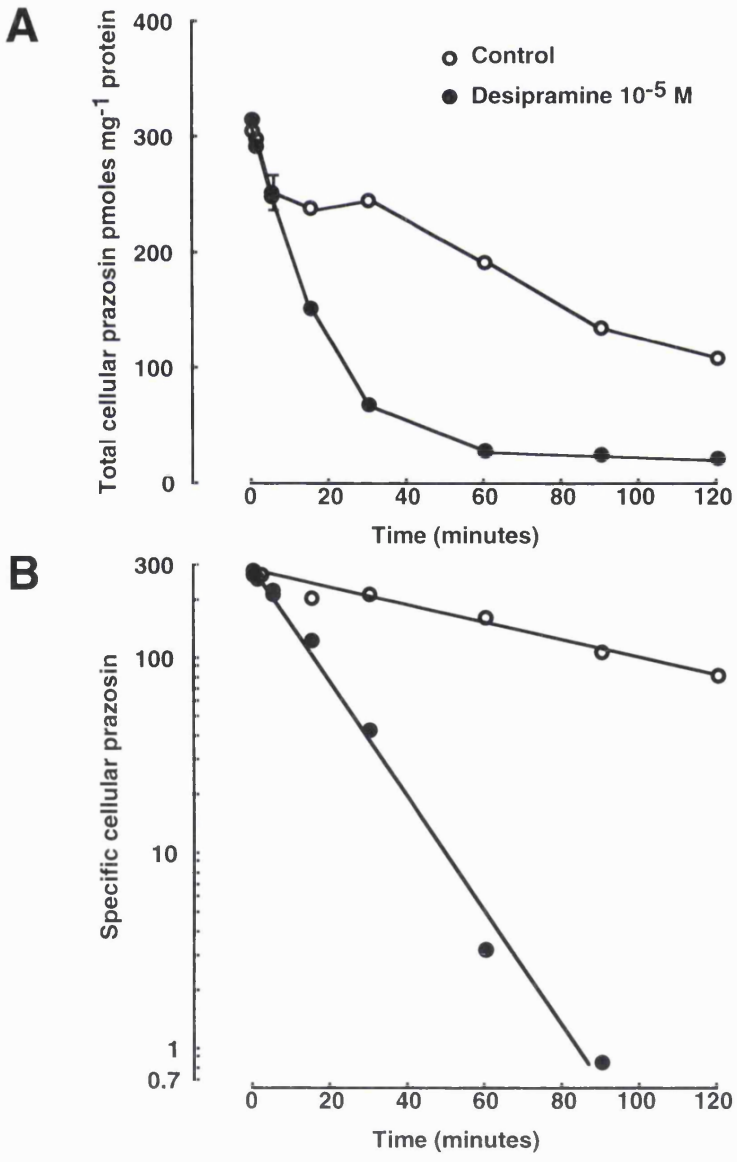


Figure 11.8.2:

A: Effect of unlabelled prazosin on the uptake of [^{3}H]prazosin in GT1-1 GnRH cells.

B. Effect of unlabelled prazosin 10^{-6} M in the release buffer on the cellular content of [^{3}H]prazosin in GT1-1 GnRH cells. All the cells were pre-loaded with [^{3}H]prazosin and unlabelled prazosin 10^{-6} M; release was then studied in the absence of drugs (control) or in the presence of unlabelled prazosin 10^{-6} M, desipramine 10^{-5} M or the combination of unlabelled prazosin and desipramine. The R^2 values for the curve fits in panel B were: control 0.9864; unlabelled prazosin 0.9834; desipramine: 0.9281; unlabelled prazosin + desipramine: 0.9993. The experiments in A and B were performed at 37^0C.

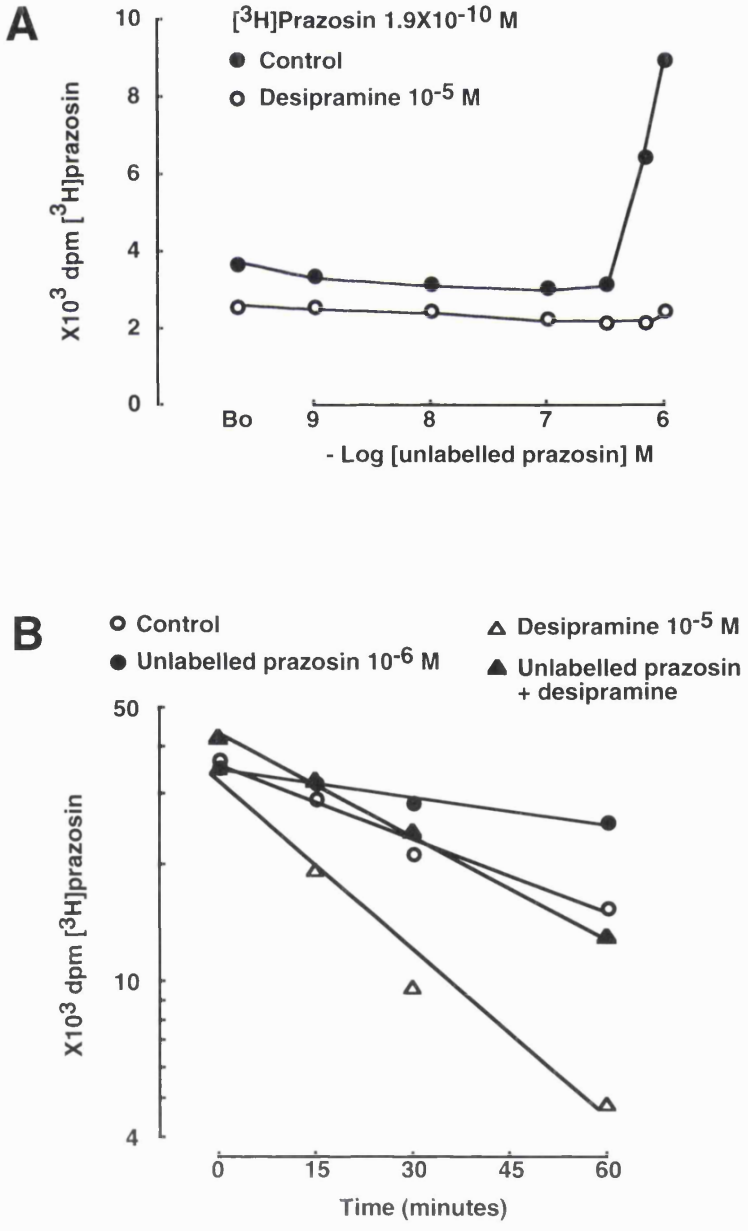


Figure 11.8.3:

Effect of the incubation temperature on the release of prazosin from GnRH cells: Release was studied at temperatures ranging from 0^0C to 37^0C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released prazosin.

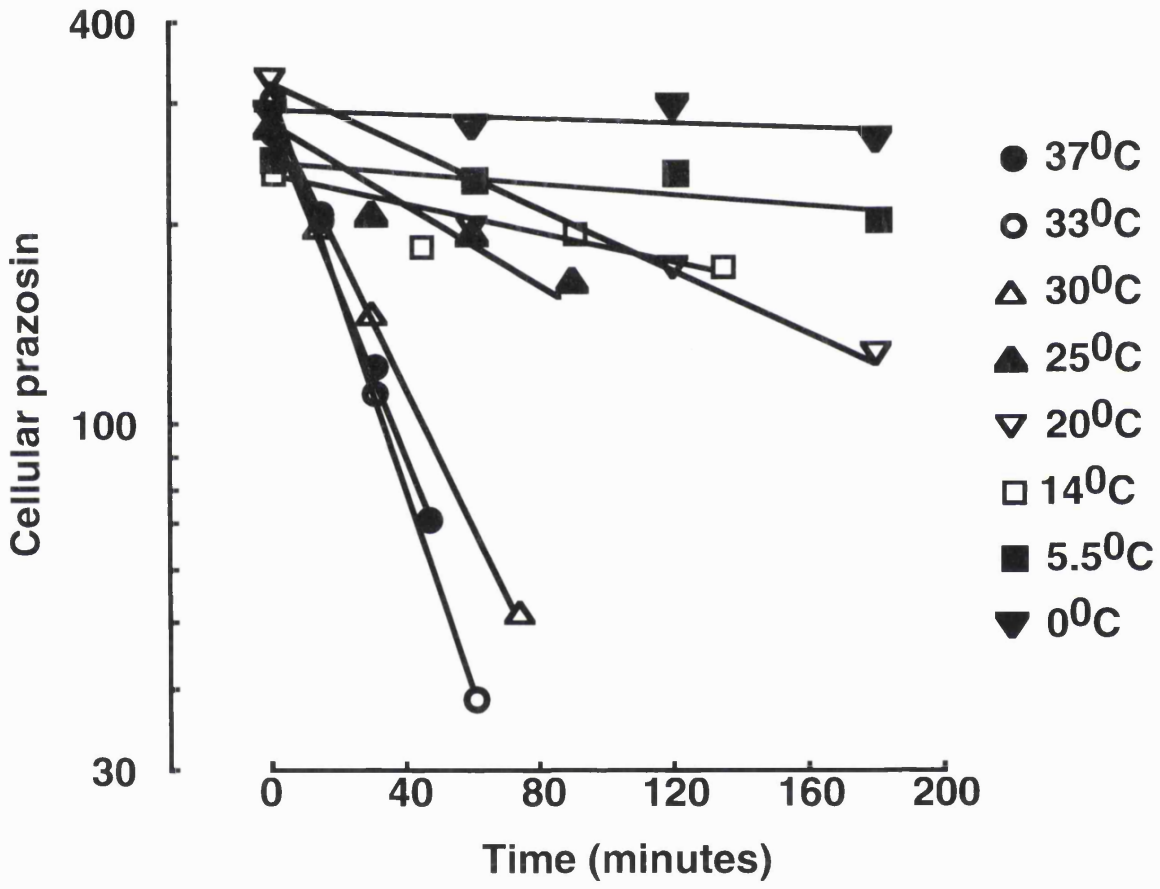


Figure 11.8.4:

Analysis of the effect of temperature on the release of prazosin from GnRH neurones: In panel A, the data from Figure 11.8.3 are presented as linear plots of temperature (^{0}C) against the release constants (sec^{-1}). In panel B, the data from Figure 11.8.3 are presented as an Arrhenius plot of temperature (K^{-1}) against the natural log of the release constant. Increasing temperature has an exponential effect on prazosin release, which is consistent with the Arrhenius equation. Calculations of Ea and Q_{10} are described in the text. The R^2 value for the curve fit in panel B was 0.9625.

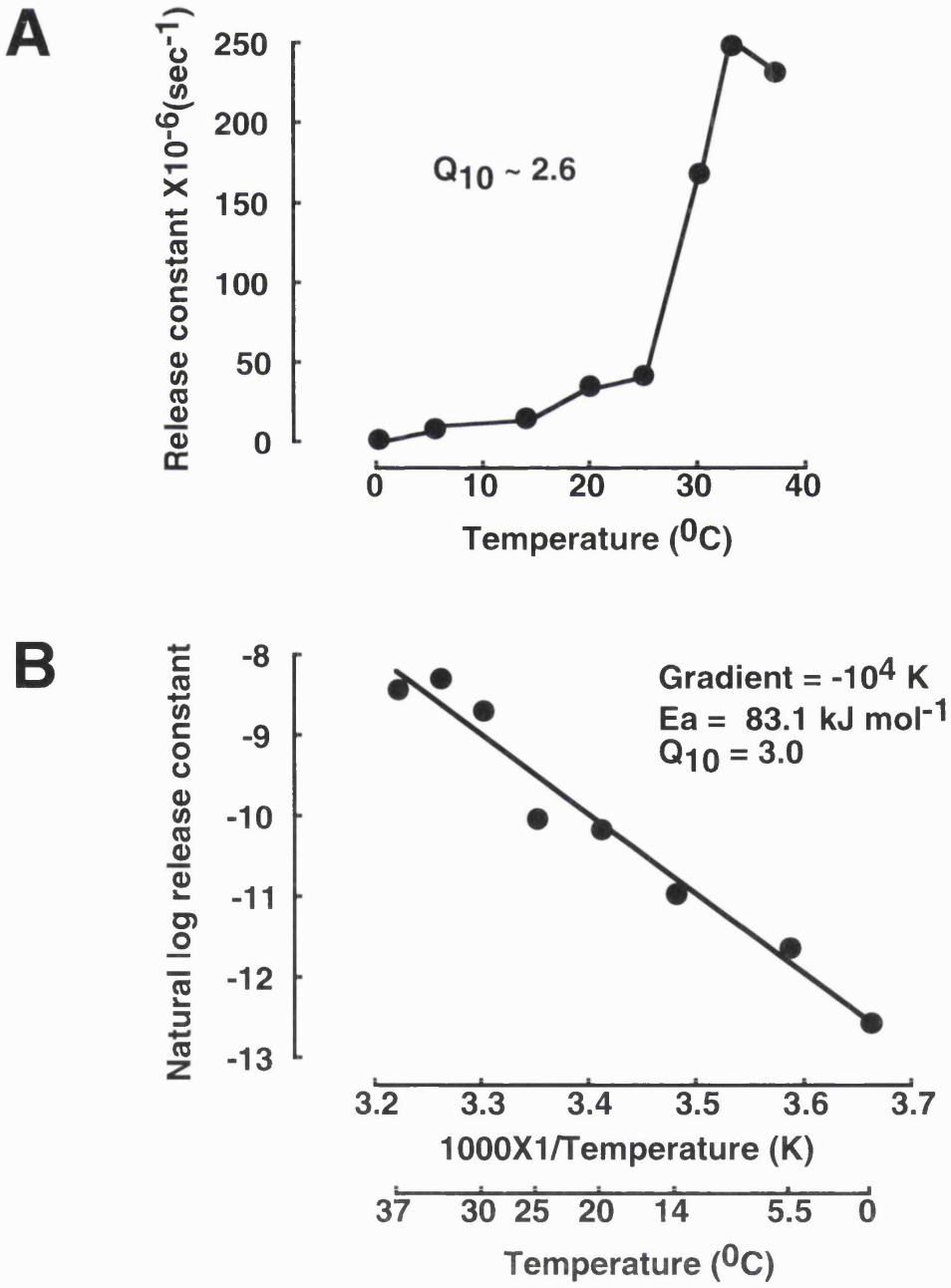


Figure 11.8.5:

Effect of the organic base chloroquine on prazosin release. Release was studied at 37^0C in the presence of desipramine 10^{-5} M. The R^2 values for the curve fits in panel A were: control 0.8901; chloroquine 0.9917. In panel B, release was studied at 15 minutes.

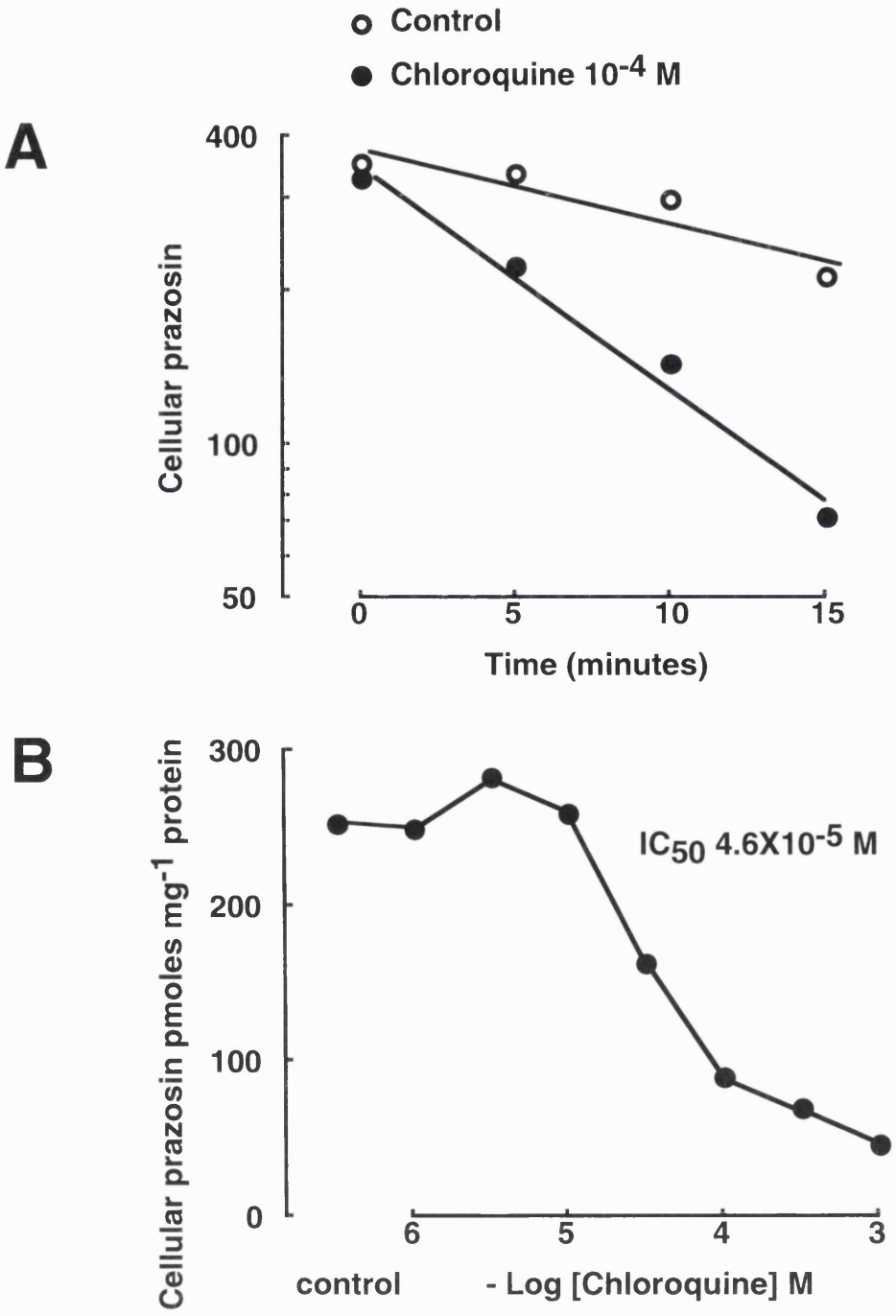


Figure 11.8.6:

Effect of monensin on prazosin release from GnRH cells:

Release was studied at 37^0C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released prazosin. The R^2 values for the curve fits in panel A were: control 0.7301; monensin 0.9974. In panel B, release was studied at 15 minutes.

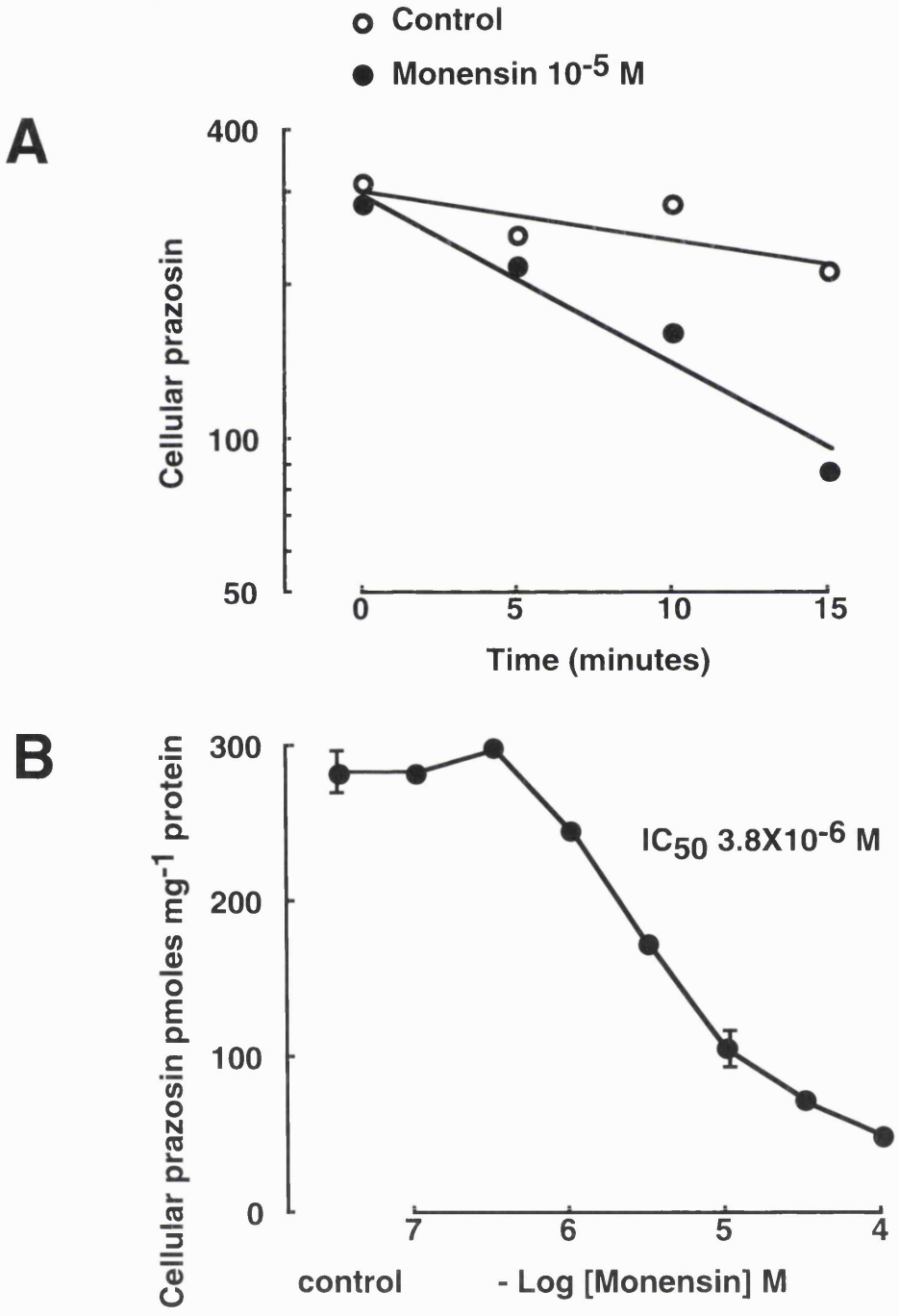


Figure 11.8.7:

Effect of the V-ATPase inhibitor bafilomycinA1 on prazosin release from GnRH cells: Release was studied at 37^0C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released prazosin. The R^2 values for the curve fits were: control 0.9935; bafilomycin 0.9951.

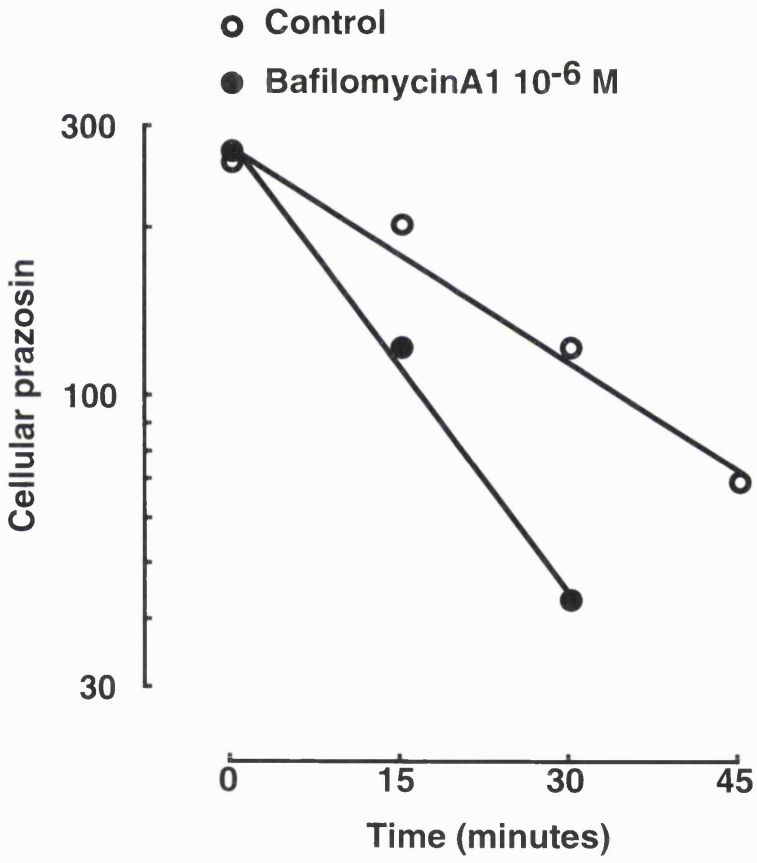


Figure 11.8.8:

Effect of extracellular pH on prazosin release from GnRH cells:

Increasing extracellular pH in the range 7.4-8.3 accelerated prazosin release in the presence of desipramine 10^{-5} M at 37^0C (Panel A). Conversely, release was slower in acidic extracellular pH (Panel B). In panel A, the R^2 values for the curve fits were: pH 7.4: 0.9835; pH 7.7: 0.9755; pH 8.3: 0.9873. In panel B, the R^2 values were: pH 7.4: 0.9703; pH 6.4: 0.9879.

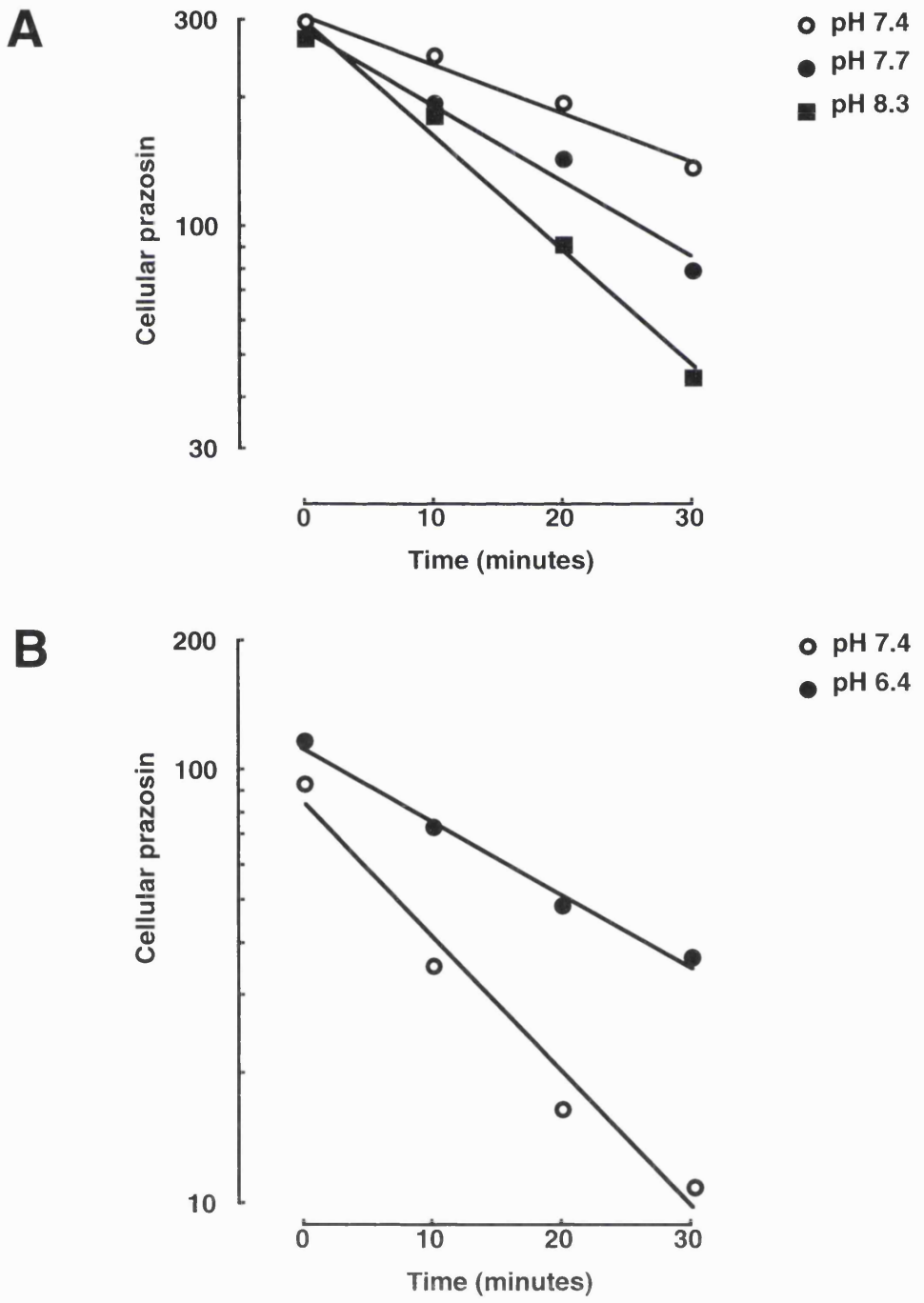
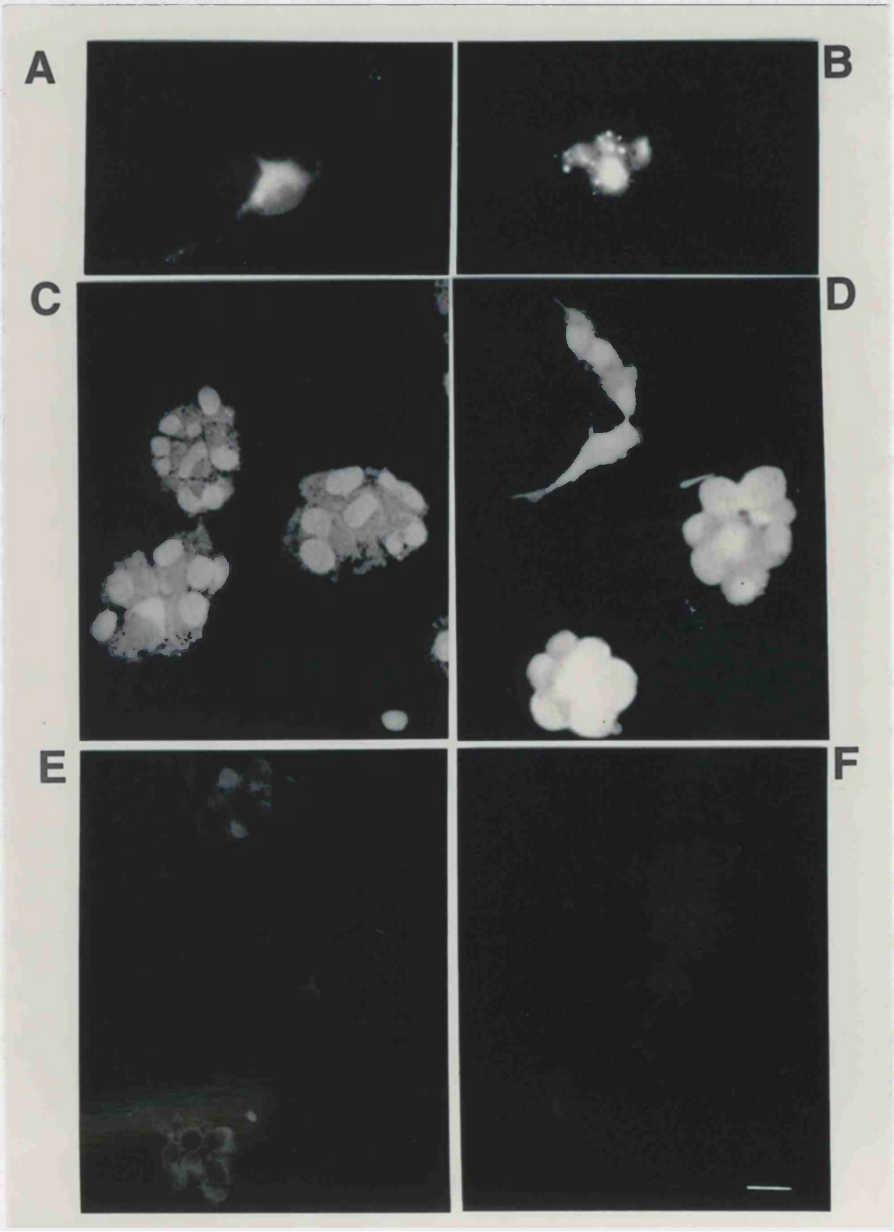


Figure 11.8.9:

A & B: Uptake: BODIPY FL prazosin accumulates in GnRH cells via Transport-P and it also binds α_{1B} adrenoceptors. These cells were incubated with BODIPY FL prazosin and unlabelled prazosin 10^{-6} M (to block binding of BODIPY FL prazosin to α_{1B} receptors). The nucleus is relatively free of fluorescence (A) and the distribution of the fluorescence is in a punctate pattern (A&B). Cells grown in the presence of FBS have few neuronal processes. *C-F: Release:* GnRH cells were exposed to BODIPY FL prazosin for 60 min at 37°C then washed and incubated for 15 min at 37°C in the presence of desipramine 10^{-5} M to inhibit re-uptake of released BODIPY FL prazosin (control; C). Release of BODIPY FL prazosin was slowed at 0°C (D), and accelerated by chloroquine (10^{-4} M at 37°C; E) and by monensin (10^{-5} M at 37°C; F). The scale bar in panel F represents 5 μm.



11.9 ISOLATION OF cDNAs FOR TRANSPORTERS IN GnRH NEURONES

The work which is described in this Section was aimed to isolate a cDNA which encodes the Transport-P carrier. Although Transport-P is clearly different from known transporters, it has functional similarities to some of the known amine transporters in the brain. Therefore, the initial work was planned to search the GnRH neurone library systematically for unknown cDNAs which may be homologous to the known carriers in the brain. The project was then extended to a strategy which is based on functional expression.

11.9.1 Cloning by homology with the SV2 orphan vesicular transporters

The accumulation of neurotransmitter amines into vesicles is mediated by the synaptic vesicle monoamine transporter in pre-synaptic neurones and by the chromaffin granule transporter in the adrenal medulla (Section 7.5.14). Despite the intense investigative activity on the synaptic vesicle 2 (SV2) proteins, the functions of these proteins have not been elucidated, and they can be regarded as "orphan" transporters (Section 7.5.16). Previous investigators isolated cDNAs which encode members of these SV2 proteins (Section 7.5.16). There were several indications that SV2 proteins are likely to function as proton-dependent transporters:

1. The SV2 proteins are located in acidic synaptic vesicles (Section 7.5.16);
2. The sequence is homologous to bacterial proton-dependent transporters (Gingrich et al, 1992);
3. There is an arginine at the beginning of the 6th membrane-spanning region, which is conserved in all proton symporters (Griffith et al, 1992).
4. There are two consensus sites for ATP binding motifs (Gingrich et al, 1992); it seemed possible that the SV2 proteins may function as transporters with intrinsic ATPase activity, and that activation of this catalytic function following ligand binding may explain the activation of Transport-P by its ligand (see Section 12.3.2.7).

The work which is described in this Section aimed to screen the GnRH neurone cDNA library for the presence of cDNAs which may encode known or unknown members of the SV2 family, and then to express the isolated cDNAs in mammalian cells. Previous investigators demonstrated that it is possible to express SV2 cDNAs in mammalian cells. When non-neuronal cells such as COS cells and CHO cells are transfected with cDNA encoding SV2, the cellular SV2 immunoreactivity appears in a punctate distribution, suggesting that the protein is expressed in small intracellular vesicles. SV2 is not detectable in the plasma membranes of these transfected cells (Bajjalieh et al, 1992; Feany & Buckley,

1993; Feany et al, 1993). Immunocytochemical studies demonstrated that the distribution of SV2 in transfected CHO fibroblasts was distinguishable from the distribution of immunocytochemical markers for the endoplasmic reticulum, Golgi apparatus, clathrin and lysosomes (Feany et al, 1993). The pattern of distribution of SV2 resembled, but was somewhat different from, the distribution of internalised transferrin, which is a marker of endocytic vesicles (Feany et al, 1993). Immunoelectron microscopy with gold particles on cellular fractions demonstrated that the SV2 antigen was located in vesicles whose average diameter was 80 nm in these transfected fibroblasts (Feany et al, 1993). These vesicles appear to be intermediate in size between the 40 nm small clear neurosecretory vesicles in nerve terminals and the 100 nm large dense core vesicles of neuroendocrine cells.

In the present work, PCR primers were designed based on the sequence of the bovine SV2A cDNA (Gingrich et al, 1992; Genbank accession number S47919); the mouse SV2A cDNA sequence was not available. Details of the primers (which are designated SV2F15 and SV2B22) are shown in Table 11.9.1. The upstream and downstream primers differ by only two bases each from the rat cDNA sequence (Bajjalieh et al, 1992; Genbank accession number L05435).

PCRs using the SV2F15 and SV2B22 primer pair generated a product of the expected size (407 bp; Figure 11.9.1). Further experiments which aimed to optimise the annealing temperature and the magnesium concentration demonstrated that it was possible to generate a specific product which could not be generated in the absence of either primer or in the absence of template (Figure 11.9.1). Using the technique of TA cloning (Section 10.12), the product of this reaction was ligated to the pCRII plasmid and two clones were sequenced manually (Section 10.13). The translated sequences of these clones were identical to the sequence of SV2A.

In order to obtain a full-sized clone, the cDNA library was screened by the procedure of hybridisation to bacterial pools of diminishing size (Section 10.14), using the SV2F15 and SV2B22 PCR product as a probe. *EcoR* I sites are present at each end of the multiple cloning site of the pCRII plasmid, but there are no *EcoR* I sites within the sequence of the insert. The PCR product was therefore detached from the pCRII plasmid by digesting the recombinant DNA with *EcoR* I. The plasmid was then separated from the insert in a 1.2% agarose gel. The 407 bp insert was cut out from the gel and extracted with the Qiaex kit

(Section 10.10). The insert was then labelled with ^{32}P using the method of random hexamers (Section 10.14.3). Plasmid pools were prepared from the library, each containing 149,000 bacteria. The bacteria were amplified overnight in the semi-solid medium to ensure representative amplification of the clones, and the plasmid DNA was then extracted from the bacteria using Qiagen kits (Section 10.14). One μg of the plasmid DNA was then digested with *Mlu* I to separate the pSVSPORT1 plasmid from the inserts and the reaction products were then electrophoresed in a 0.8% agarose gel (Section 10.14.2). The DNA was then transferred to nylon membranes which were hybridised to the 407 bp PCR product which had been labelled with ^{32}P. Several pools contained positive hybridisation bands, the largest of which was approximately 3.5 kb in pool 74 (Figure 11.9.2). That pool was then divided into sub-pools of 42,000 bacteria which were treated in an identical manner. Progressive cycles of pools of diminishing number resulted in amplification of the hybridisation signal, due to enrichment of the target molecule (Figure 11.9.2). An individual positive clone was eventually obtained and the 3.5 kb insert was sequenced manually in both directions, using 37 sequencing primers (Figure 11.9.3). The sequence of the clone (designated clone 37) is shown in Figure 11.9.4.

There were two problems with this clone:
1. Clone 37 contained a 384 bp intronic segment;
2. The 5' end of the clone was missing.

These problems were solved as follows:
1. The clone 37 cDNA contained a 384 bp segment which starts with a stop codon in position 1567 and includes several stop codons (Figure 11.9.4). This 384 bp segment interrupts the open reading frame between the 10th and 11th membrane-spanning domains. It is flanked by 5' ("donor") and 3' ("acceptor") splice sites (5' splice site: AGGTAGT; 3' splice site: CAGG). This indicates that the 384 bp segment is likely to be an intronic insert in an incompletely spliced molecule (Senapathy et al, 1990). In order to determine the relative abundance of this incompletely spliced molecule, nested PCR primers were designed to flank the 384 bp segment; the outer primer pair was designated C37F1 and C37B1 and the inner primer pair was designated C37F23 and C37B21 (Figure 11.9.4 and Table 11.9.2). Using RNA from the GT1-1 GnRH neurones as template in reverse transcriptase PCRs with the C37F23 and C37B21 primer pair, the only specific product which was generated was

220 bp, corresponding to the sequence of clone 37 without the 384 bp intronic segment. Nested PCRs were also tried in view of their greater sensitivity, but only the 220 bp product was generated (Figure 11.9.5). This indicated that most of the SV2A RNA molecules do not contain the 384 bp intronic segment. Using the GnRH neurone cDNA library as template and the C37F23 and C37B21 primer pair, the predominant product which was generated was also the 220 bp; however, a 604 bp product was also generated in a smaller amount, corresponding to the sequence of clone 37 with the 384 bp intronic segment (Figure 11.9.5). This indicated that a minority of the SV2A clones in the cDNA library contain the 384 bp segment. Presence of this clone in the library may represent either a cloning artefact or preferential amplification of a clone which contains the intronic segment.

The 220 bp PCR product was ligated to the pCR2.1 plasmid by the technique of TA cloning (Section 10.12); sequence of the 220 bp PCR product confirmed that it represents the 10th and 11th membrane spanning domains of SV2A, without the 384 bp intronic segment.

In order to excise the 384 bp intronic segment, restriction endonuclease sites for *NgoM* I and *Dra* III were identifed in positions 1226 and 2177 (respectively) of clone 37; these enzymes cut the cloned sequence at unique positions which flank the 384 bp intronic segment, but they do not cut the pSVSPORT1 plasmid (Figure 11.9.4). Following digestion with these enzymes, the cut ends of the DNA can be expected to be incompatible, and this should minimise the likelihood of re-ligation of the cut DNA. The PCR primers C37F1 and C37B1 flank the restriction endonuclease sites (Figure 11.9.4). Using these primers and the GT1-1 GnRH library as template, it was possible to generate products of the expected sizes (1214 bp and 830 bp); these products were ligated to the pCR2.1 plasmid using the technique of TA cloning. The sequence of the smaller product (830 bp) confirmed that it consists of clone 37 without the 384 bp intronic segment (not shown). This 830 bp piece was digested with *NgoM* I and *Dra* III, as was clone 37 (Figure 11.9.6); the digests were electrophoresed in agarose and the relevant segments were cut from the gel, purified and ligated to each other. The resulting DNA was introduced into *E coli* and amplified. When the DNA was tested with the C37F1 and C37B1 PCR primer pair, it was clear that all the plasmid preparations contained the 830 bp region without the 384 bp intronic segment (Figure 11.9.6). This was confirmed by sequencing the DNA manually.

2. The missing 5' end of clone 37 was obtained using the technique of rapid amplification of

cDNA ends (5'RACE; Section 10.15), using primers whose details are shown in Table 11.9.3. The 5'RACE was performed in the following steps (Figure 11.9.7):

A. RNA (1 μg) from GT1-1 GnRH neurones was denatured by heating to 70^0C for 10 minutes then reverse transcribed at 42^0C using the first gene-specific primer (SV2B5; 100 nM) and 200 Units of SuperScript II reverse transcriptase.
B. The RNA was degraded using RNAseH and the cDNA was cleaned with a spin cartridge.
C. The cDNA was tailed with dC using terminal deoxynucleotidyl transferase (10 Units).
D. The second cDNA strand was synthesised using an anchor primer which contains a sequence which is complementary to the dC tail.
E. In the same reaction, the double stranded DNA was amplified by PCR using the second gene specific primer (SV2B6) and the anchor primer.
F. The DNA was amplified again by PCR using the GSP3 (SV2B7) and an adapter primer (ABR-UAP) which is nested within the sequence of the anchor primer.

After the reactions had been optimised, two clean products (470 bp and 650 bp) were obtained. These were ligated to the pCR2.1 plasmid and sequenced manually. The sequence which was generated was then used to design PCR primers which were used to amplify the GT1-1 GnRH neurone cDNA library to generate a PCR product which was ligated to clone 37. The ligation was performed using a *Sal* I site in the pSVSPORT1 plasmid at the 5' end and a *PflM* I site in the clone 37 insert at the 3' end. *PflM* I is a methylation-sensitive enzyme and this necessitated amplifying the DNA in a methylation-deficient strain of bacteria (DM1 cells; Life Technologies). Ligation of the 5' end to clone 37 resulted in a complete SV2A cDNA whose translated sequence is shown in Figure 11.9.8. The sequence is identical to the rat SV2A protein (Bajjalieh et al, 1992; Feany et al, 1992) except in that the glutamic acid (E) in position 53 of the rat sequence is replaced by an aspartic acid (D) in this mouse sequence; in this substitution, the mouse sequence resembles the bovine SV2A sequence (Gingrich et al, 1992). In any case, this substitution is unlikely to be functionally important, as glutamate and aspartate are functionally equivalent acidic residues. Some of the features of the sequence which were described in Section 7.5.16 are shown in Figure 11.9.8.

The mouse GnRH neurone SV2A insert was cloned into the pcDNA3.1(+) plasmid which utilises the human CMV immediate early gene as a promoter for high level expression in mammalian cells. The insert was cloned into the *EcoR* I site at the 5' end and the *Not* I site at

the 3' end of the multiple cloning site of pcDNA3.1(+). Automated DNA sequence analysis demonstrated that the SV2A insert and the regulatory regions of the plasmid were intact (Figure 11.9.9).

The SV2A cDNA cloned in pcDNA3.1(+) was expressed in COS-7 cells and in GT1-1 cells by electroporation, as this technique had been shown to have no effect on the accumulation of prazosin via Transport-P (Section 10.17).The hamster smooth muscle α_{1b} adrenoceptor cDNA was used as the control for the transfection. The results in electroporated COS-7 cells were as follows:

DNA	*Unlabelled Prazosin*	*cpm [^{3}H]prazosin*	*% control*
Control (TE buffer)	None	166 ± 2	100%
	10^{-6} M	138 ± 5	83%
Hamster α_{1b} in pBC	None	926 ± 11	100%
	10^{-6} M	162 ± 2	17%
SV2A in pcDNA3.1(+)	None	163 ± 1	100%
	10^{-6} M	142 ± 2	87%

From the above, it can be seen that electroporation of α_{1b} adrenoceptor cDNA results in a great increase in the binding of [^{3}H]prazosin (at 1.5×10^{-10} M; from 166 ± 2 cpm to 926 ± 11 cpm) and that unlabelled prazosin at 10^{-6} M displaces most of the [^{3}H]prazosin, the remaining 17% being equal to the non-specific binding. However, electroporation of the SV2A DNA did not result in appearance of the prazosin paradox in the electroporated cells, as [^{3}H]prazosin binding is not increased by unlabelled prazosin (at 10^{-6} M).

The prazosin paradox was observed in peptidergic neurones. However, COS-7 cells are kidney cells which do not possess the specialised cellular apparatus which is characteristic of neurones. To address this problem, the SV2A cDNA was electroporated in GT1-1 GnRH neurones, with the aim of over-expressing the SV2A peptide in these cells. The results were as follows:

DNA	*Unlabelled Prazosin*	*cpm [^{3}H]prazosin*	*% control*
Control (TE buffer)	None	1188 ± 32	100%
	$3.33x10^{-7}$ M	900 ± 44	76%
	$6.66x10^{-7}$ M	1896 ± 163	160%
	10^{-6} M	3245 ± 200	273%
SV2A in pcDNA3.1(+)	None	1643 ± 40	100%
	$3.33x10^{-7}$ M	1249 ± 55	76%
	$6.66x10^{-7}$ M	2120 ± 76	129%
	10^{-6} M	3679 ± 224	224%

The prazosin paradox is evident in both groups of electroporated cells, as concentrations of unlabelled prazosin greater than $3.33x10^{-7}$ M increase the accumulation of [^{3}H]prazosin (at $5x10^{-10}$ M). However, electroporation of the SV2A DNA has no effect on the magnitude of the prazosin paradox in these cells. Further experiments examined the effect of electroporation of SV2A DNA on the sensitivity to antidepressants in the GT1-1 GnRH cells:

DNA	*Drug*	*cpm [^{3}H]prazosin*	*% control*
Control (TE buffer)	None	1762 ± 11	100%
	Unlabelled Praz 10^{-6} M	3287 ± 190	187%
	Desipramine 10^{-5} M	1323 ± 14	75%
	Unlab Praz + Desip	918 ± 18	52%
SV2A in pcDNA3.1(+)	None	1244 ± 10	100%
	Unlabelled Praz 10^{-6} M	2425 ± 60	195%
	Desipramine 10^{-5} M	799 ± 20	64%
	Unlab Praz + Desip	534 ± 7	43%

It can be seen from the above that in both groups of cells, unlabelled prazosin 10^{-6} M increases the accumulation of [^{3}H]prazosin (at $4x10^{-10}$ M), and that this prazosin paradox is inhibited by desipramine. However, electroporation of the SV2A DNA has no effect either

on the prazosin paradox or on the inhibitory effect of desipramine.

In summary, in the above experiments, the cells were electroporated with SV2A DNA and the α_{1b} adrenoceptor DNA was used as a control. Expression of the α_{1b} adrenoceptors in the electroporated cells suggested that under conditions in which expression has taken place, the functional properties of Transport-P are not mediated by SV2A. However, it is difficult to be certain of the interpretation of this negative result, in the absence of a functional assay for SV2A. A supportive piece of evidence is that SK-N-SH noradrenergic neurones, which can be expected to possess SV2A in their neurosecretory vesicles, do not possess Transport-P (Figure 11.1.4). This is consistent with the suggestion that SV2 proteins do not mediate the functional properties of Transport-P.

11.9.2 Cloning by homology with pre-synaptic plasma membrane amine transporters

Previous investigators isolated cDNAs which encode pre-synaptic plasma membrane carriers for noradrenaline, dopamine and serotonin (Section 7.5.13). These cDNAs are highly homologous (Figure 11.9.10). Although Transport-P is clearly different from the known pre-synaptic plasma membrane amine transporters, it has some functional similarities to this family of transporters, such as the sensitivity to antidepressants and the resistance to reserpine and steroid hormones (Table 11.4.2). It therefore seemed possible that Transport-P may be encoded by an unknown member of this group of molecules. The work which is described in this Section was therefore aimed to screen the GnRH neurone cDNA library for the presence of unknown cDNAs which may be homologous to the known pre-synaptic plasma membrane amine transporters.

The bovine dopamine transporter cDNA was used as a probe to screen the cDNA library by the method of hybridisation to plasmid pools (Section 10.14). This method has been used successfully in the present work to isolate cDNAs encoding the GnRH neurone α_{1B} adrenoceptor (Section 11.1.7), a vesicular monoamine transporter (Section 11.9.3) and an orphan vesicular transporter (Section 11.9.1). However, no hybridisation signal was detected in any of the pools (not shown).

The GT1-1 GnRH neurones are a mouse cell line and the sequence of the mouse pre-

synaptic plasma membrane noradrenaline transporter has been published (Jayanthi et al, Genbank accession number NM009209). PCR primers were designed from a translated region of the mouse noradrenaline transporter in which there is homology with the sequence of the human noradrenaline transporter and the rat dopamine transporter (Figure 11.9.11). Hybridisation of the 3' ends of the primers to the target sequence is required to enable extension by the polymerase (Section 10.11). Therefore, the 3' ends of the primers were designed to terminate in the first or the second base of an amino acid codon which is conserved in the human and mouse noradrenaline transporters and the rat dopamine transporter (Figure 11.9.12). The primers were designed to be free of adenine and thymine at the 5' ends, as these nucleotides may interfere with TA cloning (Section 10.12). Details of the primers are shown in Table 11.9.4. Two sets of primers were used, and they were utilised both in conventional PCRs and in nested PCRs, as the latter provide increased sensitivity and specificity (Section 10.11). However, no specific PCR products were detected in any of the reactions (not shown).

Thus, two techniques were used to look for cDNAs which are homologous to the plasma membrane transporters: screening the library by hybridisation has the advantage of being able to detect homologous sequences; whereas the PCRs should be able to detect rare sequences with great sensitivity. However, no sequences were detected using either of these methods. This is consistent with the data which indicated that Transport-P differs from the known plasma membrane transporters by its functional properties and by the structure of its ligands (Tables 11.4.2 and 11.5.2).

11.9.3 Cloning by homology with pre-synaptic vesicular amine transporters

Previous investigators isolated cDNAs which encode the chromaffin granule amine transporter (CGAT) of the adrenal medulla and the synaptic vesicle monoamine transporter (SVMAT) which accumulates noradrenaline, dopamine, serotonin and histamine in pre-synaptic nerve terminals (Section 7.5.14). Although Transport-P is clearly different from the known vesicular transporters, it has some functional similarities to this family of transporters, such as the dependence on the electrochemical proton gradient which is generated by V-ATPase (Table 11.4.2). It therefore seemed possible that Transport-P may be encoded by an unknown member of this group of molecules. The work which is described in this Section was therefore aimed to screen the GnRH neurone cDNA library for the presence of unknown cDNAs which may be homologous to the known vesicular amine

transporters.

There is significant homolgy in the sequences of the rat SVMAT and the rat CGAT cDNAs (Figure 11.9.13). The author designed two pairs of PCR primers which were based on the sequence of the rat SVMAT (Liu et al, 1992; Genbank accession number M97381). The primers were designed to be in nested positions; the outer primer pair was designated SVMATF8 and SVMATB12 and the inner pair was designated SVMATF17 and SVMATB26 (Figure 11.9.14). The inner primer pair was designed from a region of the SVMAT cDNA sequence in which there is homology with the sequence of the rat CGAT (Liu et al, 1992; Genbank accession number M97380; Figure 11.9.15). As hybridisation of the 3' ends of the primers to the target sequence is required for extension by the polymerase, the 3' ends of the inner primers were designed to terminate in the first or the second base of an amino acid codon which is conserved in the sequences of the rat SVMAT and rat CGAT (Figure 11.9.15). The primers were designed to be free of adenine and thymine at the 5' ends, as these nucleotides may interfere with TA cloning (Section 10.12). Details of the primers are shown in Table 11.9.5.

There were no specific products from reactions which used the outer primer pair (SVMATF8 and SVMATB12). Further, there were no specific products from nested reactions which utilised the outer primer pair to amplify the library in a first reaction, followed by a second reaction which used the inner primer pair (SVMATF17 and SVMATB26) to amplify the products of the first reaction. In contrast, reactions which used the inner primer pair to amplify the library generated a specific product of 255 bp which was thought to be of a size which is appropriate to the SVMAT. I cut this PCR product from the gel and purified it using the QiaexII kit (Figure 11.9.16; Section 10.10). It was then used as a template for further reactions which utilised the inner primer pair, resulting in a pure PCR product (Figure 11.9.16). Using the technique of TA cloning (Section 10.12), the product of this reaction was ligated to the pCR3.1 plasmid and two clones were sequenced using automated DNA sequence analysis. Surprisingly, the translated amino acid sequence was very similar to the rat CGAT sequence.

In order to obtain a full-sized clone, the cDNA library was screened by the procedure of hybridisation to bacterial pools of diminishing size (Section 10.14), using the SVMATF17 and SVMATB26 PCR product as a probe. *EcoR* I sites are present at each end of the

multiple cloning site of the pCR3.1 plasmid, but there are no *EcoR* I sites within the sequence of the insert. I detached the PCR product from the pCR3.1 plasmid by digesting the recombinant DNA with *EcoR* I. I then separated the plasmid from the insert in a 1.2% agarose gel. I cut out the 255 bp insert from the gel and extracted it with the QiaexII kit (Section 10.10), and then labelled the insert with ^{32}P using the method of random hexamers (Section 10.14.3). Plasmid pools were prepared from the library, each containing 12,800 bacteria. The bacteria were amplified overnight in the semi-solid medium to ensure representative amplification of the clones, and the plasmid DNA was then extracted from the bacteria using Qiagen kits (Section 10.10). One μg of the plasmid DNA was then digested with *Mlu* I to separate the pSVSPORT1 plasmid from the inserts and the reaction products were then electrophoresed in a 0.8% agarose gel (Section 10.14.2). The DNA was then transferred to nylon membranes which were hybridised to the 255 bp PCR product which had been labelled with ^{32}P. Figure 11.9.17 shows that one of the pools contained a hybridisation band of approximately 2500 bp. That pool was then divided into sub-pools of 1000 bacteria which were treated in an identical manner. Progressive cycles of pools of diminishing number resulted in amplification of the hybridisation signal, due to enrichment of the target molecule (Figure 11.9.17). An individual positive clone was then obtained from the sub-pool of 30 bacteria using the procedure of colony hybridisation (Section 10.14.5). The sequence of the clone, which was obtained by automated DNA sequence analysis, was almost identical to the rat CGAT DNA.

The clone was expressed in COS-7 cells by electroporation (Section 10.17). COS-7 cells which had been electroporated with the GnRH neurone CGAT accumulated greater amounts of [^{3}H]noradrenaline than control cells which had been electroporated with TE buffer (control: 16.1 ± 0.51 fmoles/mg protein; GnRH neurone CGAT: 44.32 ± 3.35 fmoles/mg protein). This confirmed that the isolated clone encodes the functional properties which are to be expected from the CGAT. However, there was no prazosin paradox in the COS-7 cells which had been electroporated with the GnRH neurone CGAT:

Electroporation	*Drug*	*Total cellular [^{3}H]prazosin (fmoles/mg protein)*
Control (TE buffer)	[^{3}H]prazosin 2x10^{-10} M	15.76 ± 0.30
	+ desipramine 10^{-5} M	13.14 ± 0.19
	+ unlabelled prazosin 10^{-6} M	13.31 ± 0.33
	+ unlab. praz. + desipramine	11.26 ± 0.11
GnRH neurone CGAT	[^{3}H]prazosin 2x10^{-10} M	17.34 ± 0.40
	+ desipramine 10^{-5} M	11.82 ± 0.11
	+ unlabelled prazosin 10^{-6} M	12.95 ± 0.41
	+ unlab. praz. + desipramine	10.67 ± 0.19

The data above indicate that Transport-P is unlikely to be encoded simply by presence of the CGAT in peptidergic neurones, and this conclusion is consistent with the previous findings that the functional properties of Transport-P are different from those of the vesicular amine transporters (Tables 11.4.2 and 11.5.2).

The synaptic vesicles of pre-synaptic (aminergic) neurones possess the SVMAT but there is no information on the transport molecules in peptideric neurones. The discovery of a molecule encoding a CGAT in peptidergic neurones was unexpected, as this molecule is normally located in the adrenal medulla. The discovery of CGAT in peptidergic neurones is relevant to this project: our previous work had indicated that peptidergic neurones are capable of accumulating small amounts of noradrenaline with high affinity (Al-Damluji et al, 1993). Initially, it was assumed that noradrenaline and prazosin may be accumulated via the same uptake process (Transport-P) in the peptidergic neurones. However, subsequent studies demonstrated that the chemical structure of noradrenaline makes it a very poor substrate for Transport-P; the phenolic hydroxyl groups and the β-hydroxyl group of noradrenaline reduce the affinity for uptake via Transport-P (Section 11.5.1). Thus, it was unclear how peptidergic neurones could accumulate noradrenaline. The discovery of CGAT in GnRH neurones provides a molecular mechanism for the accumulation of noradrenaline in the peptidegic neurones.

The significance of the uptake of noradrenaline in peptidergic neurones may be as follows: exposure of α_1 adrenoceptors to noradrenaline leads to desensitisation of the receptors (Wikberg et al, 1983; Bobik et al, 1984; Awaji et al, 1998). A post-synaptically located high affinity uptake process may serve the important function of removing noradrenaline from the vicinity of the receptors, thus maintaining the responsiveness of the post-synaptic receptors to repeated bursts of neurotransmitter released from the noradrenergic nerve teminals (Figure 7.4.8; Al-Damluji et al, 1993). In contrast, the pre-synaptic $Uptake_1$ noradrenaline transporter would presumably be less effective in removing noradrenaline from the vicinity of post-synaptic receptors, as it would have to rely on the slow diffusion of molecules of noradrenaline back across the synapse, against their concentration gradient. Clearly, a concentration gradient of transmitter molecules must exist across the synapse, otherwise forward diffusion would not take place; diffusion against a concentration gradient is energetically and kinetically unfavourable.

11.9.4 Cloning by homology with $Uptake_2$ and organic cation transporters

Previous investigators isolated a cDNA which encodes the noradrenaline $Uptake_2$ carrier in non-neuronal cells, using PCR primers which had been based on the sequences of organic cation transporters (Section 7.5.15). Although Transport-P is clearly different from the $Uptake_2$ transporter, it has some functional similarities to this family of transporters (Tables 11.4.2 and 11.5.2). It therefore seemed possible that Transport-P may be encoded by an unknown member of this group of molecules. The work which is described in this Section was therefore aimed to screen the GnRH neurone cDNA library for the presence of unknown cDNAs which may be homologous to the $Uptake_2$ or organic cation transporters.

Four sets of nested primers were designed on the sequence of the human $Uptake_2$ noradrenaline transporter (Grundemann et al, 1998; GenBank accession number AJ001417). The primers and their products covered much of the coding region of the $Uptake_2$ transporter cDNA. Details of the primers are shown in Table 11.9.6 and the positions of the primers in the $Uptake_2$ cDNA are shown in Figure 11.9.18. To increase the sensitivity and the specificity of the PCR, the primers were designed in nested positions (Figure 11.9.18; Section 10.11). Many reactions were performed, employing a range of temperatures, magnesium concentrations and DNA polymerase enzymes (AmpliTaq, *Pfu* polymerase and AmpliTaq Gold). However, no specific products were generated in any of the reactions,

using either conventional PCR or the nested method. PCR primers were also designed from a translated region of the rat organic cation transporter OCT1 in which there is homology with the sequence of the rat OCT2 transporter (Figure 11.9.19). The 3' ends were designed to terminate in the first or the second base of a conserved amino acid codon, in order to increase the chances of extension of the primer by the DNA polymerase. Two pairs of primers were designed for nested PCRs. In three of the primers, only one base was different from the sequence of the OCT2 cDNA, and in the fourth primer, (OCT1B3), 4 out of 23 bases were different from the OCT2 cDNA (Figure 11.9.19). The primers were designed to be free of adenine and thymine at the 5' ends, as these nucleotides may interfere with TA cloning (Section 10.12). Details of the primers are given in Table 11.9.7. These primers were designed by the author and were used by the author's trainee, David Housley to carry out PCRs using a protocol which was written by the author. The template was the cDNA library which had been prepared by the author. Several experiments were performed, using different reaction conditions, but no specific products were obtained, either in conventional PCRs or in nested reactions. It was therefore concluded that the GnRH neurone library is unlikely to contain a cDNA which is homologous to the $Uptake_2$ noradrenaline transporter. This is consistent with the conclusion that the functional properties and the structural requirements for ligand binding to Transport-P are distinguishable from those of $Uptake_2$ (Tables 11.4.2 and 11.5.2).

11.9.5 Expression cloning

The above work aimed to screen the GnRH neurone cDNA library for known or unknown members of the families of carriers which are known to accumulate biogenic amines. Two cDNAs were isolated, encoding SV2A and the chromaffin granule amine transporter. However, it appears that neither of these molecules is responsible for the functional properties of Transport-P. Therefore, expression cloning strategies have been devised, and one of these is being tested at the time of writing this Thesis.

Pools were prepared from the cDNA library, each containing approximately 1000 bacteria. The bacteria were amplified overnight in a semi-solid medium to ensure representative amplification of the clones (Section 10.14). Some of the bacteria from each aliquot were stored and the plasmid DNA extracted from the remainder using Qiagen kits (Section 10.10). The plasmid DNA from the pools was expressed in COS-7 cells by electroporation (Section

10.17). The GnRH neurone α_{1b} adrenoceptor cDNA was used as the positive control in the electroporation procedure. The negative control was the TE buffer in which the DNA had been dissolved. A positive pool was defined as one which causes increased accumulation of prazosin (at 10^{-6} M) which is reversible by desipramine 10^{-4} M. When a positive pool is found, a representative number of bacteria from that pool are subdivided into progressively smaller fractions, until the bacterium of interest is identified.

A mammalian cell contains about 300,000 RNA molecules and the library consists of 2.1×10^6 recombinants (Section 10.10). At the time of writing, 80 bacterial pools have been screened, representing a total of 49,600 bacteria (17% of the number of RNA molecules in a cell). Only one positive pool has been identified so far. The data from that experiment are shown in Table 11.9.8, in which the positive pool is designated "Pool 18". In general, cells which are electroporated with cDNA grow more slowly than cells which are electroporated with TE buffer, presumably due to the toxic effect of DNA on the growth of the COS-7 cells. As a result, cultures which are electroporated with cDNA contain less protein than those which are electroporated with TE buffer. When the uptake of prazosin is expressed as pmoles prazosin/mg protein, only pool 18 demonstrated increased uptake of prazosin in comparison to the controls (pool 18: 107.79 pmoles prazosin/mg protein; mean control 71 ± 4 pmoles prazosin/mg protein). The accumulation of prazosin in pool 18 is inhibited by desipramine. More pools are being prepared; if none shows greater accumulation of prazosin than pool 18, then this pool will be divided into sub-pools for screening in the same manner.

If the current expression cloning strategy does not result in identification of a positive clone, the author intends to utilise the BODIPY FL prazosin fluorescence microscopy method as a screening procedure. The advantage of the fluorescence microscopy method is that it enables detection of uptake in individual cells, which provides increased sensitivity. In this procedure, the cDNA library would be divided into pools of bacteria (see above), then each pool would be tested for the presence of a clone which encodes Transport-P, by expressing the cDNA sub-population in COS cells followed by microscopic examination for uptake of BODIPY FL prazosin. When a positive pool is identified, sub-pools will be prepared from that positive pool, containing smaller numbers of clones. Progressive cycles of pools containing diminishing numbers of clones will ultimately result in identification of an individual clone. Proton-dependent vesicular transporters have been expressed in COS cells

by electroporation and the transporter protein carried out its function of accumulating amines in these cells (Peter et al, 1994; Merickel & Edwards, 1995). Electroporation does not interfere with the function of Transport-P in peptidergic neurones (Section 10.17). The details of the procedure will be as follows:

1. Preparation of library pools and extraction of plasmid DNA: Pools containing 1000 bacteria will be prepared and amplified in semi-solid medium, to ensure representative amplification of the clones (Section 10.14). Half of each pool will be thawed and an aliquot of bacteria grown on LB ampicillin plates to calculate the number of bacteria in the pools after amplification. Plasmid DNA will be extracted using Qiagen kits (Section 10.10).

2. Electroporation of COS-7 cells: Cells will be dispersed to a density of 1.6 million/ml. 250 µl (400,000 cells) will be placed in each of 20 cuvettes. 5 µg DNA from a pool will be added to each cuvette and the cells electroporated (190 V, 1300 µF, 800 ohms). These parameters result in optimum expression of α_{1b} adrenoceptor cDNA in COS cells (Section 10.17). The cells from each electroporation will be grown in two culture dishes (200,000 electroporated cells/dish).

3. Fluorescence microscopy for Transport-P: 48 hours following electroporation, the cells will be incubated for 60 min at 37^0C in the presence of the fluorescent analogue BODIPY FL prazosin 1.77×10^{-7} M (Section 6). The duplicate culture dish will be incubated with BODIPY FL prazosin and desipramine to confirm that fluorescence is due to accumulation of BODIPY FL prazosin via Transport-P.

4. Subdivision of a positive pool: Sub-pools will be prepared from the pool corresponding to a positive culture dish, containing smaller numbers of clones. Progressive cycles using diminishing numbers of clones should result in identification of a positive clone.

5. Controls:

Positive controls:

A. Control for electroporation: GnRH neurone α_{1b} adrenoceptor cDNA in pSVSPORT1;

B. Control for Transport-P: GT1-1 GnRH neurones electroporated without DNA.

Negative control: COS-7 cells electroporated without DNA (non-specific uptake)

Definition of a positive plasmid pool: a culture dish containing cells which accumulate BODIPY FL prazosin, inhibited by desipramine.

In GT1-1 cells non-specific uptake (uptake in the presence of desipramine) was usually 18% and specific (desipramine-sensitive) uptake was therefore 82% (Table 11.7.1).

6. Calculation of expected results:

Each culture dish contains 200,000 electroporated cells

Cell survival following electroporation at the above parameters = 50% = 100,000 surviving cells/dish

Minimum transfection efficiency = 10% = 10,000 transfected cells/dish

If a pool of 1000 clones contains only one positive clone, there would be:

10,000 cells/1000 clones = 10 positive (fluorescent) cells/dish

Figure 11.9.1:

Polymerase chain reactions using primers which are based on the sequence of the SV2A cDNA (SV2F15 and SV2B22 primer pair). Details of the primers are in Table 11.9.1. The template was the GnRH neurone cDNA library (0.4 μg/reaction) and the primers were used at concentrations of 1 μM. Magnesium chloride concentration was 1.5 mM and each reaction included AmpliTaq DNA polymerase 2.5 Units and dNTPs at 200 μM each. The reaction conditions were as follows: denaturing at 95^{0}C for 1 minute; annealing at 58^{0}C for 1 minute; extension at 72^{0}C for 2 minutes. The reactions were performed as "hot start" using Ampliwax beads. The photograph shows an ethidium bromide stained 2% agarose gel. The reaction in lane 1 contained all the components which are listed above; in lane 2, the downstream primer (SV2B22) was excluded; in lane 3, the upstream primer (SV2F15) was excluded; in lane 4, the template was excluded. In lane 1, there is a specific product of 407 bp which cannot be generated in the absence of either primer or in the absence of template.

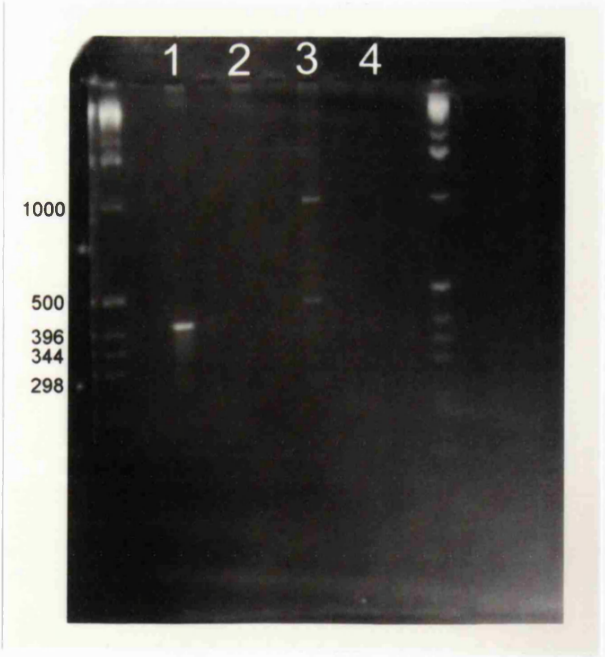


Figure 11.9.2:

Screening the GnRH neurone library for the SV2A cDNA:

The cDNA library consists of $2x10^6$ recombinants. The library was divided into pools, each containing 149,000 bacteria. The bacteria were amplified in semi-solid medium and plasmid DNA was extracted and digested to separate the plasmid from the insert. One µg of digested DNA from each pool was then run in an agarose gel and transferred to a nylon membrane. The SV2F15/SV2B22 PCR product was labelled with ^{32}P and used as a hybridisation probe. In pool 74, there is a faint hybridisation band of 3500 bp. Sub-pools were prepared from that positive pool, each containing 42,000 bacteria and treated in the same manner. In sub-pool 16, the same hybridisation band is seen but is now amplified, due to enrichment of the target molecule. The procedure was repeated using further sub-pools containing diminishing numbers of clones, resulting in progressive amplification of the signal. An individual, positive clone was eventually obtained.

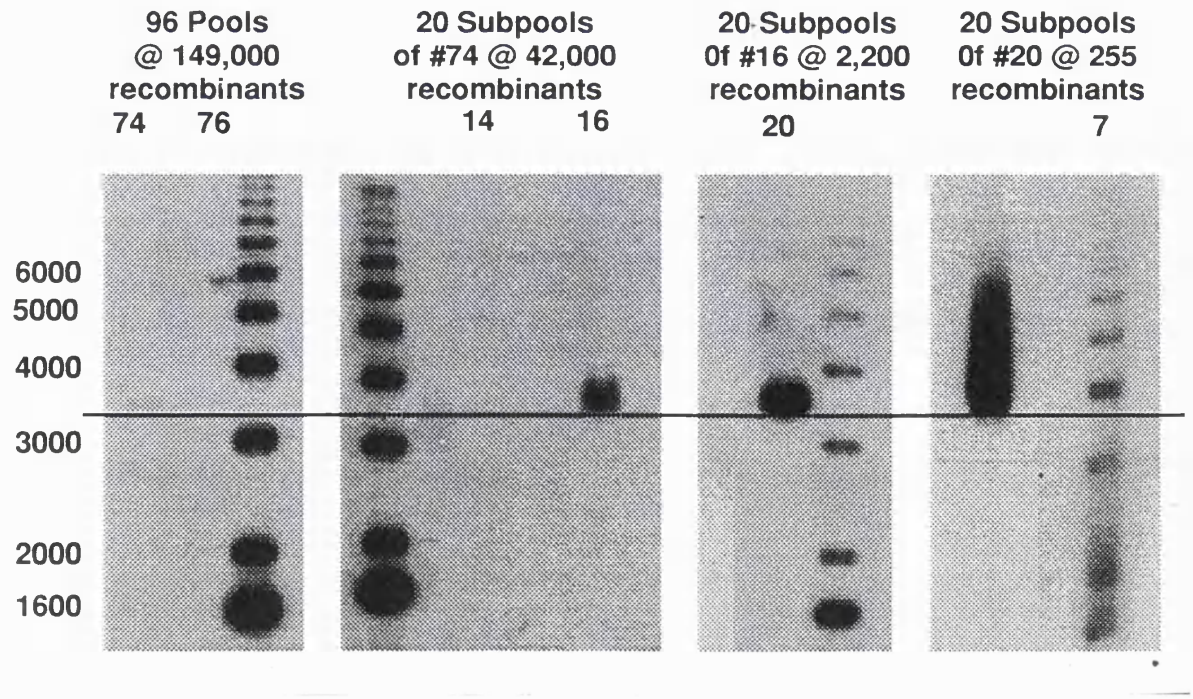


Figure 11.9.3:

Arrangement of the sequencing reactions which were used to sequence the GnRH neurone SV2A cDNA. The insert was sequenced manually on both strands, as indicated by the directions of the arrows.

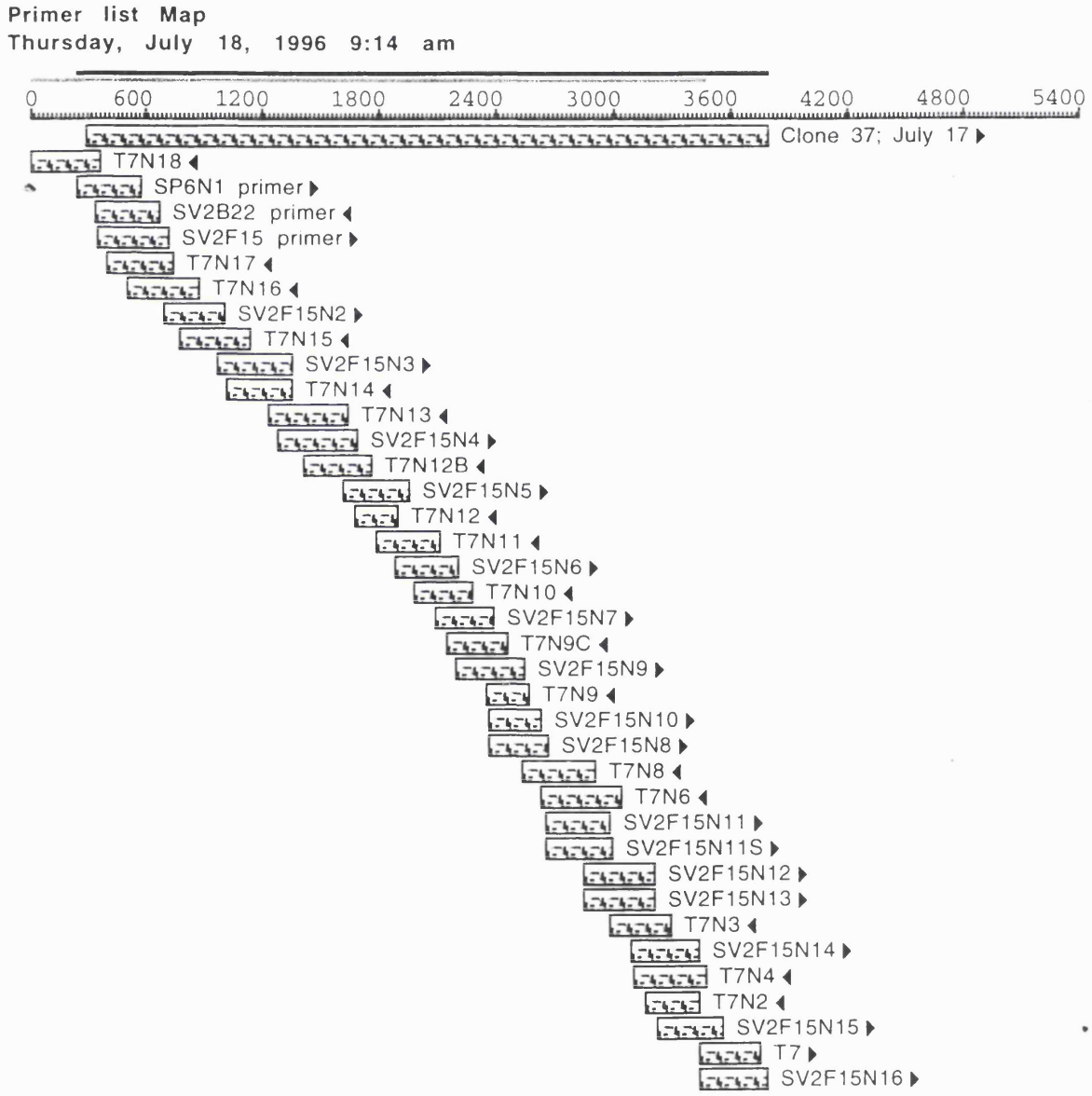


Figure 11.9.4: Sequence of the SV2A clone which was obtained by screening the library. The 5' end is missing and this was later obtained by 5'RACE. There is a 384 bp intronic segment (underlined). Also shown are the positions of the *NgoM* I and *Dra* III sites and the PCR primers which were used to excise the intron and replace it appropriately.

```
   1 CATGGCCGCT TCCAGTGGAC ACTCTACTTC GTGCTGGGTC TGGCGCTGAT GGCAGATGGT
  61 GTAGAGGTCT TTGTGGTGGG CTTTGTGCTG CCCAGTGCTG AGAAAGACAT GTGCCTGTCG
 121 GACTCCAACA AAGGCATGCT AGGCCTCATT GTGTACCTGG GCATGATGGT GGGGGCCTTC
 181 CTCTGGGGAG GCCTGGCTGA TCGGCTGGGT CGGAGACAGT GTCTGCTCAT CTCACTCTCA
 241 GTCAACAGCG TCTTCGCCTT CTTCTCGTCC TTCGTCCAGG GTTATGGAAC CTTTCTCTTC
 301 TGCCGCCTCC TTTCCGGGGT CGGGATTGGC GGTTCCATCC CCATAGTCTT CTCCTATTTT
 361 TCGGAGTTTC TGGCGCAGGA GAAACGTGGG GAGCATTTGA GCTGGCTCTG TATGTTCTGG
 421 ATGATCGGTG GAGTGTATGC AGCTGCAATG GCCTGGGCCA TCATCCCTCA CTATGGGTGG
 481 AGTTTCCAGA TGGGCTCTGC TTACCAGTTC CACAGCTGGA GGGTGTTTGT CCTCGTGTGT
 541 GCCTTTCCCT CTGTGTTTGC CATCGGGGCT CTGACTACGC AGCCAGAGAG TCCCCGCTTC
 601 TTCCTAGAGA ATGGGAAGCA TGACGAAGCC TGGATGGTAC TGAAGCAGGT TCACGACACC
 661 AACATGCGAG CCAAGGGCCA CCCTGAGCGC GTCTTCTCAG TGACCCACAT TAAAACGATT
 721 CATCAAGAGG ATGAATTGAT TGAGATCCAG TCTGACACAG GAACCTGGTA CCAGCGCTGG
 781 GGAGTACGGG CTTTGAGCCT TGGGGGTCAG GTTTGGGGGA ATTTCCTCTC CTGCTTCAGT
 841 CCAGAGTATC GGCGCATCAC GCTGATGATG ATGGGTGTGT GGTTCACCAT GTCTTTCAGC
 901 TACTATGGTT TGACTGTCTG GTTTCCCGAC ATGATCCGCC ATCTCCAGGC TGTGGACTAT
 961 GCAGCCCGAA CCAAAGTGTT CCCAGGGGAG CGCGTGGAGC ATGTGACGTT TAACTTCACA
1021 CTGGAGAATC AGATCCACCG AGGGGGACAG TACTTCAATG ACAAGTTCAT CGGGCTGCGT
1081 CTGAAGTCAG TGTCCTTTGA GGATTCCCTG TTTGAGGAGT GTTACTTTGA AGATGTTACA
1141 TCCAGCAACA CATTCTTCCG CAACTGCACG TTCATCAACA CTGTGTTCTA TAACACTGAC
1201 CTATTTGAGT ACAAGTTCGT GAACACCCGG CTGGTGAACA GCACGTTCCT GCACAATAAG
1261 GAAGGCTGCC CGCTAGACGT GACGGGGACA GGCGAAGGTG CCTACATGGT GTACTTTGTC
1321 AGCTTCTTGG GGACACTGGC TGTGCTTCCT GGAAACATTG TGTCTGCTCT GCTCATGGAC
1381 AAGATTGGCA GGCTCAGAAT GCTTGCTGGT TCCAGTGTGT TGTCCTGTGT GTCCTGCTTC
1441 TTCCTGTCTT TTGGGAACAG CGAGTCAGCC ATGATCGCTC TGCTCTGCCT TTTTGGGGGA
1501 GTTAGCATCG CATCCTGGAA CGCGCTGGAC GTGCTGACCG TTGAGCTCTA CCCTTCCGAC
1561 AAGAGGTAGT TTGGAGTGTG GTGGGCGAGC AGGGGAGGAG CTACAGGACT GGGGAGAAGC
1621 GAGGAAGAGA GCTGAGAGAA TATCCTTAGG GAAGAGAATA GGGTGGAGGA TAAGGATGTT
1681 GTTGGGGTAG GACACACATC CATGCTGAGC TTTTCCTTAA CAGCTGGGAG CTTTTGTCTT
1741 CACTTCTGGA ACTGCTAATT ACTAACCTCT GCTTCTGTCT CCTTTCTCTC TTGTCTCCCA
1801 ACGTGTCTGT TGCCCCACCC CCTCCTGTCA CTTCTCCATC CTTCCTTCCC GCTCCCGCAC
1861 ACTGTTTTCC TCTGACCCCA CTTTGCCTTC CCACCTGCCC ATCTTCCCTC TTCTGGACCG
1921 CCACCGCGCC GTGGCTTGGC ACTGAGCAGG ACTACTGCCT TTGGCTTCCT GAATGCCCTG
1981 TGTAAGCTGG CAGCTGTGCT GGGGATCAGC ATCTTCACAT CCTTTGTGGG CATCACCAAG
2041 GCTGCTCCCA TTCTCTTTGC CTCGGCTGCT CTTGCCCTTG GTAGCTCTCT GGCTCTGAAG
2101 CTGCCTGAGA CCCGGGGACA GGTGCTGCAG TGAGGGATGG GGGCTGTCTC AGGGGCTTTA
2161 GGGATGGCAG GCACACTGTG AGACCAATAA TTCCTTTTAT CCCTACCCTG CCCTGCTGTC
2221 CTGGTCCTCA CTCCTGTGTT TGGTGTCTTA GCTGTGTGTG CCTGTGTGCA TGTGTGTGAC
2281 CCTGATGGGC AGGGACTACA GGGACGGTCC CTTCGTTCCA CTTTTGGGAT GAGGTACTCC
2341 TTACCTGCTG CCACCCTCAA CTTTGCACAA GGAGAAGGCT GCGCTGCATC CTTCTCTCCC
2401 CCAGTGTTAG CAGGGGGGCT GTTTCCCTGC TCCGAGGGTT CCGGAACTTC TGCCTTCCCC
2461 TTCTTTCCCT CCGCCTAGGC CCTGGTGAAC CACAGGTATG GAGTTATAAT GGGGGCTGAG
2521 GCTTGGACCA AAAGAACTTC TTGAGTGGGA AGGCCTTGGG TGCCCTCTAG CCTCCCAAGG
2581 ATGCTGGGGA GTAGCAATAA ACCTCAGCCC TCTGGCCACC ATTTTCACCT CAATTCAGGC
2641 TACAAGTGTG AAGCCTGGAT TTAATTTATG GAATTAGTTT TCTGAGTTTT ATTTATATGT
2701 AAGTTCTGAG GCAGCTTAGC TGGACTGTGT GTGGATGTAT ACATACACTC GTGTGTGTGC
2761 GTGCGCATGT GTGTGTGTGT GTACCATGGG GTAGGGGTAC CACTCTACTG TCTAATATAA
2821 GCCAAGAGTA GTAGTTTCAG TGAACACACA CAACACTGTT TTTCTATAGT ACCTCCCAGA
2881 ATCTTGTATC TGTGCTGGGG CTGGAGGCAG AACCCCTAGG CCAGGCTGGG ATGAGTCCTG
2941 CAATCTTGGG GGACCTGAGG GCACCTGACA AGGACTCACT CTCTCCTTGC TCTAGAGAGG
3001 TTCTACCCAC TAGCCACAGC CCTCCATCTG ACCTGTTCAC ACAGGCAGTG GACCAGAGGA
3061 AAGAAAGGGA GAATAACCAG GCACGTATGG TCAAACCAGC AGGTCCGAAA GCACAAGGAG
3121 CTGGGGCAGG AAGCAGGGGT CCGTCCCTAA CCCTTCTCAA AAAGGCTGGG TCGTGAGGGA
3181 CCCCTAATGC AGGGACCAGA AGCCTCAGTT TCCCCGTTTT GCCCTTCCAC AGAACAGCCT
3241 TGTAGGTAGA GCTGCCCCCG TCCTACCCTA CTCTGTGTGG CTGCTTTCTT TGGTACTCTC
3301 TCCCCACTCC CAACGTAGCT GTGACGTGTT GTAGTTTTTA GCTGTTTGTA AAATGTTAAA
3361 AAAAAAAAAA GTTAAAAGGA AGTTAAACTA ACCACAAAAA AAAAAAAAAA
```

C37 F1 — Ngo MI — 384 bp INSERT — C37 F23 — C37 B21 — Dra III — C37 B1 — STOP

Figure 11.9.5: the intronic segment in the SV2A clone

A. RNA PCR: the reverse transcription was at 60^0C and utilised 250 ng GnRH neurone RNA, the C37B1 primer (1 μM), rTth DNA polymerase/reverse transcriptase 5 Units, $MnCl_2$ (1 mM) and dNTPs (200 μM). The first PCR utilised rTth, $MgCl_2$ (2.5 mM), the C37F1 and C37B1 primer pair and the other components as above. Reaction conditions were: denaturing: 95^0C for 10 sec; annealing and extension: 63^0C for 15 sec. The second (nested) PCR utilised as template 10 μl of the first reaction product, AmpliTaq DNA polymerase 2.5 U, the C37F23 and C37B21 nested primer pair (1 μM each), $MgCl_2$ (1.5 mM) and dNTPs (200 μM). Reaction conditions were: denaturing: 95^0C for 30 sec; annealing: 60^0C for 30 sec; extension: 72^0C for 1 min. The reaction in lane 1 contained all the components listed above; in lane 2, the downstream primer (C37B21) was excluded; in lane 3, the upstream primer (C37F23) was excluded; in lane 4, the template was excluded. B. PCR using the GnRH neurone library as template and the C37F23 and C37B21 primer pair. The reaction conditions were as described above, except that $MgCl_2$ was at 2.5 mM.

In the RNA PCR (A), the only specific product is 220 bp, corresponding to clone 37 without the 384 bp intronic segment. The 220 bp product cannot be generated in the absence of either primer or in the absence of template. In the library DNA PCR (B), the predominant product was also 220 bp; however, a 604 bp product was also generated in a smaller amount, corresponding to clone 37 with the 384 bp intronic segment.

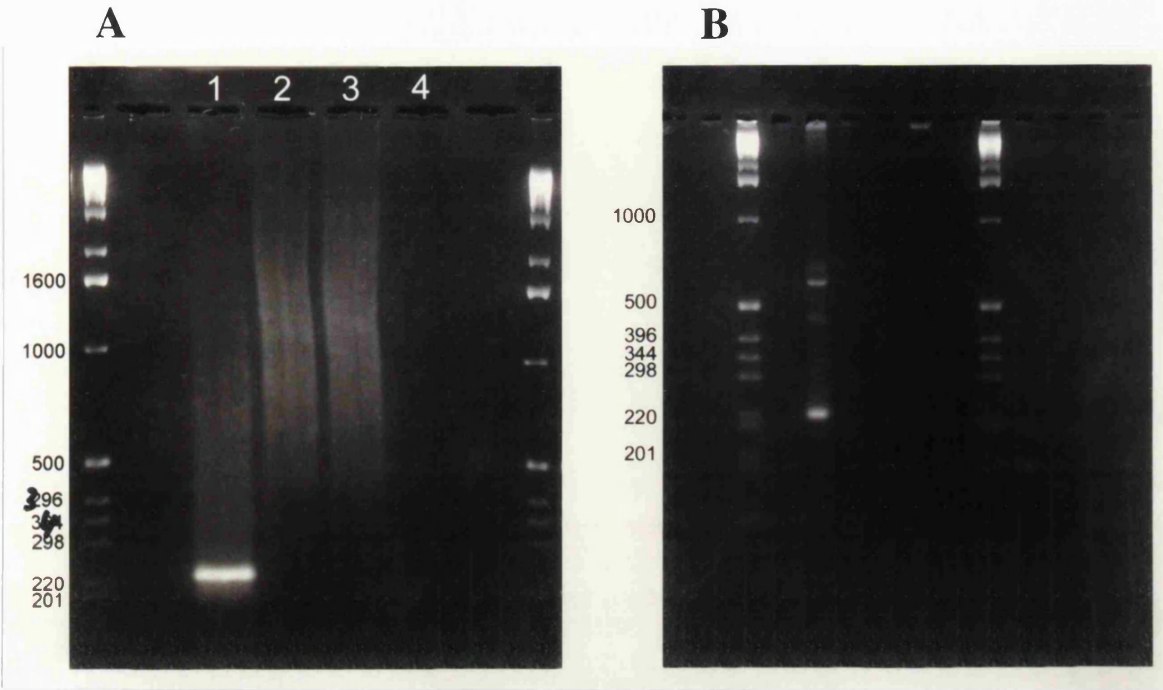


Figure 11.9.6: removing the intronic segment in the SV2A clone

A. Lane 1 shows the undigested 830 bp segment which does not contain the intron, generated using the C37F1 and C37B1 PCR primer pair. Lane 2 shows the same DNA following digestion with *NgoM* I and *Dra* III. Lane 3 shows clone 37 DNA digested with *NgoM* I and *Dra* III.

B. The appropriate pieces of DNA were cut from the gel; these DNA fragments were then purified and ligated.

C. Testing the DNA from the ligates by PCR with the C37F1 and C37B1 primer pair. Lanes 1-10 contain PCR amplifications of DNA from 10 ligate minipreps, all of which have the 830 bp segment, without the intron. Lane 11 contains PCR amplification of DNA from the 830 bp segment as a control, demonstrating that the PCR products in lanes 1-10 are of appropriate size. Lane 12 contains PCR amplification of DNA from clone 37, demonstrating the larger 1213 bp PCR product which contains the intron.

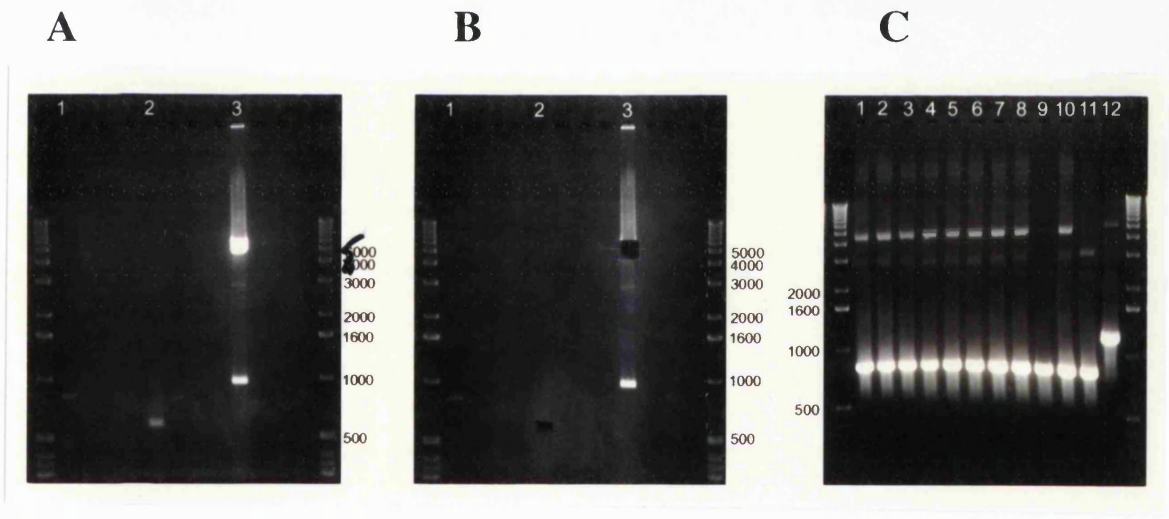


Figure 11.9.7:

Outline plan of 5'RACE experiment to obtain the missing 5' end in the SV2A clone. Details are described in the text.

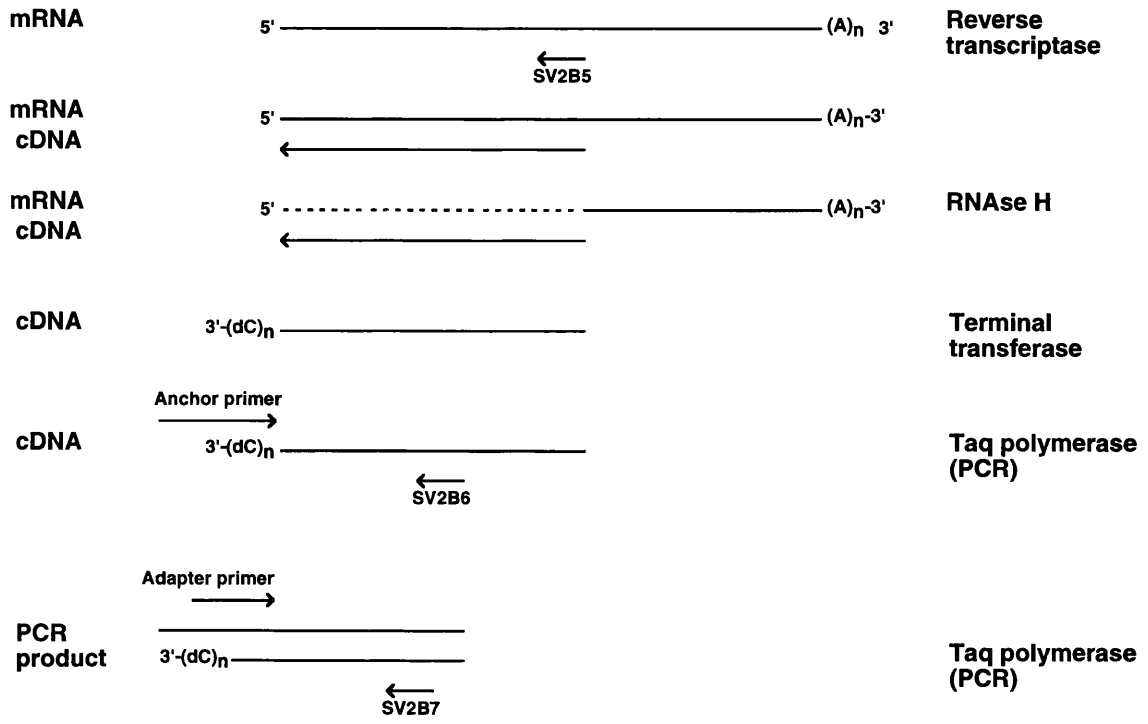


Figure 11.9.8:

Translated sequence of SV2A which was obtained from GnRH neurones. The sequence is identical to the sequences which were obtained by previous investigators. Membrane spanning regions are underlined. The dashed lines indicate regions which are conserved in transporters for sugars, citrate and tetracycline. The sites for asparagine-linked glycosylation on the luminal side are marked by arrows. The consensus sites for ATP binding motifs are marked "+". The basic amino acid, arginine, before the 6th membrane-spanning region is conserved in all proton symporters (marked by an asterisk).

MEEGFRDRAAFIRGAKDIAKEVKKHAAKKVVKGLDRVQDEYSRRSYSRF 49

EEEDDDDDFPAPADGYYRGEGAQDEEEGGASSDATEGHDEDDEIYEGEY 98

QGIPRAESGGKGERMADGAPLAGVRGGLSDGEGPPGGRGEAQRRKDREE 147

LAQQYETILRECGHGRFQWTLYFVLGLALMADGVEVFVVGFVLPSAEKD 196

MCLSDSNKGMLGLIVYLGMMVGAFLWGGLADRLGRRQCLLISLSVNSVF 245

AFFSSFVQGYGTFLFCRLLSGVGIGGSIPIVFSYFSEFLAQEKRGEHLSWLC 297

MFWMIGGVYAAAMAWAIIPHYGWSFQMGSAYQFHSWRVFVLVCAFPSV 345

FAIGALTTQPESPRFFLENGKHDEAWMVLKQVHDTNMRAKGHPERVFSV 394

THIKTIHQEDELIEIQSDTGTWYQRWGVRALSLGGQVWGNFLSCFSPEYRR 445

ITLMMMGVWFTMSFSYYGLTVWFPDMIRHLQAVDYAARTKVFPGERVE 493

HVTFNFTLENQIHRGGQYFNDKFIGLRLKSVSFEDSLFEECYFEDVTSSNTF 545

FRNCTFINTVFYNTDLFEYKFVNSRLVNSTFLHNKEGCPLDVTGTGEGAY 595

MVYFVSFLGTLAVLPGNIVSALLMDKIGRLRMLAGSSVLSCVSCFFLSFGN 646

SESAMIALLCLFGGVSIASWNALDVLTVELYPSDKRTTAFGFLNALCKLAA 697

VLGISIFTSFVGITKAAPILFASAALALGSSLALKLPETRGQVLQ* 742

Figure 11.9.9: The SV2A cDNA in pcDNA3.1(+). The cloning sites and the plasmid regulatory regions are intact.

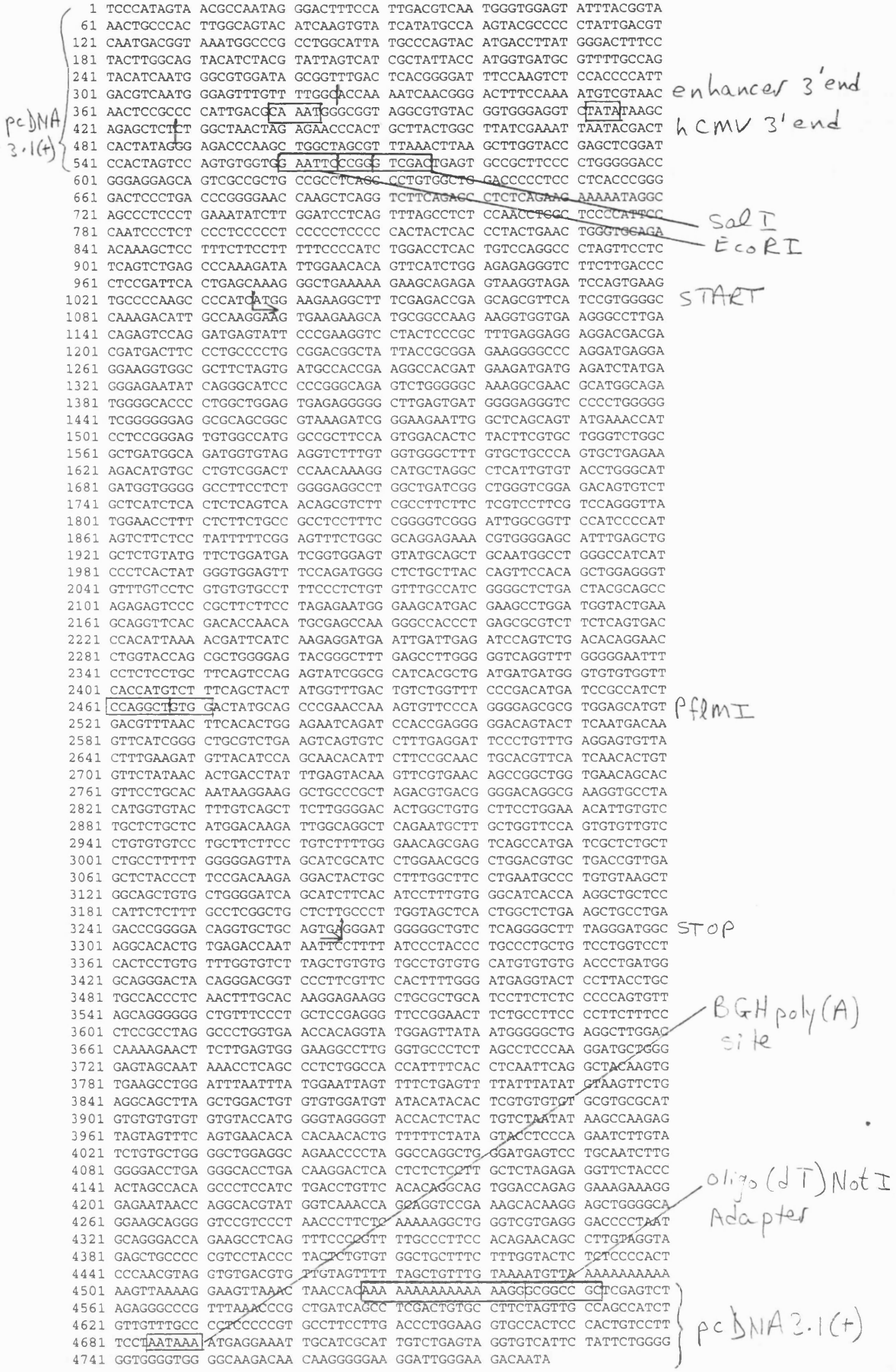
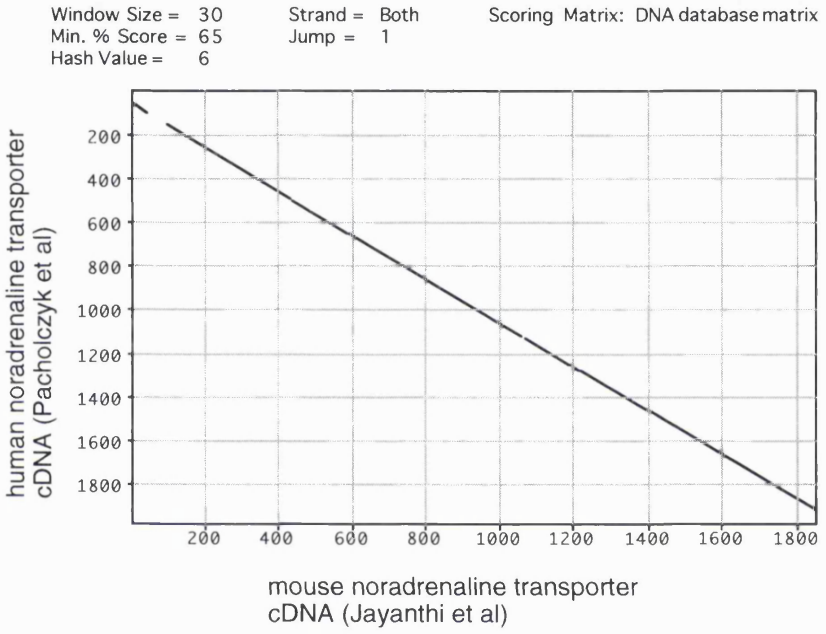


Figure 11.9.10:

Comparison of the sequences of cDNAs which encode the plasma membrane mouse noradrenaline transporter (Jayanthi et al, Genbank accession number NM009209), the human noradrenaline transporter (Pacholczyk et al, 1991; Genbank accession number M65105) and the rat dopamine transporter (Kilty et al, 1991; Genbank accession number M80233). The comparisons were done using the Pustell DNA matrix which is available as a function in the MacVector sequence analysis software on a Macintosh computer. The parameters which were used are indicated and were identical in both comparisons.



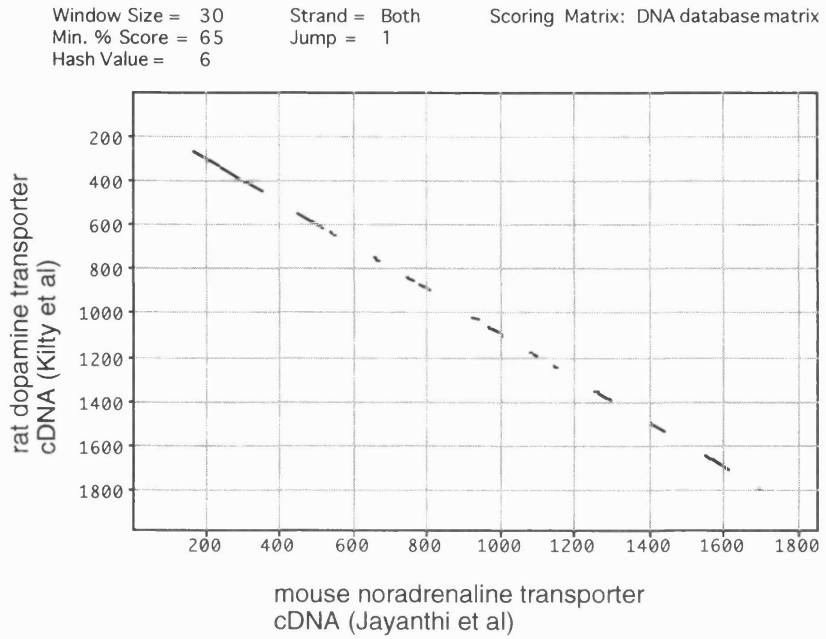


Figure 11.9.11:

Positions of the nested PCR primers in the translated cDNA sequence of the mouse plasma membrane noradrenaline transporter (Jayanthi et al, Genbank accession NM009209).

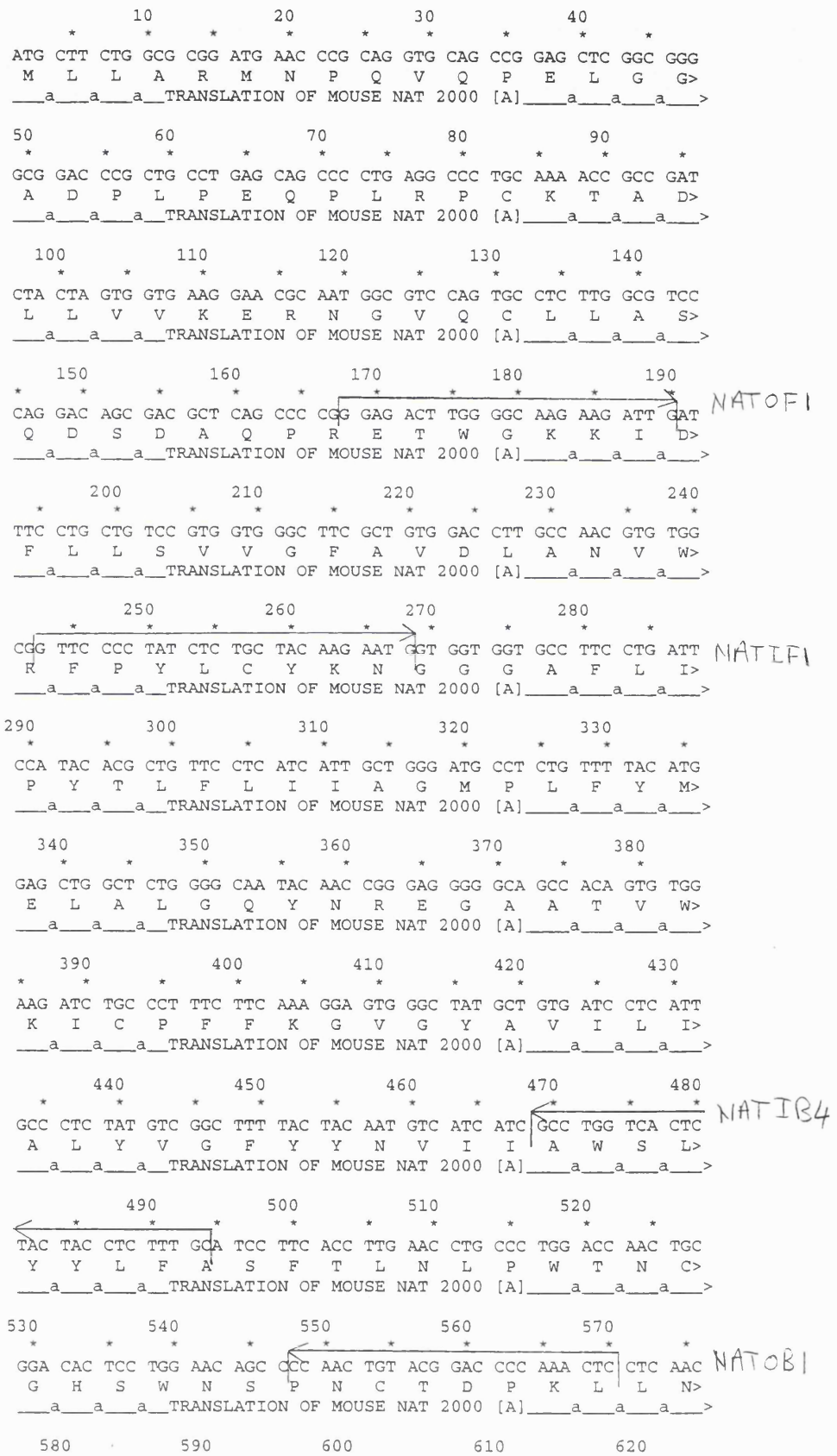


Figure 11.9.12:

Positions of the nested PCR primers in the cDNA sequences of the mouse noradrenaline transporter (Jayanthi et al, Genbank accession NM009209) and the human noradrenaline transporter (Pacholczyk et al, 1991; Genbank accession M65105). In order to increase the chances of detecting a product, the 3' ends of the primers were designed to terminate in the first or the second base of an amino acid codon which is conserved in the human and mouse noradrenaline transporters and the rat dopamine transporter (see Section 10.11).

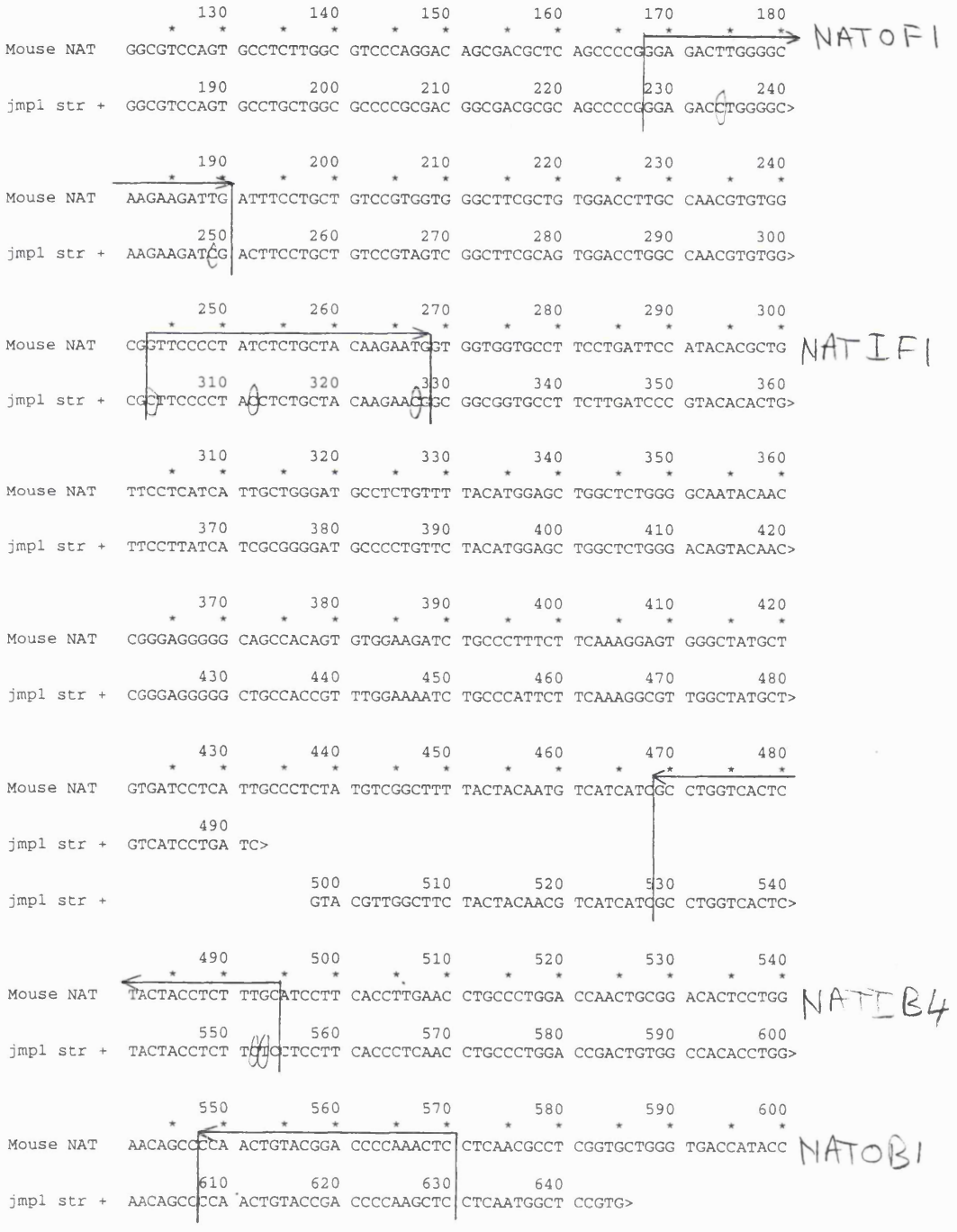


Figure 11.9.13:

Comparison of the sequences of cDNAs which encode the rat synaptic vesicle monoamine transporter (SVMAT; Liu et al, 1992; Genbank accession number M97381) and the rat chromaffin granule amine transporter (CGAT) of the adrenal medulla (Liu et al, 1992; Genbank accession number M97380). The comparisons were done using the Pustell DNA matrix in the MacVector sequence analysis software on a Macintosh computer. The parameters which were used are indicated.

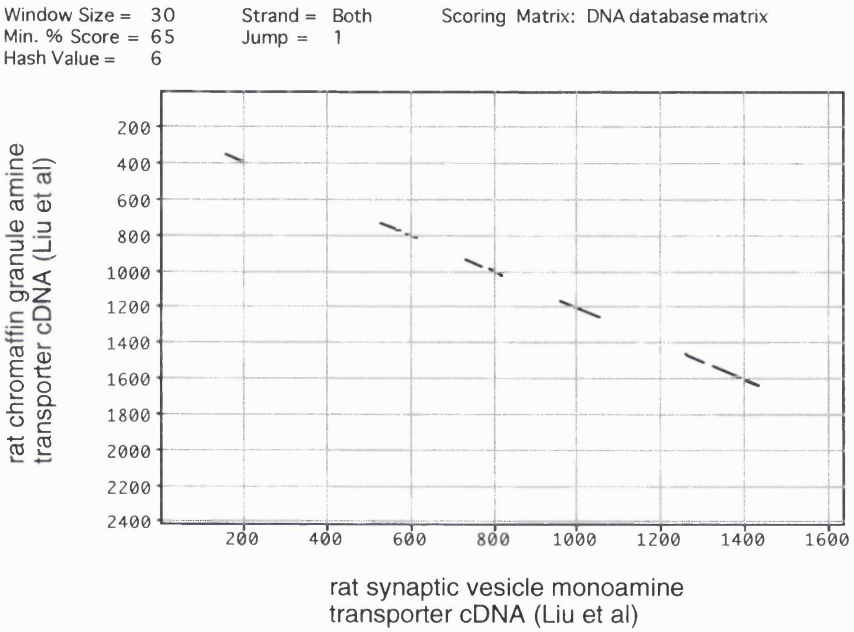


Figure 11.9.14:

Positions of the nested PCR primers in the sequence of the rat synaptic vesicle monoamine transporter (SVMAT; Liu et al, 1992; Genbank accession number M97381).

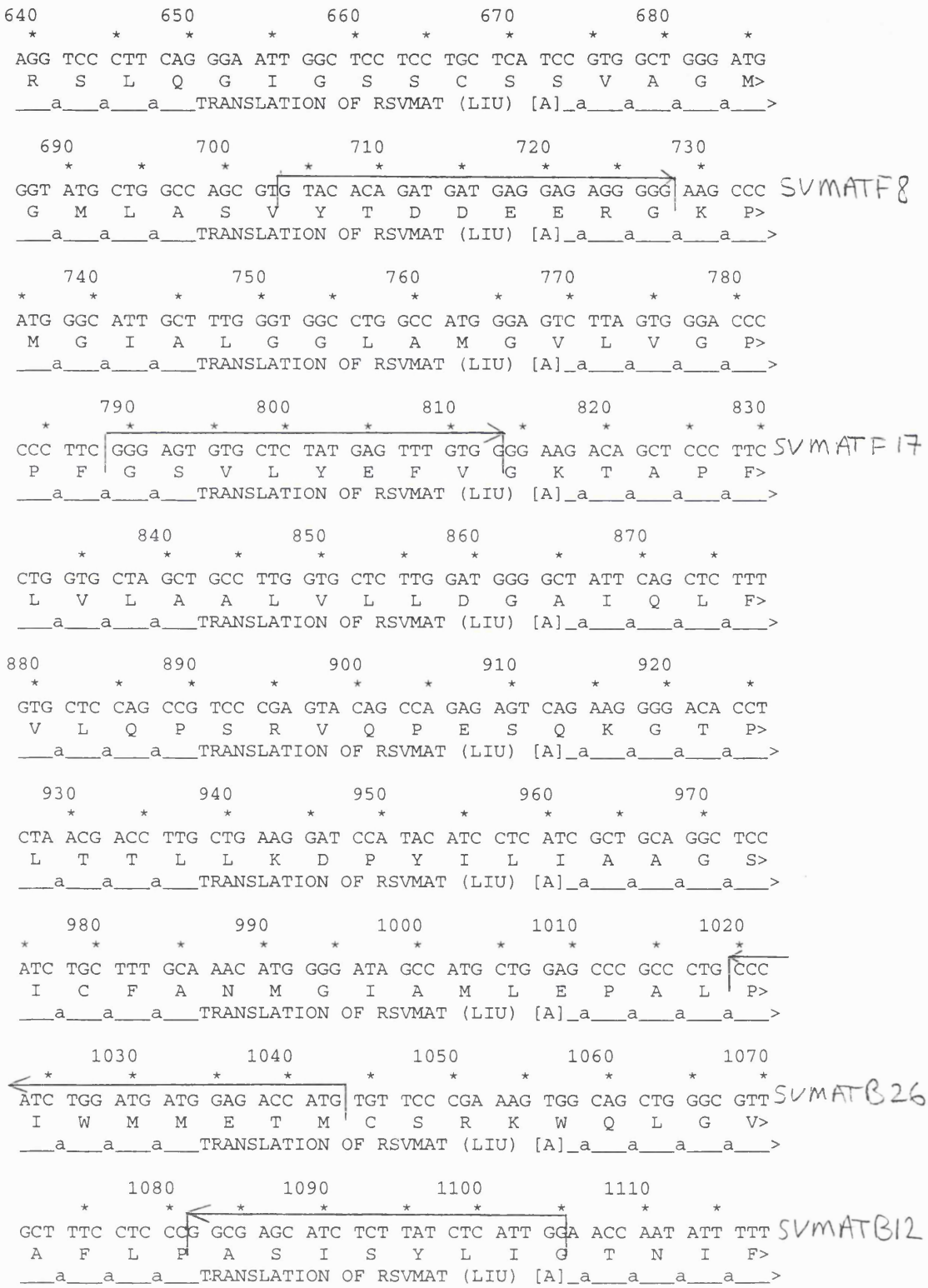


Figure 11.9.15:

Positions of the nested PCR primers in the cDNA sequences of the rat synaptic vesicle monoamine transporter (SVMAT; Liu et al, 1992; Genbank accession number M97381) and the rat chromaffin granule amine transporter (CGAT; Liu et al, 1992; Genbank accession number M97380). The inner primer pair was designed from a region of the SVMAT cDNA sequence in which there is homology with the sequence of the rat CGAT. In order to increase the chances of detecting a product, the 3' ends of the inner primers were designed to terminate in the first or the second base of an amino acid codon which is conserved in the SVMAT and CGAT sequences (see Section 10.11).

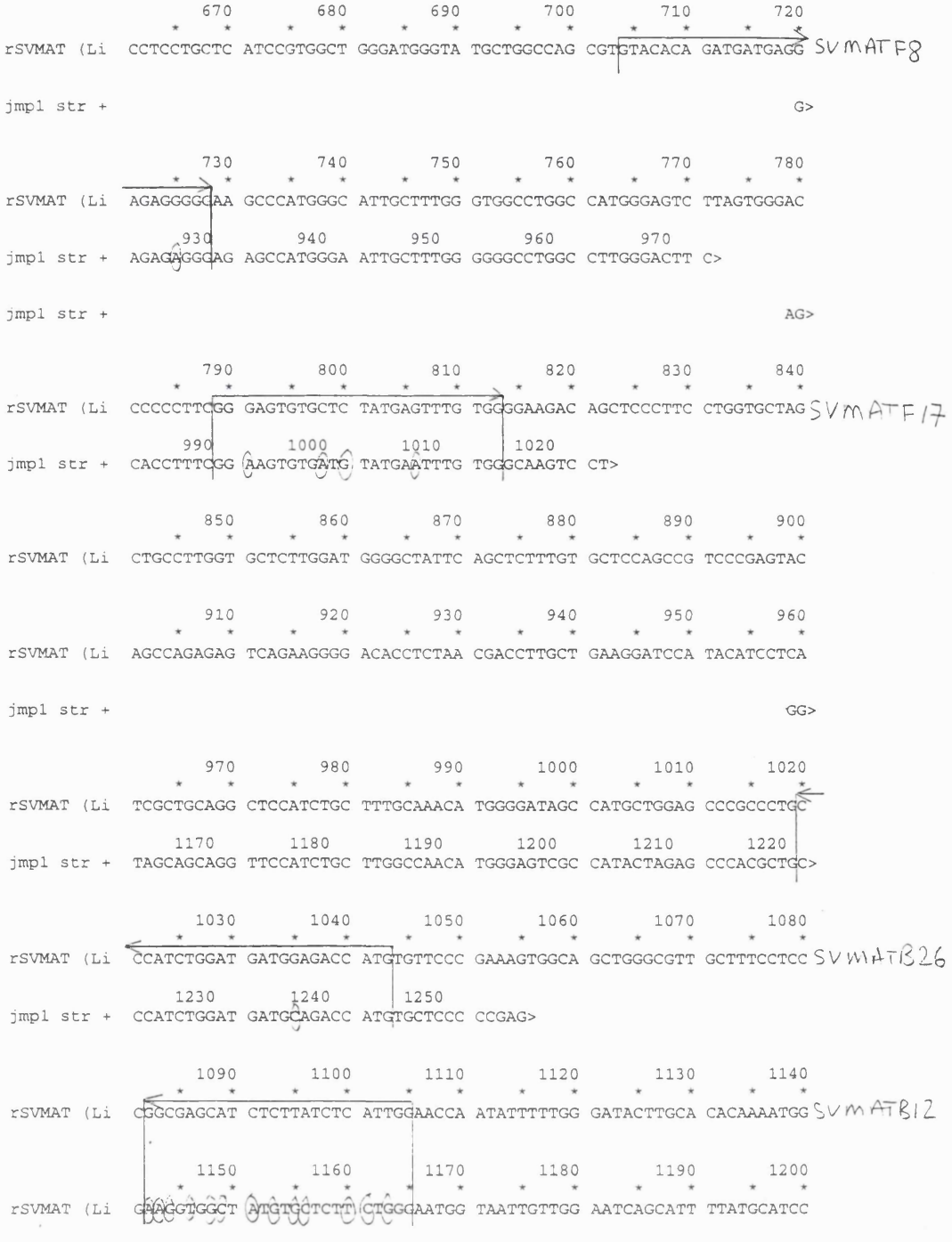


Figure 11.9.16:

Polymerase chain reactions using primers which are based on the sequence of the SVMAT. In A, each reaction contained the GnRH neurone cDNA library (0.5 µg) as template, the SVMATF17 and SVMATB26 primer pair (1 µM each), $MgCl_2$ (2.5 mM), dNTPs (200 µM), PCR buffer II and AmpliTaq DNA polymerase (2.5 U). The reactions were performed as "hot start" using AmpliWax. The total volume was 75 µl. Reaction conditions were: denaturing at 95^0C for 30 seconds; annealing at 57.8^0C for 30 seconds; extension at 72^0C for 60 seconds; 35 cycles. A PCR product of 255 bp is present in small amounts in each lane and there is much smearing due to the presence of large amounts of non-specific products. The 255 bp PCR product was cut from the gel (B) and purified from the agarose using glass beads. The purified PCR product was then used as a template for a second PCR which utilised the same primer pair, resulting in a pure PCR product (C). The reaction in panel C was performed by the author's trainee, David Housley, according to a protocol which had been written by the author. The PCR primers were designed by the author.

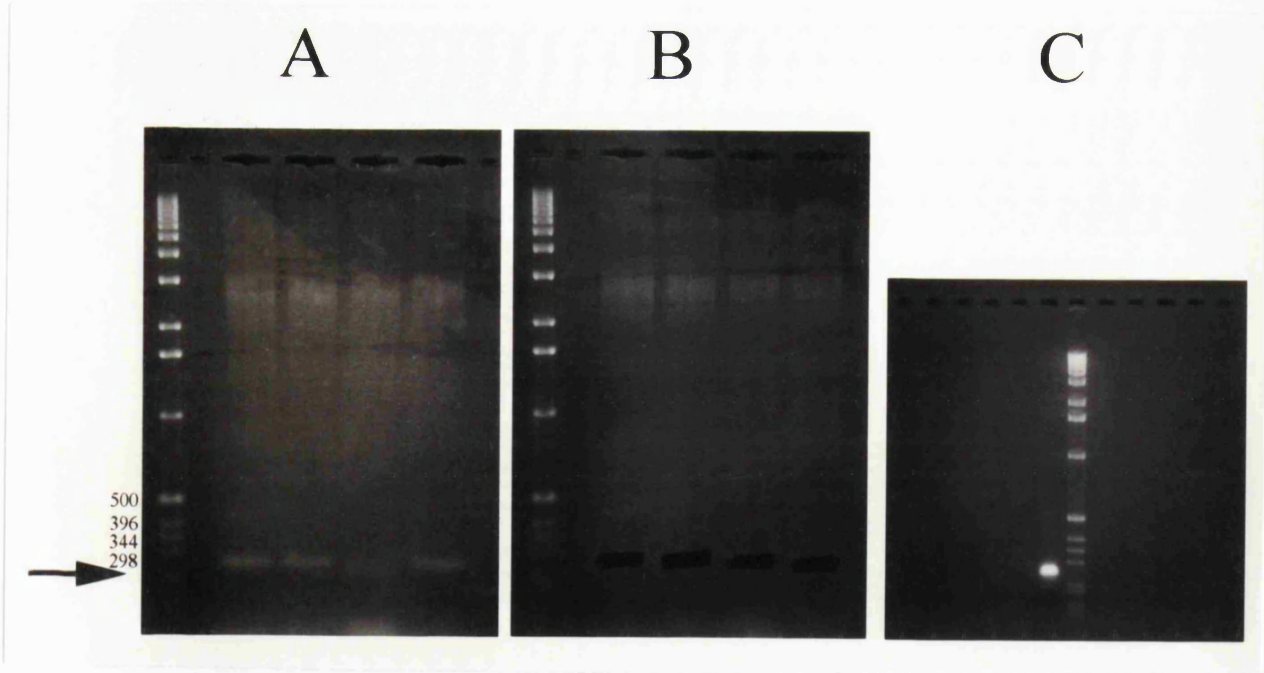


Figure 11.9.17: Screening the library for the GnRH neurone CGAT cDNA

The library was divided into pools, each containing 12,800 bacteria. The bacteria were amplified and DNA extracted and digested to separate the plasmid from the insert. One μg of digested DNA from each pool was run in a gel and transferred to a membrane. The SVMATF17/SVMATB26 PCR product was labelled with ^{32}P and used as a probe. One of the pools was positive, demonstrating a hybridisation band of 2500 bp. Sub-pools were prepared from that positive pool, each containing 1,000 bacteria and treated in the same manner. One of the sub-pools was positive, demonstrating the same hybridisation band which was now amplified, due to enrichment of the target molecule. The procedure was repeated using further sub-pools containing diminishing numbers of clones, resulting in progressive amplification of the signal. Finally, colony hybridisation was used to obtain an individual, positive clone. The procedures in A and B were performed by the author's trainee, David Housley, according to a protocol which had been written by the author.

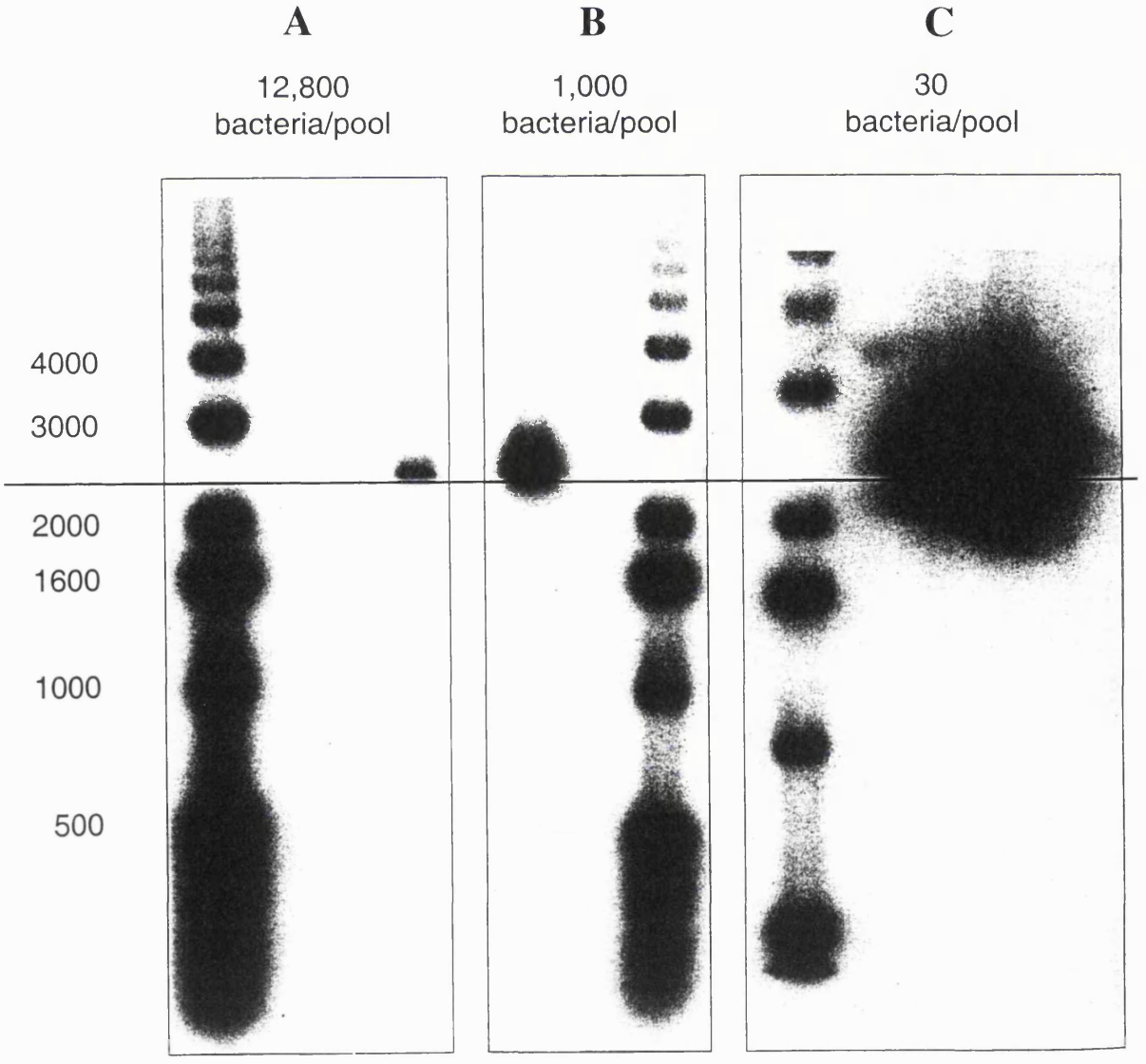


Figure 11.9.18:

Positions of the nested PCR primers in the sequence of the human Uptake$_2$ noradrenaline transporter (Grundemann et al, 1998; GenBank accession number AJ001417).

```
START    1 CTGCGGGCGG CGGGCGGCGG GCGCACCATG CCCTCCTTCG ACGAGGCGCT GCAGCGGGTG
        61 GGCGAGTTCG GGCGCTTCCA GAGGCGCGTG TTTTTGCTGC TGTGCCTGAC GGGCGTCACC
       121 TTCGCCTTCC TCTTCGTCGG CGTGGTCTTC CTGGGCACGC AGCCCGACCA CTACTGGTGC
       181 CGCGGGCCAA GTGCCGCGGC GCTGGCCGAG CGCTGCGGCT GGAGCCCGGA GGAGGAGTGG
       241 AACCGCACGG CGCCCGCCTC CCGCGGCCCA GAGCCCCCCG AGCGCCGCGG CCGCTGCCAG
       301 CGCTACCTCC TGGAGGCGGC CAACGACAGC GCCTCCGCCA CTAGCGCTCT CAGCTGCGCG
       361 GACCCACTCG CCGCCTTCCC CAACCGTTCG GCTCCCCTTG TGCCGTGCCG CGGCGGCTGG
U2F1   421 CGCTACGCCC AGGCCCACTC CACCATCGTC AGCGAGTTTG ACCTTGTCTG TGTCAATGCG
       481 TGGATGCTGG ACCTCACCCA AGCCATCCTG AACCTCGGCT TCCTGACTGG AGCATTCACC
U2F9   541 TTAGGCTATG CAGCAGACAG GTATGGCAGG ATCGTCATTT ACTTGCTATC CTGCCTTGGT
       601 GTTGGCGTCA CTGGGGTTGT GGTGGCCTTT GCACCAAACT TCCCTGTGTT TGTGATCTTC
       661 CGCTTCCTGC AAGGTGTATT TGGAAAGGGG ACGTGGATGA CTTGCTACGT GATTGTGACA
U2B11  721 GAAATAGTAG GTTCGAAACA AAGGAGGATT GTGGGAATCG TGATTCAAAT GTTCTTTACC
U2B13  781 CTTGGAATCA TAATTCTCCC TGGAATTGCC TACTTCATCC CCAACTGGCA AGGAATCCAG
U2F46  841 TTAGCCATCA CGCTGCCCAG CTTTCTCTTC CTCCTTTATT ACTGGGTGGT CCCTGAGTCT
U2F48  901 CCCCGTTGGC TGATTACTCG GAAGAAAGGA GATAAAGCAT TACAGATCCT GAGACGCATT
       961 GCTAAGTGCA ATGGGAAATA CCTCTCATCA AATTACTCAG AGATCACTGT TACAGATGAG
      1021 GAAGTTAGTA ATCCATCCTT TTTAGATCTG GTGAGAACTC CCCAAATGAG GAAATGCACA
U2B27 1081 CTTATTCTTA TGTTTGCTTG GTTCACAAGC GCAGTGGTGT ATCAAGGACT TGTCATGCGC
      1141 CTGGGAATTA TAGGGGGCAA CCTCTATATA GACTTTTTCA TCTCGGGCGT GGTGGAACTG
      1201 CCAGGAGCTC TCTTGATCTT ACTAACCATT GAGCGCCTTG GACGACGCCT CCCCTTTGCG
U2B32 1261 GCAAGCAATA TAGTGGCAGG GGTGGCATGC CTTGTCACTG CGTTCTTACC AGAAGGAATA
      1321 GCATGGTTGA GGACCACAGT GGCTACATTG GGAAGACTAG GGATAACCAT GGCCTTTGAA
      1381 ATTGTTTATT TGGTAAATTC AGAATTGTAC CCAACAACAT TACGAAATTT CGGAGTTTCG
      1441 CTCTGTTCAG GTCTGTGTGA TTTTGGGGGA ATCATAGCCC CATTTCTGCT CTTTCGGCTA
      1501 GCAGCCGTGT GGCTAGAACT ACCTCTGATC ATCTTTGGTA TCCTGGCATC CATCTGTGGT
      1561 GGCCTTGTGA TGCTTTTGCC TGAAACCAAG GGTATTGCCT TGCCAGAGAC AGTGGATGAT
      1621 GTAGAAAAAC TTGGCAGTCC ACATTCCTGT AAATGTGGCA GGAATAAGAA AACCCCAGTT
STOP  1681 TCCCGCTCTC ACCTTTGAGG CCCCCGACAA AGACAGAAAG AAGGAGCTAT CCAGGAGCTG
      1741 ATCCTCCTTG CAAAGCTGTG CCTTGCAGAG ATGCACGTGT GCATTTCAGC TACATCATGC
      1801 CGCGCTGTTG TAATACTGTA TAAAGACCTC AATCTATCCA GAGTATTTTT ATATAATGTT
      1861 GGATGAGTTA GGATTTGTAA TGCTGTTGAA GTTTCTGGGA ACACATAATA TGTAGCCAGT
      1921 TTAACAAAGA AGCTGTCAGG TGCACAGCCC TTCCTGGGTT TTTTTCTTGT GTTCCCTGTG
      1981 GTCTCTGACC CATTAGGCTA AAGAGAGACA AGAGAAGCCC CCAACCTGAT TCTCATGACA
      2041 GCTCCATCAA GAATGTGGGA TGTGCCGACC AAGGATTTGA GAAAGTTGTA CAGAAATGTG
      2101 TTCATCAAAT CTGGTCAAGG GACTAAGCTC CTAGCTGACC ATTCATTCTG AAGATTGCAT
      2161 GGAGGATGAA CATCTGGGAA TCCTGTTAAT GAGAAGGCTG AATCACAGGC ACCTGGGCCA
      2221 AAGGGTGTGA GCATTCATGT TCTCTGCTCA CCTTGGTTTC CGCACACCTT CGCAATGTGA
      2281 ACAGGTCAGG AGTCCCTCCC GTCCACCTCC TCTGTAACAG CTGGGGTTCC AGGCATGGTT
      2341 TAGGCCCTGT TCCAGCAATA AGAACCAATC TGCTGTACAA TCTGAGGACT TGGCTGTGTT
      2401 ATTTACAAAA TGATGCTGTG GTTCTGAGAT TATTTGGGAC ATTTTTGGCT CTCCTTTAGT
      2461 GGACACCTAG AGCCACAGAT TCCCTTCTTT ACTAAACAAA TCCCATGGAT TCTGATTTCT
      2521 GGGTCTTAGG ATTTTAAAAG TGAAGGGATA TTTTTCTTAT ATTTGTGAGT TCAGTTCCGA
      2581 TGGTGCCCGT GGTCAAAAGC GAAAAACATG GACAATTCCT ATTCATTCTT AGCACTTTGA
      2641 CATGTCTTGG GGAAAAGCTT TACATTTTAA TTTAAAAGAA AGATCAATTA TATCCATGCT
      2701 TAACAGGATC AGCAGGAGCT TTATAAATGA CTTTACAGAG ACTAATAAGG GATTGATCTT
      2761 TCTTTTTTTG TTATCGAGGC TTTTGAAATG TGGAACTTGT GTGTTCTGCT TTATATGTTA
      2821 TATTCAATAT CTTTTCAGAT GCAGTCTATA TTTTATGCTG AGTTTTAAAA ATGAAATACT
      2881 TTATGCAAAC AGGCAAAATT GGTACCAAAG GGAAACATTA ACCATGAGGA AGAGCATTTT
      2941 TCTAAGGAGA ACAGGTGACA ATATACACAT GTCGCGTAAT CGTAAAATGA GCATCTTAGT
      3001 CTTTAAAACA CATCAGAATT GAATACGAAT AATCTATTTG TCGATGAAAT AAACACAACT
      3061 CTTTGAGGAT TTGAGACTAC ATTCACCCTT TATTCACAGT CACTTGCAGT TTTGCTTTTC
      3121 TCTCCATTTC TCTGCTGTAA GATGACTGTT GCATTGTTGA ATTGTATTTT GAGTGGATAT
      3181 TTTTGTTTGG TAACAATTAA AATTTTAAAT CGT
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Figure 11.9.19:

PCR primers were designed from a translated region of the rat organic cation transporter OCT1 (Grundemann et al, 1994; GenBank accession number X78855) in which there is homology with the sequence of the rat OCT2 transporter (Okuda et al, 1996; GenBank accession number D83044). The 3' ends were designed to terminate in the first or the second base of a conserved amino acid codon. Two pairs of primers were designed for nested PCRs. In three of the primers, only one base was different from the OCT2 sequence, and in the fourth primer, (OCT1B3), 4 out of 23 bases were different from the OCT2 cDNA

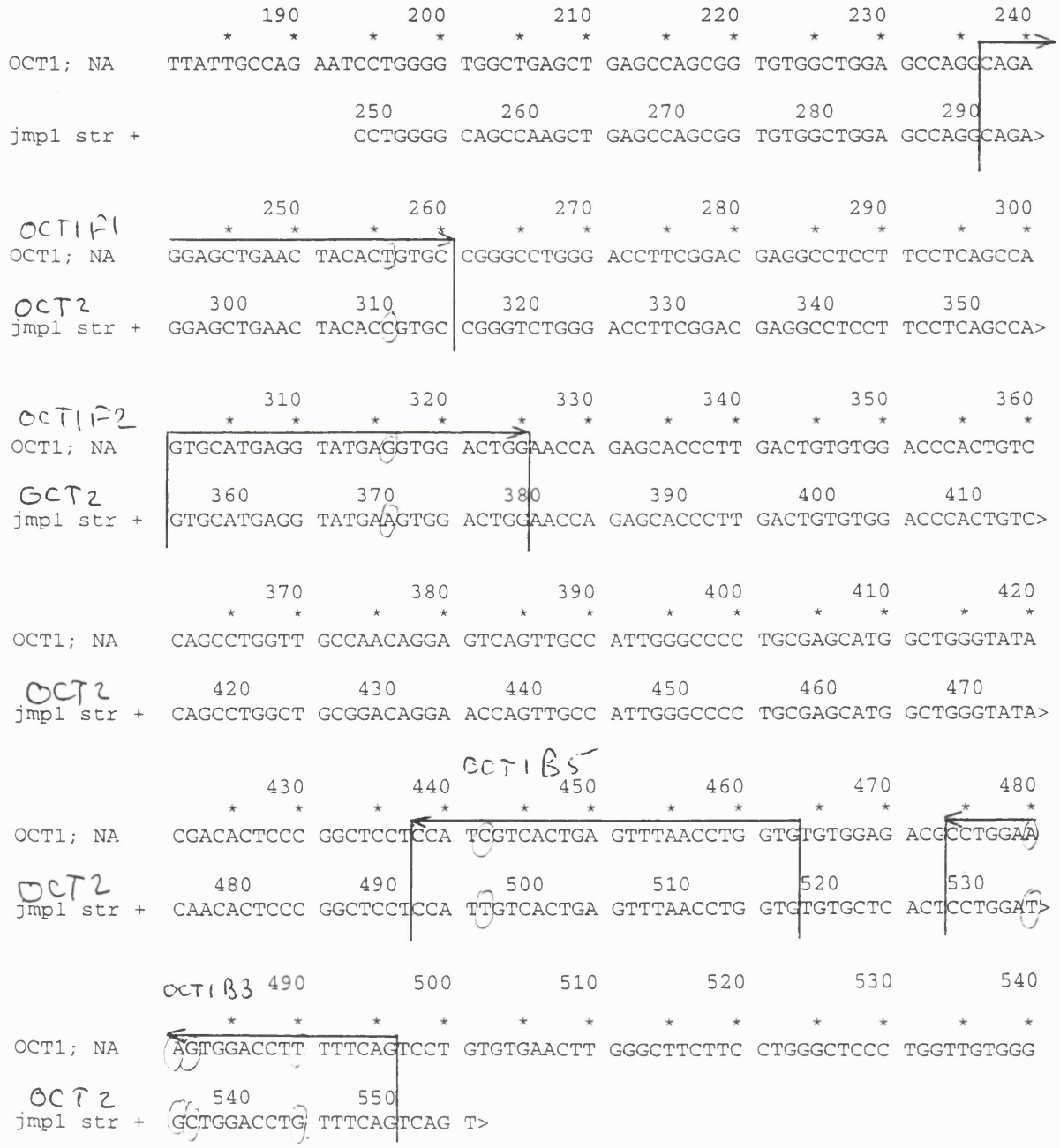


Table 11.9.1:
Design of PCR primers based on the sequence of the bovine SV2A cDNA (Gingrich et al, 1992; Genbank accession number S47919).

Analysis settings:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
500 to 2500	20 to 25	55-80	300 - 700	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
SV2F15	923-946	24	forward	50.0	55.8
5'- GGACACTCTATTTCGTGCTTGGTC -3'					
SV2B22	1329-1308	22	backward	50.0	57.0
5'- CATCCAAAACATGCAGAGCCAG -3'					

Optimal annealing temperature: 58.3°C
PCR product:
407 base pairs (923-1329)
54.5% G + C content
Tm: 80.6°C

Table 11.9.2:
Design of PCR primers based on the GnRH neurone SV2A cDNA. The primers were used to analyse the 384 bp intronic segment and to replace it with an appropriate segment.

Analysis settings for outer primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
1000 to 2500	20 to 40	70-80	950 - 1500	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
C37F1	1057-1088	32	forward	50.0	70.2
5'- AATGACAAGTTCATCGGGCTGCGTCTGAAGTC -3'					
C37B1	2270-2238	33	backward	51.5	70.9
5'- TGCACACAGGCACACACAGCTAAGACACCAAAC -3'					

Optimal annealing temperature: 63.2°C
PCR product:
1214 base pairs (1057-2270)
53.9% G + C content
Tm: 81.4°C

Analysis settings for nested primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
1000 to 2500	18 - 25	55 - 80	500 - 1500	NS	45 - 55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
C37F23	1449-1470	22	forward	54.5	60.1
5'- TTTTGGGAACAGCGAGTCAGCC -3'					
C37B21	2052-2031	22	backward	54.5	59.8
5'- AATGGGAGCAGCCTTGGTGATG -3'					

Optimal annealing temp 60.0°C
PCR product:
604 base pairs (1449-2052)
55.0% G + C content
Tm 81.3°C

Table 11.9.3:
Design of the 5'RACE primers which were used to obtain the missing 5' end of the GnRH neurone SV2A cDNA. The gene specific primers were derived from the sequence of an intermediate PCR product designated "clone 2 contig Nov 7". The gene specific primers and the abridged Adapter primer were designed by the Author. The Anchor and Adapter primers were included in the 5'RACE kit (Life Technologies).

Analysis settings for gene-specific primers (GSP):

Sequence scanned	*Primer size*	*Tm (°C)*	*3' dinucleotide*	*%G+C*
1 to 531	18 to 25	60-80	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs sequence*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
SV2B5 (GSP1)	400-381	20	backward	55.0	64.6
5'- TCTTCCCGATCTTTACGCCG -3'					
SV2B6 (GSP2)	261-239	23	backward	47.8	61.6
5'- TGCCCTGATATTCTCCCTCATAG -3'					
SV2B7 (GSP3)	87-63	25	backward	52.0	63.5
5'- GGGAATACTCATCCTGGACTCTGTC -3'					

Upstream primers:

Primer	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
Anchor	48	forward		
5'- CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG -3'				
Adapter	32	forward		
5'- CUACUACUACUAGGCCACGCGTCGACTAGTAC -3'				
Abridged adapter	20	forward	65.0	66.9
5'- GGCCACGCGTCGACTAGTAC -3'				

Table 11.9.4:
Design of PCR primers based on the sequence of the mouse plasma membrane noradrenaline transporter (Jayanthi et al, Genbank accession number NM009209).

Analysis settings for outer primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
150 to 800	20 to 30	55-80	350 - 600	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
NATOF1	168-190	23	forward	52.2	57.9
5'- GGAGACTTGGGGCAAGAAGATTG -3'					
NATOB1	569-548	23	backward	54.5	56.6
5'- GAGTTTGGGGTCCGTACAGTTGG -3'					

Optimal annealing temperature: 58.3°C
PCR product:
402 base pairs (168-569)
54.0% G + C content
Tm: 80.4°C

Analysis settings for nested primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
200 to 800	20 - 30	55 - 80	200 - 400	NS	45 - 55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
NATIF1	243-268	26	forward	46.2	55.8
5'- GTTCCCCTATCTCTGCTACAAGAATG -3'					
NATIB4	494-469	26	backward	53.8	57.8
5'- GCAAAGAGGTAGTAGAGTGACCAGGC -3'					

Optimal annealing temp 56.7°C
PCR product:
252 base pairs (243-494)
51.6% G + C content
Tm 78.4°C

Table 11.9.5:
Design of PCR primers based on the sequence of the rat synaptic vesicle monoamine transporter (SVMAT; Liu et al, 1992; Genbank accession number M97381).

Analysis settings for nested primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
500 to 1400	20 to 30	55-80	200 - 400	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
SVMATF17	790-814	25	forward	52.0	57.9
5'- GGGAGTGTGCTCTATGAGTTTGTGG -3'					
SVMATB26	1044-1022	24	backward	47.8	55.0
5'- CATGGTCTCCATCATCCAGATGGG -3'					

Optimal annealing temperature: 57.8°C
PCR product:
255 base pairs (790-1044)
56.1% G + C content
Tm: 80.2°C

Analysis settings for outer primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
500 to 1400	20 - 30	55 - 80	300 - 500	NS	45 - 55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
SVMATF8	705-728	25	forward	54.2	58.0
5'- GTACACAGATGATGAGGAGAGGGGG -3'					
SVMATB12	1105-1082	24	backward	50.0	58.0
5'- CCAATGAGATAAGAGATGCTCGCC -3'					

Optimal annealing temp 59.7°C
PCR product:
401 base pairs (705-1105)
57.1% G + C content
Tm 81.6°C

Table 11.9.6:
Design of four sets of nested PCR primers based on the sequence of the human Uptake2 noradrenaline transporter (Grundemann et al, 1998; GenBank accession number AJ001417).

Analysis settings:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
100 to 1500	25 to 30	60-80	200 - 450	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
U2F1	437-462	26	forward	53.8	63.3
5'- CCACTCCACCATCGTCAGCGAGTTTGAC -3'					
U2B13	823-798	26	backward	53.8	65.4
5'- GTTGGGGATGAAGTAGGCAATTCCAGGG -3'					

Optimal annealing temperature: 59.0°C
PCR product:
387 base pairs (437-823) 49.4% G + C content Tm: 78.4°C

U2F9	518-544	27	forward	51.9	61.5
5'- GCTTCCTGACTGGAGCATTCACCTTAG -3'					
U2B11	760-735	26	backward	46.2	61.7
5'- CGATTCCCACAATCCTCCTTTGTTTC -3'					

Optimal annealing temperature: 57.8°C
PCR product:
243 base pairs (518-760) 49.8% G + C content Tm: 77.5°C

U2F46	855-884	30	forward	46.7	62.7
5'- GCCCAGCTTTCTCTTCCTCCTTTATTACTG -3'					
U2B32	1288-1261	28	backward	53.6	67.7
5'- CATGCCACCCCTGCCACTATATTGCTTGC -3'					

Optimal annealing temperature: 58.3°C
PCR product:
434 base pairs (855-1288) 47.2% G + C content Tm: 77.7°C

U2F48	904-929	26	forward	50.0	62.0
5'- CGTTGGCTGATTACTCGGAAGAAAGG -3'					
U2B27	1139-1114	26	backward	50.0	60.5
5'- CGCATGACAAGTCCTTGATACACCAC -3'					

Optimal annealing temperature: 55.2°C
PCR product:
236 base pairs (904-1139) 41.9% G + C content Tm: 74.2°C

Table 11.9.7:
Design of nested PCR primers based on the rat OCT1 cDNA.

Analysis settings for outer primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
195 to 500	18 to 25	55-80	200 - 300	NS	45-55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
OCT1F1	238-261	24	forward	54.2	55.7
5'- CAGAGGAGCTGAACTACACTGTGC -3'					
OCT1B3	496-474	23	backward	47.8	55.8
5'- CTGAAAAAGGTCCACTTTCCAGG -3'					

Optimal annealing temperature: 58.8°C
PCR product:
259 base pairs (238-496)
58.7% G + C content
Tm: 81.4°C

Analysis settings for nested primer pair:

Sequence scanned	*Primer size*	*Tm (°C)*	*Product size*	*3' dinucleotide*	*%G+C*
195 to 500	18 - 25	55 - 80	150 - 300	NS	45 - 55

Maximum consecutive bonds allowed:

primer vs primer (any)	*primer vs primer (G-C only)*	*3'-ends*	*3'-end vs product*
4	2	2	5

Primer	*Position*	*Bases*	*Orientation*	*%G+C*	*Tm (°C)*
OCT1F2	302-325	24	forward	54.2	59.7
5'- GTGCATGAGGTATGAGGTGGACTGG -3'					
OCT1B5	463-439	25	backward	48.0	56.6
5'- CACCAGGTTAAACTCAGTGACGATGG -3'					

Optimal annealing temp 57.8°C
PCR product:
162 base pairs (302-463)
58.0% G + C content
Tm 79.5°C

Table 11.9.8: Expression cloning for Transport-P: COS-7 cells were electroporated with library pools, TE buffer (negative control) or alpha-1b DNA (positive control), followed by assay for uptake or binding of prazosin

Well	DNA	Drugs	CPM	pmoles/well	Protein mg/ml	Protein mg/well	pmol/mg	Specific pmoles/mg	Specific %
1	TE	Control	5709	83.45	0.627	1.255	66.50	7.87	12
2		Desipramin	3759	54.95	0.469	0.937	58.63		
3	Pool 1	Control	6164	90.10	0.539	1.078	83.60	20.69	25
4		Desipramin	4218	61.66	0.490	0.980	62.92		
5	Pool 2	Control	5505	80.47	0.513	1.026	78.44	13.33	17
6		Desipramin	3590	52.48	0.403	0.806	65.12		
7	Pool 3	Control	5684	83.08	0.600	1.200	69.24	18.94	27
8		Desipramin	2910	42.54	0.423	0.846	50.31		
9	Pool 4	Control	5252	76.77	0.499	0.998	76.90	26.95	35
10		Desipramin	3265	47.72	0.478	0.956	49.95		
11	Pool 5	Control	5472	79.98	0.507	1.014	78.91	18.64	24
12		Desipramin	3915	57.23	0.475	0.949	60.28		
13	TE	Control	5682	83.05	0.618	1.237	67.17	10.17	15
14		Desipramin	4131	60.38	0.530	1.059	57.00		
15	Pool 6	Control	4492	65.66	0.522	1.044	62.89	17.26	27
16		Desipramin	2687	39.28	0.430	0.861	45.63		
17	Pool 7	Control	4321	63.16	0.488	0.977	64.65	7.10	11
18		Desipramin	3245	47.43	0.412	0.824	57.55		
19	Pool 8	Control	4011	58.63	0.521	1.041	56.32	5.63	10
20		Desipramin	2826	41.31	0.408	0.815	50.68		
21	Pool 9	Control	4919	71.90	0.481	0.962	74.77	14.45	19
22		Desipramin	3729	54.51	0.452	0.904	60.32		
23	Pool 10	Control	4735	69.21	0.467	0.934	74.09	14.86	20
24		Desipramin	3253	47.55	0.401	0.803	59.23		
25	TE	Control	5891	86.11	0.539	1.078	79.90	23.49	29
26		Desipramin	3782	55.28	0.490	0.980	56.41		
27	Pool 11	Control	4232	61.86	0.427	0.855	72.37	23.78	33
28		Desipramin	2547	37.23	0.383	0.766	48.59		
29	Pool 12	Control	4010	58.61	0.389	0.778	75.31	10.59	14
30		Desipramin	2797	40.88	0.316	0.632	64.72		
31	Pool 13	Control	3725	54.45	0.560	1.120	48.59	9.24	19
32		Desipramin	2038	29.79	0.378	0.757	39.35		
33	Pool 14	Control	4281	62.58	0.498	0.995	62.88	19.52	31
34		Desipramin	2363	34.54	0.398	0.797	43.36		
35	Pool 15	Control	4259	62.25	0.485	0.971	64.13	16.07	25
36		Desipramin	2790	40.78	0.424	0.849	48.06		
37	Alpha-1b	[3H]Praz	8005		0.534	1.069			
38		Praz 10-6	4007		0.426	0.852			
39	Pool 16	Control	4754	69.49	0.493	0.986	70.47	21.26	30
40		Desipramin	2816	41.16	0.418	0.836	49.21		
41	Pool 17	Control	5228	76.42	0.444	0.888	86.03	35.48	41
42		Desipramin	2871	41.97	0.415	0.830	50.54		
43	Pool 18	Control	5672	82.91	0.385	0.769	107.79	58.00	54
44		Desipramin	2901	42.40	0.426	0.852	49.79		
45	Pool 19	Control	4736	69.23	0.492	0.983	70.42	23.28	33
46		Desipramin	2609	38.14	0.404	0.809	47.15		
47	Pool 20	Control	4451	65.06	0.560	1.120	58.07	4.35	7
48		Desipramin	2591	37.87	0.353	0.705	53.72		
		TC	54396						
Control (TE B0) mean			5761	84	0.595	1.190	71	14	19
Control (TE B0) SD			114	2	0.049	0.097	8	8	9
Control (TE B0) SEM			66	1	0.028	0.056	4	5	5

12. GENERAL DISCUSSION

12.1 ASSESSMENT OF THE HYPOTHESIS

Classically, a radiolabelled ligand binds to its receptor and can be displaced by added unlabelled ligand in a simple equilibrium process. When the α_1 adrenergic ligand [^{3}H]prazosin binds to peptidergic neurones it is displaceable by unlabelled prazosin in concentrations up to 10^{-7} M. However, at greater concentrations of unlabelled prazosin, there is a paradoxical increase in the binding of [^{3}H]prazosin (Al-Damluji & Krsmanovic, 1992; Al-Damluji et al, 1993; Figure 7.4.1). The following hypothesis was formulated to explain these findings:

A. Displacement of [^{3}H]prazosin is from α_1 adrenoceptors in the peptidergic neurones;

B. The increase in radioactivity (the prazosin paradox) is due to the presence in peptidergic neurones of an unusual uptake process which is activated by its ligand (prazosin). This uptake process was designated Transport-P.

The hypothesis has been examined as follows:

A. Displacement of [^{3}H]prazosin is from α_1 adrenoceptors in the peptidergic neurones:

The work which is described in Section 11.1 demonstrates that immortalised GnRH neurones express an α_1 adrenoceptor gene and identifies its molecular sub-type as α_{1B}. The initial identification was based on the sequences of multiple PCR products which were generated using as template the GnRH neurone RNA or cDNA library. Further work resulted in isolation of an individual clone whose sequence was of an α_{1B} adrenoceptor. When expressed in COS-7 cells, [^{3}H]prazosin binds to this receptor and is displaceable by unlabelled prazosin. Further, when the α_{1B} adrenoceptor was over-expressed in the GnRH neurones, [^{3}H]prazosin was displaced by unlabelled prazosin in the same concentration range as in the control GnRH neurones (Figure 11.1.10). This indicated that presence of these receptors in GnRH neurones is sufficient to account for the displacement of [^{3}H]prazosin by unlabelled prazosin in the concentration range 10^{-9} to 10^{-7} M.

Binding of a ligand to its receptor may be followed by internalisation of the receptor. The

following evidence indicated that the prazosin paradox was not due to internalisation of ligand-α_1 adrenoceptor complexes:

1. COS-7 cells transfected with hamster smooth muscle or mouse GnRH neurone α_{1b} adrenoceptor cDNA expressed abundant α_{1B} adrenoceptors, as indicated by the great increase in [^{3}H]prazosin binding. However, there was no prazosin paradox in these transfected cells (Figures 11.1.8 and 11.1.15).

2. DDT_1 MF-2 smooth muscle cells express native α_{1B} adrenoceptors and are known to internalise these receptors upon exposure to the agonists adrenaline, noradrenaline and phenylephrine (Cornett & Norris, 1982; Fratelli & DeBlasi, 1987; Cotecchia et al, 1988). As in the transfected COS-7 cells, there was no prazosin paradox in DDT_1 MF-2 cells (Figure 11.1.3).

3. Internalisation of ligand-receptor complexes usually follows binding of an agonist to its receptor. The adrenergic agonists adrenaline, noradrenaline, methoxamine and phenylephrine displaced [^{3}H]prazosin from α_{1B} adrenoceptors in DDT_1 MF-2 cells, but the prazosin paradox was not seen in any of these experiments (Figure 11.1.9).

4. The prazosin paradox was unaffected by over-expression of α_{1B} adrenoceptors in the peptidergic neurones, indicating that it is mediated by a different component of the GnRH neurones (Figure 11.1.10).

5. The structural properties of ligands for Transport-P are different from those of ligands for α_1 adrenoceptors (Table 11.5.2).

In the initial work, multiple PCR products were generated using primers which were derived from homologous regions of the α_1 adrenoceptor cDNAs. The sequences of all these PCR products were consistent with the α_{1B} adrenoceptor subtype. Further, screening the cDNA library yielded only one clone whose sequence was that of an α_{1B} adrenoceptor; these

findings indicate that the only α_1 adrenoceptor which exists in the GnRH neurones is the α_{1B} subtype.

The conclusion from these experiments is that presence of α_{1B} adrenoceptors in the peptidergic neurones accounts for the displacement of [^{3}H]prazosin by unlabelled prazosin, but that the α_{1B} adrenoceptors are not responsible for the prazosin paradox.

B. The increase in radioactivity (the prazosin paradox) is due to the presence in peptidergic neurones of an unusual uptake process which is activated by its ligand (prazosin):

1. The prazosin equilibrium curve: Analysis of the accumulation of the total amount of prazosin (labelled and unlabelled) demonstrates that the paradoxical increase in accumulation of [^{3}H]prazosin is due to an exponential increase in the accumulation of prazosin; this is manifested by a sigmoidal shape of the prazosin equilibrium curve (Figure 11.4.1). A sigmoidal shape is typical of a cooperative effect, in which binding of a ligand increases the affinity of a protein for the ligand. This is discussed further in Section 12.3.

2. The effects of antidepressants: Tricyclic antidepressants and their newer derivatives are known to inhibit the re-uptake of amines by the pre-synaptic plasma membrane transporters. The antidepressants inhibited the paradoxical increase in accumulation of [^{3}H]prazosin at concentrations of unlabelled prazosin greater than 10^{-7} M (Figure 11.2.5). As the antidepressants inhibit the pre-synaptic plasma membrane transporters, the findings were consistent with the hypothesis that the prazosin paradox is due to uptake of the radioligand via a specific transporter.

The antidepressants inhibited the accumulation of [^{3}H]prazosin when the radioligand was used at $2x10^{-9}$ M (Figure 11.2.5), indicating that uptake takes place at these low extracellular concentrations of prazosin. The blockade of uptake by the antidepressants is competitive (Figure 11.2.6). This competitive action suggests that prazosin and the antidepressants may act on the same site in the GnRH neurones. The work which is described in Section 11.2 also included the first demonstration of specific binding sites for antidepressants in post-

synaptic neurones (Figure 11.2.3); these binding sites are presumably the transporter molecules which are the sites of action of prazosin and the antidepressants in the peptidergic neurones.

Although the antidepressants and prazosin are likely to act on the same site in the GnRH cells, unlabelled imipramine did not increase the binding of [^{3}H]imipramine (Figure 11.2.2). This indicates that the antidepressants do not activate the uptake process. This is attributable to the structural properties of the antidepressant molecules, as is discussed below (Section 12.3.2.6).

3. The paradoxical increase in accumulation of [^{3}H]prazosin was not seen in membrane preparations of the GnRH neurones, indicating that it requires intact cells or storage organelles (Al-Damluji et al, 1993). This is consistent with the hypothesis that the prazosin paradox is attributable to accumulation of the radioligand via an uptake process in the peptidergic neurones. Clearly, the prazosin paradox is not due to a non-specific accumulation of prazosin in cell membranes.

4. Antidepressant-sensitive prazosin uptake was not seen in pre-synaptic noradrenergic neurones or in non-neuronal cells, indicating that it is a specialised function of peptidergic neurones. Thus, desipramine had no effect on the accumulation of [^{3}H]prazosin in SK-N-SH noradrenrgic neurones, COS-7 kidney cells or in DDT$_1$ MF-2 smooth muscle cells. Further, there was no antidepressant-blockable prazosin paradox in any of these cells (Figures 11.1.3 and 11.1.4).

5. The prazosin paradox is temperature-sensitive and energy-dependent. Cooling the GnRH cells inhibited the incorporation of [^{3}H]prazosin and abolished the paradoxical increase in accumulation of [^{3}H]prazosin (Figure 11.1.1). This indicated that the increase in accumulation of [^{3}H]prazosin is due to an energy-requiring, active uptake process. Uptake of prazosin was completely inhibited by the V-ATPase inhibitor, bafilomycinA1 (Figure 11.3.3), suggesting that uptake requires energy from an electrochemical proton gradient generated by a V-ATPase ion pump. Thus, enhanced prazosin accumulation at higher concentrations of the drug may be into an acidified intracellular compartment, such as

neurosecretory vesicles. Further evidence for this hypothesis was obtained from the findings that uptake of prazosin is inhibited by increases in extracellular pH (Table 11.3.1), and by the monovalent carboxylic ionophore monensin (Figure 11.3.4) and the organic base chloroquine (Figure 11.3.5). In contrast, chloroquine had no comparable inhibitory effect on the accumulaion of prazosin in COS-7 cells (Figure 11.3.5). This confirmed that accumulation of prazosin in GnRH cells is unlikely to be via a lysosomotropic effect, in which amphiphilic amines accumulate non-specifically into acidified vesicles such as mitochondria or lysosomes which are present in all eukaryotic cells; the uptake of prazosin is more likely to be into some specialised compartment in peptidergic neurones, such as neurosecretory vesicles or internalised clathrin-coated pits.

Further evidence for the localisation of the amines in acidic stores was obtained from experiments which examined the effects of pharmacological manipulations on the release of these amines; release of prazosin was accelerated by bafilomycinA1, chloroquine, monensin and by increasing extracellular pH (Section 11.8). This is consistent with the conclusion that Transport-P accumulates amines in acidified intracellular vesicles. Clearly, intracellular acidity is required for retention of amines which are accumulated via Transport-P in peptidergic neurones.

6. The GnRH neurones accumulated the fluorescent analogue BODIPY FL prazosin, providing visual evidence for amine uptake in peptidergic neurones. The compound accumulated in a granular distribution, indicating a vesicular location (Section 11.7). As in the case of prazosin, accumulation of BODIPY FL prazosin was inhibited in the cold and by the organic base chloroquine. This indicated that prazosin and its analogue are internalised by the same antidepressant-sensitive, proton-dependent uptake process (Transport-P).

Hypothalamic cells from fetal rats also accumulated BODIPY FL prazosin by a desipramine-sensitive process (Figure 11.7.9). Most of the intensely labelled cells appeared to be neurones, whereas glial cells were not intensely labelled (Figures 11.7.9 and 11.7.10). These findings provided further evidence that accumulation of prazosin is a specialised function of hypothlamic neurones.

7. Following uptake into peptidergic neurones, the ligands can be released from acidified cellular stores by an active, temperature-dependent process. The kinetics of temperature

dependence conformed to the Arrhenius equation in which there is an exponential increase in reaction rate with temperature (Figure 11.8.4). This is typical of cellular processes such as enzymatic reactions (Price & Stevens, 1989; Cornish-Bowden, 1995). In contrast, simple diffusion along a concentration gradient increases linearly with temperature, as described in the Fick-Einstein and the Stokes-Einstein equations (Stein, 1986). Release of prazosin from GnRH neurones is clearly not simply by a process of passive diffusion down a concentration gradient. In common with other biological systems, release of prazosin from GnRH neurones requires an activation energy. The values which were obtained for the activation energy (Ea) and for the temperature quotient (Q_{10}) were within the range of values which are expected in a biological system (Price & Stevens, 1989).

Many transporters are capable of reversing the direction of transport if the concentrations of their substrates are reversed. However, release of prazosin and BODIPY FL prazosin was not blocked by desipramine 10^{-5} M. As this concentration of desipramine completely blocks Transport-P (Figure 11.2.6), the findings indicate that release of prazosin occurs by a mechanism other than Transport-P. Future work will aim to determine the mechanisms of release of amines from the peptidergic neurones.

8. There are specific structural requirements for accumulation of ligands via Transport-P. The compounds which have the greatest affinity for Transport-P consist of a basic amine and a carbon skeleton which consists of 18-20 carbons which are arranged in a condensed cyclic structure. Halogens increase affinity for Transport-P. These structural properties determine the affinity of ligands for accumulation via Transport-P, but they are insufficient for activation of the uptake process; that requires a furan group as part of the cyclic structure (Section 11.5). Thus, accumulation via Transport-P and activation of the uptake process are dependent on the presence of specific structures which presumably conform to a molecular arrangement within a membrane carrier molecule.

Thus, a considerable body of evidence is consistent with the hypothesis that the prazosin paradox is due to an unusual active uptake process. The remaining Sections in this Thesis discuss possible mechanisms of this process and its possible significance.

12.2 COMPARISON OF TRANSPORT-P TO OTHER AMINE UPTAKE PROCESSES IN THE BRAIN

Uptake processes for amines were known to exist in pre-synaptic nerve terminals and in glial cells. Transport-P differs from these uptake processes as follows (Table 12.1):

Comparison of Transport-P to pre-synaptic plasma membrane transporters:

Pre-synaptic plasma membrane transporters utilise the electrochemical gradient of sodium ions which is generated by the Na^+/K^+ATPase (sodium pump) to accumulate molecules of neurotransmitter from the extracellular space into the cytoplasm (Amara & Kuhar, 1993; Lester et al, 1994). These plasma membrane transporters can be blocked by typical antidepressants (Glowinski & Axelrod, 1964; Giros et al, 1994; Barker et al, 1994). Transport-P resembles pre-synaptic plasma membrane transporters in its sensitivity to antidepressants but it differs as follows:

1. Transport-P is located in post-synaptic peptidergic neurones (Section 11.7) and does not exist in pre-synaptic noradrenergic neurones (Figure 11.1.4).
2. In pre-synaptic neurones, increasing concentrations of unlabelled neurotransmitter amine do not cause a paradoxical increase in accumulation of the radiolabelled ligand (Figures 11.1.2 and 11.2.7). Thus, pre-synaptic re-uptake is not activated by its ligand.
3. Pre-synaptic re-uptake is linked to a P-ATPase (Na^+/K^+ATPase; sodium pump) whereas Transport-P is linked to V-ATPase which is specific to vesicles (Section 11.3).
4. Transport-P is independent of sodium which is required for pre-synaptic plasma membrane uptake (Section 11.3; Figure 11.3.1).
5. Potency at Transport-P is reduced by the presence of phenolic hydroxyl groups in the ligands, whereas these phenolic hydroxyl groups increase potency at pre-synaptic plasma membrane re-uptake (Section 11.5.1.3; Figure 11.5.6).
6. Potency at Transport-P is unaffected by the presence of phenolic methoxyl groups in the ligands, whereas these phenolic methoxyl groups reduce potency at pre-synaptic plasma membrane re-uptake (Section 11.5.1.3; Figure 11.5.8).

Comparison of Transport-P to pre-synaptic vesicular transporters:

Pre-synaptic vesicular transporters utilise the electrochemical gradient of protons which is generated by V-ATPase to accumulate neurotransmitter amines from the cytoplasm to the interior of neurosecretory vesicles (Schuldiner et al, 1995; Liu & Edwards, 1997). Transport-P resembles pre-synaptic vesicular transporters in its dependence on protons and

V-ATPase but it differs as follows:

1. Transport-P is located in post-synaptic peptidergic neurones (Section 11.7) and does not exist in pre-synaptic noradrenergic neurones (Figure 11.1.4).
2. Transport-P is unaffected by reserpine which inhibits pre-synaptic vesicular transporters (Section 11.4.2.2; Figure 11.4.4).
3. Potency at Transport-P is reduced by the presence of phenolic hydroxyl groups in the ligands, whereas these phenolic hydroxyl groups increase potency at pre-synaptic vesicular uptake (Section 11.5.1.3; Figure 11.5.6).
4. Transport-P does not accumulate serotonin or histamine which are accumulated by pre-synaptic vesicular amine transporters (Section 11.4; Table 11.4.1).
5. Cocaine inhibits Transport-P but has no effect on pre-synaptic vesicular transporters (Section 11.4.2.1; Figure 11.4.3).

Comparison of Transport-P to $Uptake_2$ in glial cells:

The uptake process in non-neuronal cells, including glial cells ($Uptake_2$) is insensitive to antidepressants and reserpine and is independent of protons but is blocked by steroid hormones (Iversen & Salt, 1970; Salt, 1972; Schomig et al, 1992; Russ et al, 1996). Transport-P differs from $Uptake_2$ as follows:

1. Transport-P is located in neurones (Section 11.7) and does not exist in non-neuronal cells such as glia, muscle or kidney cells (Figures 11.7.10, 11.1.3 and 11.1.4).
2. Transport-P is unaffected by steroids which inhibit $Uptake_2$ (Section 11.4.2.3).
3. $Uptake_2$ is independent of protons which are required for Transport-P (Section 11.3.2).
4. Potency at Transport-P is unaffected by phenolic methoxyl groups in the ligands, whereas these methoxyl groups increase potency at $Uptake_2$ (Section 11.5.1.3; Figure 11.5.8).
5. Potency at Transport-P is enhanced by α-methyl and reduced by β-hydroxyl groups in the ligands, whereas these substitutions have the opposite effect on the potency of ligands at $Uptake_2$ (Section 11.5.1.2; Figure 11.5.4).
6. Transport-P does not accumulate serotonin, histamine, isoprenaline or MPP^+ which are accumulated by $Uptake_2$ (Section 11.4; Table 11.4.1; Figure 11.5.3).

Transport-P has properties which differ from those of known uptake processes in the brain, and is therefore likely to be encoded by an unknown transport protein.

Table 12.1:

Comparison of Transport-P to other amine uptake processes in the brain

	Transport-P	*Plasma membrane*	*Vesicular*	*Uptake$_2$*
Location	Post-synaptic	Pre-synaptic	Pre-synaptic	Non-neuronal
Amines activate	Yes	No	No	No
Energy source	V-ATPase	P-ATPase	V-ATPase	?
Ion dependence	H^+	Na^+	H^+	None
Antidepressants	Block	Block	No effect	No effect
Reserpine	No effect	No effect	Blocks	No effect
Steroids	No effect	No effect	No effect	Block
Substitutions in substrates				
Phenolic OH^-	Inhibit	Enhance	Enhance	Inhibit
Phenolic OCH_3	No effect	Inhibit	Inhibit	Enhance
β–OH^-	Inhibit	Inhibit	Inhibit	Enhance
α-methyl	Enhance	Enhance	Enhance	Inhibit
Amino methyl	Inhibit	Inhibit	No effect	Enhance

12.3 THE MECHANISM OF UPTAKE VIA TRANSPORT-P

12.3.1 The ligand

The work which is described in Section 11.5 represents significant progress in understanding the molecular properties of ligands for Transport-P.

Transport-P accumulates amines of a certain size and structure. There is an absolute requirement for the amine which may be primary, secondary or tertiary. The work which is described in Section 11.5.2 demonstrated that the amine must be basic. This conclusion was supported by evidence from an extensive series of compounds, including analogues of aniline, naphthalene, fluorene, pyridine and amides (Figures 11.5.9 and 11.5.10). Neutral amines were inactive, as were quaternary and guanyl amines which are permanently positively charged (Figure 11.5.3). Thus, the amine must be able to acquire and to shed its charge, in order to be accumulated via Transport-P. A possible mechanism which explains these findings is illustrated in Figure 12.3.1. It is possible that the Transport-P carrier may possess a negatively charged pocket to which the basic amine binds. The carrier may also possess a hydrophobic region which must be traversed by the ligand. In order to be accumulated via Transport-P, the ligand must first acquire a proton, becoming positively charged and thus binding to the negatively charged pocket. The ligand would then lose the proton, becoming neutral, and this enables it to traverse the hydrophobic region in the carrier. Thus, neutral amines would not be able to bind to the carrier, and quaternary and guanyl amines would bind but would not be able to traverse the hydrophobic region in the Transport-P carrier.

Among the phenylethylamines, the compounds which possess a hydrophobic phenyl group have the greatest potency at Transport-P (Section 11.5.1.3). Thus: a) potency was reduced by phenolic hydroxyl groups which are hydrophilic (Figure 11.5.6); b) potency was increased by phenolic chlorines which are hydrophobic (Figure 11.5.7); c) potency was unaffected by phenolic methoxyl groups which are neutral (Figure 11.5.8). The requirement for a hydrophobic phenyl group is consistent with the above model: in order to traverse the hydrophobic region in the Transport-P carrier, the phenyl group would have to be hydrophobic (Figure 12.3.1). The slight reduction of potency by a β-hydroxyl group and enhancement of potency by an α-methyl group in the side chain (Section 11.5.1.2) are consistent with this model.

Compounds which have the greatest affinity for Transport-P consist of a condensed cyclic structure of 18-20 carbons (Section 11.5.3). This conclusion was based on an extensive series of aliphatic and aromatic amines whose carbon skeletons ranged in size from 1 to 20 atoms (Table 11.5.3). Among aromatic compounds, increasing size was associated with increased potency at Transport-P. This is consistent with the above model, as the larger compounds can be expected to be more hydrophobic.

Small aliphatic amines (consisting of less than 5 carbons) could not be accumulated via Transport-P, presumably because they are relatively hydrophilic. Aliphatic amines consisting of a single chain of 5 to 13 carbons were accumulated, presumably because of their greater lipophilicity. Among the compounds which consist of 5 to 12 carbons, the structure of the carbon skeleton has little effect on accumulation via Transport-P (Table 11.5.3). Inability of Transport-P to distinguish between the stereoisomers of amphetamine, ephedrine and norephedrine (Section 11.5.1.2) is consistent with this conclusion. Aliphatic amines consisting of a single chain of 14 or more carbons could not be accumulated, despite their greater lipophilicity. These experiments were performed in the presence of unlabelled prazosin 10^{-6} M, at which concentration Transport-P is activated. It is possible that the Transport-P carrier is comprised of a pore which can accumulate compounds up to a certain size; this maximum size is equivalent to an alipahtic chain of 13 carbons. Further evidence for this suggestion is provided by the finding that aliphatic amines consisting of 16 to 18 carbons arranged in two or three chains were accumulated via Transport-P (Table 11.5.3). Among these two-chain aliphatic amines, dioctylamine was the largest compound which could be accumulated, and didecylamine was inactive. This provides further evidence for the restriction on the size of ligands which can be accumulated via Transport-P.

Whereas the aliphatic amines octadecylamine (C18), N-methyloctadecylamine (C19) and didecylamine (C20) were inactive at Transport-P, their aromatic counterparts desipramine (C18), nortriptyline (C19), imipramine (C19), amitriptyline (C20) and trimipramine (C20) were fully active (Table 11.5.3). Aromatic compounds are condensed structures; the findings are therefore consistent with the hypothesis that when Transport-P is activated, it can only accumulate compounds which are smaller than a certain size. Further evidence for this hypothesis is provided by the finding that desipramine (C18) was much more potent than the equivalent branched compound trihexylamine (C18), despite the greater lipophilicity

of trihexylamine (Table 11.5.3).

Pentylamine was the smallest compound which could be accumulated via Transport-P. The LogP of this compound is 1.5 and this may be the minimum lipophilicity of ligands which can be accumulated via Transport-P. However, it is clear from the above that while lipophilicity is a permissive property, it is not the only determinant, as the larger aliphatic amines which are highly lipophilic could not be accumulated via Transport-P. Further, among aliphatic compounds which are accumulated via Transport-P, increasing size reduced the affinity for Transport-P; thus, dipentylamine and dihexylamine were much more potent than trihexylamine, despite the greater lipophilicity of trihexylamine (Table 11.5.3).

The furan group in prazosin is necessary for activation of Transport-P, as demonstrated by the much weaker effect of the non-aromatic tetrahydrofuran analogue (Figure 11.5.11). Further, substitution of the furan with another aromatic group such as benzene greatly reduces the potency of these compounds in activating Transport-P (Figure 11.5.11).

12.3.2 The transporter

The above findings suggest that activation of Transport-P by the furan ring of prazosin opens a pore which admits basic amines of a certain molecular size. The nature of this pore remains speculative, as the work which is described in Section 11.9 has not resulted in isolation of a cDNA which encodes Transport-P. However, using the functional data which are available so far, it is possible to make some predictions regarding the nature of the pore and to limit the alternatives. The activation of Transport-P by a concentration of prazosin which is above a certain threshold suggests that the Transport-P carrier may be an allosteric membrane protein to which prazosin binds in a cooperative manner. Cooperativity is defined as increasing affinity for the ligand, as ligand binding proceeds (Baldwin & Chothia, 1979). This is manifested by a sigmoidal curve, as was seen for the uptake of prazosin (Figure 11.4.1).

12.3.2.1 The principles of cooperativity and allosteric change

The concept of allostery was introduced by Monod and his colleagues in Paris to explain the cooperative effects of regulatory metabolites on enzymes and other proteins (Monod et al, 1963). The direct experimental evidence for Monod's hypothesis was provided by Perutz in his work on haemoglobin in Cambridge. Monod et al (1963) defined allostery as a

"specifically inducible conformational alteration of protein structure" involving "the breaking, or formation, or substitution of bonds between subunits in the protein" which "frequently involve alterations of quaternary structure". According to Perutz (1990), cooperative binding arises in proteins which consist of two or more structures in equilibrium; the cooperativity arises by a change in equilibrium between the alternative structures at successive binding steps of the ligands. The structures are stabilised predominantly by electrostatic interactions in the form of hydrogen bonds between amino acid side chains of opposite charge; binding of the ligand alters the interactions between these side chains (Perutz, 1990). Monod et al (1965) suggested that in one structure, the subunits would be constrained by strong bonds that would resist the structural change which is required for ligand binding; they called this structure T for tense. In the other structure, the constraints would be relaxed, allowing ligand binding; they called this structure R for relaxed. It was proposed that an allosteric activator would stabilise the protein in the R state, increasing the affinity for ligand binding; conversely, an allosteric inhibitor would favour the T state, reducing the affinity for ligand binding (Monod et al, 1965). It was already known that many functional proteins (including haemoglobin and many enzymes) consist of independent subunits which are linked not by rigid covalent bonds but by reversible electrostatic interactions; it was therefore proposed that the structural basis of the change from the T to the R state is likely to involve a movement of the protein subunits along an axis of symmetry (Monod et al, 1965).

Monod et al (1965) postulated that the symmetry of the protein would be conserved during the allosteric transition; in other words, the movement from the T to the R structure would be concerted rather than sequential, and all the binding sites would be either in the T state or in the R state. The data from the haemoglobin oxygen dissociation curve could be fitted to such a model (Monod et al, 1965). Koshland et al (1966) examined several other models which involved allosteric transition states and they found that the data from the haemoglobin oxygen dissociation curve could be fitted equally well to such transitional state models. Subsequent work on crystal structure demonstrated that there are only two stable states of haemoglobin, which was impressive confirmation of Monod's prediction. However, as the crystal structures of more proteins are being solved, it is becoming clear that concerted transitions do not apply to all allosteric effects, and that proteins use a variety of structural movements to promote the cooperative binding of their ligands (Perutz, 1990).

12.3.2.2 Cooperativity and allosteric regulation in phosphofructokinase

Blangy, Buc & Monod (1968) provided a mathematical description of the allosteric principle, based on the kinetics of the interaction of various ligands with the glycolytic enzyme phosphofructokinase. This is the third enzyme in the glycolytic pathway; it catalyses the transfer of the γ phosphate from ATP to fructose-6-phosphate, to form the bisphosphate (fructose-1,6-diphosphate). The interaction of this enzyme with the substrate fructose-6-phosphate is cooperative, in that a plot of the substrate concentration *vs* the initial velocity of the reaction was sigmoidal (Figures 12.3.2A & B). The Hill coefficient, which describes the cooperativity of the interaction of the enzyme with fructose-6-phosphate, was 3.8 (Figure 12.3.2C). The most striking aspect of the mathematical analysis was that the Hill coefficient was reduced both by activators and inhibitors of the enzyme (Figure 12.3.2D). With increasing concentrations of the activator adenosinediphosphate (ADP), the curves become less sigmoidal, finally becoming a parabola (Figure 12.3.2A); conversely, with increasing concentrations of the inhibitor phosphoenolpyruvate, the curves become less sigmoidal, finally becoming a parabola (Figure 12.3.2B). Thus, both the activator and the inhibitor lower the Hill coefficient from 3.8 to 1, but whereas ADP increases the affinity of the enzyme for its substrate, the inhibitor decreases it (Figure 12.3.2D; Blangy et al, 1968). This was consistent with the allosteric hypothesis (Monod et al, 1965) which proposed that an allosteric activator would stabilise the protein in the R state, increasing the affinity for the ligand, and that an allosteric inhibitor would favour the T state, reducing the affinity for ligand binding (see above). It therefore appears that the Hill coefficient is 1 when the protein is completely converted into either the R state or the T state (Blangy et al, 1968).

The data could best be fitted to a mathematical model in which the protein is a tetramer. In their analysis, Monod and his colleagues assumed that the transition between the R and T states is complete, ie, there are no intermediate states in which the protein exists as a hybrid of the R and T states (Blangy et al, 1968). However, the authors found that the results could also be fitted to a mathematical model in which the protein is a hexamer which exists not only as R_6 and T_6, but also in a hybrid state such as R_3T_3.

Blangy et al (1968) found that whereas phosphofructokinase interacts with one of its substrates, fructose-6-phosphate, in a cooperative manner, the interaction of the enzyme with its other substrate, ATP, is non-cooperative. Thus, the rate-concentration curves with respect to ATP are hyperbolic and can be fitted in the Lineweaver-Burk representation of

kinetic behaviour, from which a Michaelis constant can be calculated. This K_m for ATP was independent of the concentration of fructose-6-phosphate, and ATP had no effect on the Hill coefficient for fructose-6-phosphate. However, ADP acted as a competitive inhibitor with respect to ATP. These data were interpreted as indicating that the enzyme has separate binding sites for fructose-6-phosphate (which is cooperative) and for ATP (which is non-cooperative), ie, the R and T states of the protein have the same affinities for ATP (Blangy et al, 1968).

Blangy et al (1968) predicted the structure and the allosteric regulation of phosphofructokinase with no knowledge of the amino acid sequence or the three dimensional architecture of this enzyme. Evans and his colleagues in Cambridge solved the crystal structure of the enzyme and provided direct experimental evidence for Monod's predictions regarding this enzyme (Schirmer & Evans, 1990). Phosphofructokinase is indeed a tetramer, consisting of four identical subunits; each pair of subunits is rigidly linked together to form a dimer (Figure 12.3.3; Schirmer & Evans, 1990). Each subunit consists of two domains, a larger one which binds the substrate ATP and a smaller one which binds the other substrate, fructose-6-phosphate. The allosteric effectors (ADP and phosphoenolpyruvate) bind to a separate effector site. The allosteric transition consists of a rotation of one dimer relative to the other by 7^o. The ATP binding site is formed almost entirely by the large domain, whereas the fructose-6-phosphate site consists of residues from both domains and from the other dimer. The ATP binding site remains almost unchanged during the allosteric transition, and this accounts for the lack of cooperativity of ATP which was observed by Blangy et al (1968), expressed as equal affinity of ATP for the T and R states. In contrast, the binding site for fructose-6-phosphate is drastically altered during the allosteric transition: in the R state, the phosphate group of fructose-6-phosphate binds to the positively charged guanidinium group of an arginine in the other dimer; in the T state, this guanidinium group swings out and is replaced by the negatively charged carboxyl group of a glutamate residue. In this way, the negatively charged phosphate group of fructose-6-phosphate can no longer bind to the enzyme, and this accounts for the large decrease in the affinity of the T state for fructose-6-phosphate. When the allosteric effectors bind to their separate site, a hinge movement takes place, and the width of the site is then determined by the size of the effector molecule: the smaller phosphoenolpyruvate induces a narrow cleft which is associated with the T structure, whereas the larger ADP induces a wider cleft which is associated with the R structure. Thus, the allosteric transition in this enzyme is

accomplished predominantly by a change in quaternary structure, with minimal change in tertiary structure, as had been predicted by Monod.

In their mathematical analysis, Blangy et al (1968) predicted that the transition between the R and T states is complete, ie, there are no intermediate states in which the protein exists as a hybrid of the R and T states (see above). This was confirmed by studies on the crystal structure of the enzyme: in the R structure, there is a layer of water molecules between the two dimers; when the dimers rotate, the water molecules are removed and hydrogen bonds form between the two dimers. All the water molecules must be either present or absent, and the alternative contacts would be disrupted in an intermediate conformation. Therefore, intermediate states between the T and R structures are unstable (Schirmer & Evans, 1990).

12.3.2.3 Cooperativity and allosteric regulation in haemoglobin

The binding of oxygen to haemoglobin is described by a sigmoidal curve which does not fit the saturable adsorption model of Michaelis and Menten (1913). Hill (1910) formulated an equation which described the data (see below). Monod and his colleagues used Hill's formulation to express the cooperativity of the binding process, as described above. The oxygen affinity of haemoglobin rises with increasing oxygen saturation; this cooperativity ensures that haemoglobin functions as a transport protein, being either fully saturated (in the lungs) or fully desaturated (in peripheral tissues). Figure 12.3.4 shows the equilibrium curves for uptake of oxygen by haemoglobin in the presence of various allosteric effectors. The data are presented as Hill plots, ie, log substrate concentration *vs* log (fraction bound sites/fraction free sites). The slope of each curve at the midpoint is the Hill coefficient, which is the measure of the degree of cooperativity (Imai, 1982; Perutz, 1990). The allosteric effectors tend to shift the haemoglobin equilibrium curves towards one or other of the extremes which are defined by a Hill coefficient of 1, as in the case of the effects of the allosteric regulators on phosphofructokinase.

Human haemoglobin is a tetramer of two α globin chains and two β globin chains. Each chain is associated with one haem molecule. Globin keeps the iron in the ferrous state which is the only form which can bind reversibly with oxygen. The mechanism of cooperativity in human haemoglobin is based on an equilibrium between two alternative quaternary structures of the tetramer, the deoxygenated or T structure and the oxygenated or R structure (Perutz, 1990). The transition from the R to the T structure involves a rotation of the dimer

$\alpha_1\beta_1$ relative to the dimer $\alpha_2\beta_2$ by 12-15°. The contacts $\alpha_1\beta_1$ and $\alpha_2\beta_2$ remain rigid. The transition brings a histidine residue closer to the iron, enabling a molecule of oxygen to bind both to the histidine and to the iron (Perutz, 1990). Binding of one oxygen molecule initiates the transition from the T state to the R state, increasing the affinity of haemoglobin for the binding of subsequent oxygen molecules.

12.3.2.4 Allosteric change in membrane pores

The principles of allosteric conformational change have been demonstrated in membrane proteins which form pores. There are several structural mechanisms whereby membrane pores can be opened, as is summarised below.

"Gated" ion channels are opened upon binding of appropriate ligands or by changes in voltage or pH. The nicotinic acetylcholine receptor consists of a ring of five subunits which are designated α, α, β, γ and δ. These subunits are arranged around a central channel which allows the entry of Na^+ into the cells and exit of K^+. The subunits are bent or 'kinked' about their midpoints, so they can touch each other at the level of the inner leaflet of the plasma membrane; this forms the "gate" of the channel (Figure 12.3.5A; Unwin, 1995; Miyazawa et al, 1999). The kinks are points of flexure between two α helical segments. In the absence of acetylcholine, the kinks in the five subunits point inwards and touch each other and the "gate" is therefore closed. Acetylcholine binds to a distant site which is located at the interface of two subunits, one of which is an α subunit. The quaternary amine of acetylcholine binds not to a negatively charged residue, but to a group of five aromatic residues which form a box which is open at the end where the ligand enters; binding of the quaternary amine is via cation-π interactions (Brejc et al, 2001). When acetylcholine binds to the receptor, the α subunits rotate clockwise, turning the other subunits along with them. The rotation of the subunits causes the kinks to point to the side, so the channel "gate" is open. The kink in each subunit contains a leucine residue; in the closed state, the constricted part of the channel is formed by these leucine residues whose hydrophobic side chains form a ring which prevents the passage of ions through the channel (Figure 12.3.5A; Unwin, 1995). In the open state, the parts of the helices which face the channel are occupied by serine and threonine residues whose polar side chains allow the passage of ions through the

channel (Figure 12.3.5A; Unwin, 1995).

Mammalian potassium channels are homo-tetramers, ie, they consist of four identical subunits, each of which is comprised of six membrane spanning domains. In *Streptomyces lividans,* each subunit is comprised of two membrane spanning domains and the four subunits are arranged in the shape of an inverted tepee, with a wide extracellular orifice and a narrow intracellular orifice (Figure 12.3.5B; Doyle et al, 1998). The extracellular part of the channel contains the "selectivity filter" which prevents the entry of sodium ions. The selectivity filter is composed of a rigid ring of carbonyl oxygen atoms; the geometry of this negatively charged ring is such that dehydrated potassium ions can fit into the ring, whereas dehydrated sodium ions are too small (Doyle et al, 1998). These voltage-activated channels contain an unusual amino acid sequence, the S4 segment, which consists of a basic residue (arginine or lysine) at every third position. When the gate is closed, the S4 segment forms a helix within the plasma membrane. When intracellular voltage increases as a result of the influx of Na^+, the positively charged S4 segment is believed to move across the plasma membrane to the extracellular side; such a movement may involve a change in the secondary structure, such that the segment may change from a helix to a loop (Aggarwal & MacKinnon, 1996). The intracellular part of the channel contains the "gate" mechanism. In the *Streptomyces lividans* potassium channel, the second membrane-spanning domain rotates counterclockwise (viewed from the exterior), swinging away from the central axis of the channel. This widens the entrance to the pore at the intracellular surface of the channel. This opens the channel for entrance of potassium ions from the cytoplasm. The rotation does not change the positions of the extracellular ends of the second membrane-spanning domain, so the diameter of the extracellular orifice remains unchanged (Figure 12.3.5C; Perozo et al, 1999). Depolarisation of the cell interior opens these potassium channels; the channels then close by a process which is called "inactivation". The mechanism of inactivation appears to be attributable to movement of the amino terminal of one of the four subunits into the central cavity, where it blocks the channel (Zhou et al, 2001).

Voltage-sensitive sodium channels initiate action potentials in nerves, muscles and other excitable cells. These channels are activated by voltage and become rapidly inactivated thereafter. The α-subunit of this channel is a protein which consists of 24 membrane-spanning domains which are arranged as four homologous groups, each comprised of six membrane-spanning domains (Catterall, 2001). In each homologous group, the fourth

membrane spanning domain is positively charged and these domains act as the voltage sensor. According to a low resolution 3-D density map, the four homologous groups are arranged symmetrically around a central axis which does not consist of a single pore, but is split into four branches, each of which traverses the plasma membrane (Sato et al, 2001). The "gate" is formed by the short loop which connects domains III and IV; this loop is located at the intracellular exit of the pore. The precise mechanism whereby voltage opens the pore of the sodium channel and the gate shuts it are not known.

Another mechanism of allosteric change in membrane proteins is exemplified by gap junctions. These are channels which allow diffusion of large molecules (up to 1000 Da) between cells. These channels close in the presence of increased concentrations of H^+ or Ca^{2+}. The gap junction consists of six identical subunits, each containing four membrane-spanning α-helices. Each subunit is in the shape of a rod and the six units are arranged symmetrically around an aqueous pathway. In the absence of calcium, the six rods are in a tilted posture around a central channel. In the presence of calcium, the six rods stand upright by 7^o and this movement closes the channel (Figure 12.3.5D; Unwin, 1987).

12.3.2.5 Allosteric change in transport enzymes

A further example of allosteric change in membrane proteins is the rotational movement which is exemplified by the mitochondrial ATPase, which is believed to apply to related proteins such as the V-ATPase. These proteins possess Walker motifs which bind and hydrolyse ATP in a reversible manner. The hydrolysis or synthesis of ATP is coupled to movement of protons through a channel in the protein structure. A plot of the ATP concentration *vs* the catalytic activity of the protein is described by a parabola and the data conform to the Michaelis-Menten model (Yasuda et al, 2001). These proteins were described in Section 7.5.8.

Walker motifs are also present in proteins which are designated ATP-binding casette (ABC) transporters. These include eukaryotic proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR) and the multiple drug resistance (MDR) pump. Prokaryotic proteins in this group include nutrient pumps such as histidine permease. Each pump contains two nucleotide binding domains and twelve membrane-spanning domains. The prokaryotic pumps consist of multiple subunits which are encoded by separate genes,

whereas in the eukaryotic pumps, the entire structure is contained in a single protein. ABC transporters mediate energy-dependent transport processes; binding of a substrate to the transporter activates the ATPase and the energy is used to transport the substrate across the cell membrane (Sarkadi et al, 1992; Al-Shawi & Senior, 1993; Liu et al, 1997).

The crystal structure of the ATP-binding subunit of *Salmonella typhimurium* histidine permease has been described (Hung et al, 1998). Each ATP-binding domain is in the shape of an "L" which includes the Walker A motif. The adenine base of ATP is stacked against the aromatic group of a tyrosine residue ("Tyr-16" in Figure 12.3.6). The β-phosphate of ATP binds to the Walker A motif and the γ-phosphate forms a hydrogen bond with a serine residue. The γ-phosphate of ATP is linked via a water molecule ("Wat-407" in Figure 12.3.6) to an aspartate (Asp-178) which is highly conserved in ABC transporters. Glu 179 and Gln 100 which are also highly conserved, form hydrogen bonds with another water molecule ("Wat-437") which interacts with the γ-phosphate of ATP through a hydrogen bond which is parallel to the bond between the γ-phosphate and the β-oxygen; this is the bond which breaks during the hydrolysis of ATP. Mutagenesis experiments confirmed the significance of the individual amino acids which are described above, as point mutations of these residues caused a loss of binding efficacy (Hung et al, 1998). Presumably, binding of ATP is followed by a movement in the arms of the "L" conformation, resulting in a break in the bond between the γ-phosphate and the β-oxygen of ATP. Movement of the arms of the "L" could open the channel which allows entry of histidine across the membrane (Welsh et al, 1998).

12.3.2.6 Analysis of Transport-P in terms of allosteric change

Figure 11.4.1 shows the relationship between the extracellular concentration of prazosin and the specific uptake of prazosin in GT1-1 GnRH peptidergic neurones at equilibrium, represented as a linear plot. The shape of the curve is sigmoidal and the data do not fit the Michaelis-Menten model. Clearly, cooperativity may be a mechanism of the prazosin paradox in peptidergic neurones.

It is envisaged that the Transport-P carrier forms a pore which allows the entry of small

amounts of prazosin. At concentrations greater than 10^{-7} M, prazosin facilitates its own entry via the Transport-P pore. This is done by a specific chemical interaction between the furan group of prazosin and the Transport-P carrier. In the absence of knowledge of the structure of the Transport-P carrier, the following interactions have been considered:

A. Allosteric change in a membrane pore:

1. Opening of a gated pore: In the allosteric mechanisms which are utilised to open membrane pores, the pore is envisaged as being either open or shut. This does not accord with the data on the uptake of prazosin via Transport-P. If it is assumed that exceeding the threshold (10^{-7} M) opens the gated pore, then it would be expected that when the gate is open, there would be an abrupt increase in the entry of [^{3}H]prazosin and that further increases in the concentration of unlabelled prazosin would displace [^{3}H]prazosin; however, this is not the case. Progressive increases in the concentrations of unlabelled prazosin above 10^{-7} M cause further, progressive increases in the accumulation of [^{3}H]prazosin (Figures 11.1.1 and 11.2.7); saturation is not seen until the concentration of unlabelled prazosin reaches $2x10^{-6}$ M, above which the uptake of [^{3}H]prazosin is inhibited, presumably due to competition between [^{3}H]prazosin and unlabelled prazosin (Figure 11.4.1). Therefore, the gating mechanisms which have been described for the ligand-gated cation channels and the voltage-gated ion channels (see above) are unlikely to provide a full explanation for the mechanism of uptake of prazosin in peptidergic neurones.

2. A second explanation for the prazosin paradox is that prazosin causes progressive widening of the pore. In that case, it can be expected that there would be a loss of specificity when the gate is wide open; in particular, it would be expected that in the presence of prazosin at 10^{-6} M, the peptidergic neurones would accumulate prazosin analogues which do not possess a furan group. However, the reverse was in fact observed; prazosin at 10^{-6} M reduced the accumulation of BODIPY FL prazosin (at $1.77x10^{-7}$ M; Figure 11.7.6). Thus, activation of Transport-P is not accompanied by loss of specificity for the ligand, and progressive widening of a pore is an unsuitable model. In this regard, Transport-P is distinguishable from the P2X receptors for ATP, in which repeated applications of ATP cause widening of the cation channel which is integral to the receptor, allowing the entry of

very large cations non-selectively (Surprenant et al, 1996; Khakh, 2001).

3. A third possibility is that activation of Transport-P involves progressive widening of a pore which has a selectivity filter. In the case of the voltage gated K^+ channel, opening the pore allows K^+ to leave the cells without allowing Na^+ to enter the cells. The proposed molecular mechanism of this filter was described above. If such a filter exists in the Transport-P carrier, then BODIPY FL prazosin can be expected to be able to pass through this filter. In that case, widening of the pore would be expected to allow the entry of greater amounts of BODIPY FL prazosin. However, this was not seen, and the reverse was in fact observed (Figure 11.7.6). Therefore, at present, this model also seems unlikely.

4. A fourth and most likely explanation is that prazosin increases its own affinity for Transport-P by exposing additional binding sites which are "constrained" in the absence of prazosin. This would be analogous to the binding of oxygen to haemoglobin which is described above; the first oxygen molecule binds with low affinity because all four ferrous atoms are in the T structure; however, binding of the first oxygen molecule favours the equilibrium towards the R structure in which the ferrous atoms are more accessible, thus increasing the affinity for binding of the second, third and fourth oxygen molecules. However, the proposed mechanism for Transport-P would differ from haemoglobin as follows: in the case of haemoglobin, there are only two states (T and R). This would not account for the progressive increase in accumulation of [^{3}H]prazosin in the presence of increasing concentrations of unlabelled prazosin, as described above. Therefore, the proposed mechanism for Transport-P would involve intermediate states in which the number of available binding sites can be increased progressively. Such a mechanism would explain the progressive, paradoxical increase in accumulation of [^{3}H]prazosin (Figures 11.1.1, 11.2.7 and 11.4.1) and would account for the preservation of the specificity of binding, despite the increased flux of prazosin upon activation of the uptake process.

B. Activation of a transport ATPase:

Activation of Transport-P requires cellular energy (Section 11.3). It is therefore possible that binding of prazosin at concentrations greater than 10^{-7} M may activate a transport ATPase. This could in turn provide the energy for the allosteric change which would increase the affinity for prazosin; phosphorylation of the protein would be a plausible mechanism which

is amenable to examination by further experiments.

ABC transporters mediate energy-dependent transport processes. These transporters possess the conserved Walker motifs which bind ATP and catalyse its hydrolysis to ADP. Binding of a substrate to the transporter activates the ATPase and the energy is used to transport the substrate across the cell membrane. ABC transporters include the CFTR pump, MDR pump and the histidine permease. The SV2A cDNA which was isolated from the GnRH neurone library also contains Walker motifs (Section 11.9.1). In the *Salmonella typhimurium* histidine permease, a plot of ATP concentration *vs* ATPase activity appears to have a sigmoidal shape, indicating that hydrolysis of ATP takes place in a cooperative manner (Liu et al, 1997). However, uptake of histidine is not cooperative; the histidine uptake curve is parabolic and the data fit the Lineweaver-Burk representation which describes the Michaelis-Mentin model (Ames & Lever, 1970; Kustu & Ames, 1974; Prossnitz et al, 1989; Liu & Ames, 1997). Therefore, the known ABC transporters are unlikely to have the functional properties which would explain the observations on the uptake of prazosin in the peptidergic neurones. Nevertheless, it is possible that the functional properties of Transport-P may be mediated by some unknown transport ATPase.

Summary of possible molecular mechanisms of Transport-P:

Mechanism	*Objection*
Opening of a gated pore	Prazosin paradox is progressive
Progressive widening of a pore	Selectivity is preserved
Pore widening with a selectivity filter	No increase in uptake of prazosin analogues
Progressive exposure of constrained sites	None at present
Activation of a transport ATPase	None at present
Known ABC transporter	Uptake by ABC transporters not cooperative

It is proposed that prazosin binds to the Transport-P carrier protein in at least two sites: the basic amine and the furan group. According to allosteric theory, the low ligand affinity of the T state is due to increased number of bonds between the subunits (Monod et al, 1965). It is therefore proposed that binding of the furan group of prazosin results in the breaking of bonds in the carrier protein; this "relaxes" the T state of the carrier and allows binding of further prazosin molecules to sites which were hitherto inaccesible in the constrained T structure of the carrier. The allosteric transition which follows the binding of the first

prazosin molecules therefore increases the affinity for binding of subsequent molecules, and this is seen as the paradoxical increase in accumulation of [^{3}H]prazosin. By analogy with human haemoglobin, the overall structure of the carrier is preserved during the allosteric transition, so there is no loss of specificity of the uptake process.

In their mathematical analysis of phosphofructokinase, Blangy et al (1968) found that the data could best be fitted to a mathematical model in which the protein undergoes a complete transition between the R and T states, ie, there are no intermediate states in which the protein exists as a hybrid of the R and T states. However, the authors found that the results could also be fitted to a mathematical model in which the protein is a hexamer which exists not only as R_6 and T_6, but also in a hybrid state such as R_3T_3. In the case of Transport-P, the existence of such hybrid states may explain why there is a progressive increase in the binding of [^{3}H]prazosin as the concentration of unlabelled prazosin is increased. It is proposed that the Transport-P carrier possesses multiple binding sites for prazosin; above a certain thershold (equivalent to an extracellular prazosin concentration of 10^{-7} M), binding of prazosin exposes, progressively, hitherto constrained sites. It therefore appears that once the threshold is exceeded, the allosteric change is likely to be sequential rather than concerted.

In the absence of knowledge of the structure of the Transport-P carrier, it is not possible to state whether it consists of a single protein or of multiple units. Many allosteric proteins are oligomers which consist of interacting subunits. However, cooperativity may arise in a single unit without a change in quaternary structure and this has been demonstrated in several enzymes (Cornish-Bowden & Cardenas, 1987). Acetylcholine esterase is a monomeric enzyme whose crystal structure has been resolved and some aspects of its chemistry are understood at the atomic level (Taylor & Radic, 1994). The catalytic site is situated in a deep and narrow gorge within the molecule; the quaternary amine of acetylcholine binds to the aromatic group of a tryptophan residue in a π-cation interaction. Binding of certain inhibitors to a distant site at the entrance to the gorge is believed to cause movement of the aromatic group of the tryptophan to a position which blocks access of acetylcholine to the active site. This allosteric mechanism in the monomeric molecule is believed to be the mechanism of action of some acetylcholine esterase inhibitors (Ordentlich et al, 1995).

Prazosin was not accumulated by the SK-N-SH noradrenergic neurones which possess the pre-synaptic $Uptake_1$ noradrenaline transporter and the synaptic vesicle monoamine transporter (Figure 11.1.4). Further, there was no evidence of a desipramine-sensitive prazosin paradox in these SK-N-SH neurones. Therefore, the cooperative effect of prazosin is not exerted on all transport proteins; this effect of prazosin is likely to be exerted on a specific transport protein which exists in peptidergic neurones, and which has been designated Transport-P.

Antidepressants and phenylethylamines (such as verapamil) are substrates for the uptake process but they do not activate Transport-P. Thus, these compounds are competitive inhibitors of the uptake of prazosin and they accumulate in the peptidergic neurones, but they do not increase the accumulation of [^{3}H]prazosin (Sections 11.2 & 11.6). This is analogous to the interaction of the two substrates for phosphofructokinase which are described above: the enzyme interacts with one of its substrates, fructose-6-phosphate, in a cooperative manner, but the interaction of the enzyme with its other substrate, ATP, is non-cooperative (Blangy et al, 1968). Therefore, ligands for Transport-P can be divided into two groups:
Group A compounds activate Transport-P and are internalised by Transport-P in peptidergic neurones. These compounds interact with the carrier in a cooperative manner. Their accumulation in the peptidergic neurones is described by a sigmoidal function which is likely to represent an allosteric transition in the carrier. These compounds bind to the carrier via the basic amine and they activate the carrier via the furan group. Prazosin is the prototype of this group. The chemical properties of these compounds are the subject of a collaborative study with Professor CR Ganellin FRS.

Group B compounds are internalised by Transport-P in peptidergic neurones, but they do not activate the uptake process. This group includes phenylethylamines (such as verapamil) and the antidepressants. These compounds are incapable of activating the uptake process as they do not possess the furan group. Thus, the accumulation curves in the peptidergic neurones are hyperbolic and can be described by the Michaelis-Menten model. The chemical properties of this group of compounds are as follows:

1. They have a basic amine which is neither permanently neutral nor permanently charged.
2. Compounds which have the greatest affinity for Transport-P are condensed cyclic structures of 18 to 20 carbons.
3. Phenolic halogens increase the affinity of these compounds for Transport-P.

4. Phenolic hydroxyl groups reduce affinity for Transport-P.
5. Phenolic methoxyl groups have no effect on affinity for Transport-P.

According to allosteric theory, "no direct interactions of any kind need occur between the substrate of an allosteric protein and the regulatory metabolite which controls its activity" (Monod et al, 1963). This has been corroborated in the case of haemoglobin, as the heterotropic ligands (protons, CO_2 and 2,3-diphosphoglycerate) bind to sites which are distant from the haem iron (Perutz, 1990). At present, it is not possible to state whether the competitive action of the Group B compounds is exerted by binding to the same site as prazosin, or whether they exert their effects by binding to another site at which they exert an inhibitory allosteric effect. This will be addressed in future using more detailed kinetic analysis of the effects of these compounds on the uptake of prazosin, and by mutagenesis studies of the Transport-P carrier protein, when its sequence finally becomes available.

12.3.2.7 Proposed molecular mechanism of Transport-P

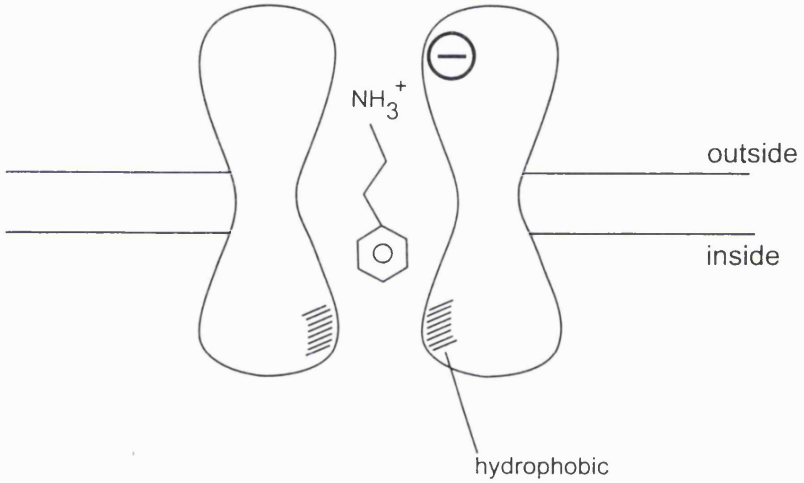
In summary, it is proposed that Transport-P is an allosteric membrane protein which is activated by its ligand prazosin when the concentration of prazosin exceeds the threshold of 10^{-7} M. Prazosin binds to the Transport-P carrier protein in at least two sites: the basic amine and the furan group. Binding of the furan group of prazosin results in the breaking of bonds in the carrier protein; this "relaxes" the T state of the carrier and allows binding of further prazosin molecules to sites which were hitherto inaccesible in the constrained T structure of the carrier. The allosteric transition which follows the binding of the first prazosin molecules therefore increases the affinity for binding of subsequent molecules, and this is seen as the paradoxical increase in accumulation of [^{3}H]prazosin. The overall structure of the carrier is preserved during the allosteric transition, so there is no loss of specificity of the uptake process. The allosteric change is likely to be sequential rather than concerted; this would explain the progressive increase in the binding of [^{3}H]prazosin as the concentration of unlabelled prazosin is increased. In addition to compounds such as prazosin which interact with the carrier in a cooperative manner, Transport-P also interacts with phenylethylamines and antidepressants. These compounds are incapable of activating the uptake process as they do not possess the furan group. The accumulation of the Group B compounds can be described by the Michaelis-Menten model. At present, it is unknown whether the competitive action of the Group B compounds is exerted by binding to the same site as prazosin, or

whether they exert their effects by binding to another site at which they exert an inhibitory allosteric effect. This will be addressed in future studies on Transport-P.

The analysis in this Section has limited the alternatives for the molecular nature of the Transport-P carrier, and should help to guide further experiments.

Figure 12.3.1:

Model of the uptake of phenylethylamines via Transport-P. It is proposed that the Transport-P carrier may possess a negatively charged pocket to which the basic amine binds. The carrier may also possess a hydrophobic region which must be traversed by the ligand. In order to be accumulated via Transport-P, the ligand must first acquire a proton, becoming positively charged and thus binding to the negatively charged pocket. The ligand would then lose the proton, becoming neutral, and this enables it to traverse the hydrophobic region in the carrier. Thus, neutral amines would not be able to bind to the carrier, and quaternary and guanyl amines would bind but would not be able to traverse the hydrophobic region in the Transport-P carrier.



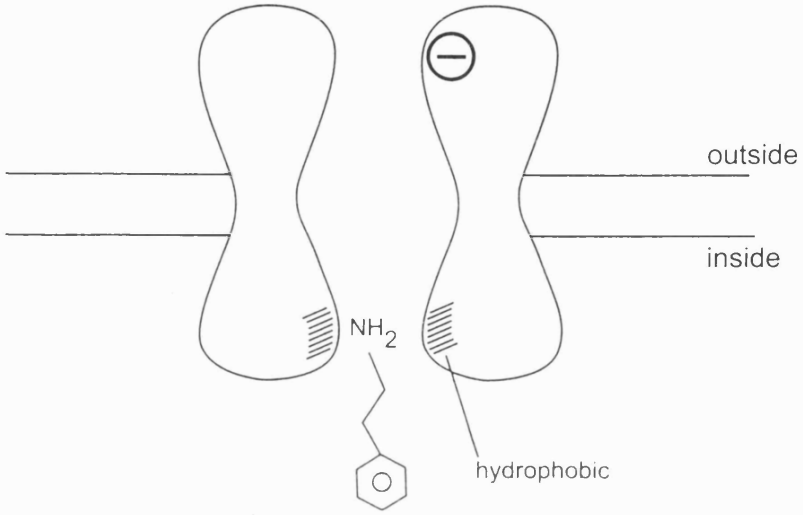


Figure 12.3.2:

Kinetics of the allosteric interactions of phosphofructokinase (from Blangy et al, 1968):

A & B. Initial velocity of the reaction as a function of the concentration of the substrate, fructose-6-phosphate, with or without different concentrations of the allosteric activator ADP (Panel A) and the allosteric inhibitor, phosphoenolpyruvate (Panel B).

C. Data from A and B plotted as v/V_{max} (vertical axis) *vs* log substrate concentration. The Hill coefficient is the slope of the curve at $v/V_{max} = 0.5$, ie, at 50% of maximal initial velocity. V_{max} was calculated by plotting the data according to Lineweaver & Burk (1934), ie, 1/S *vs* 1/V, using only points corresponding to high substrate concentration.

D. Variation of the Hill coefficient (vertical axis) as a function of substrate concentration corresponding to half maximal intial velocity. Each point corresponds to a curve in C.

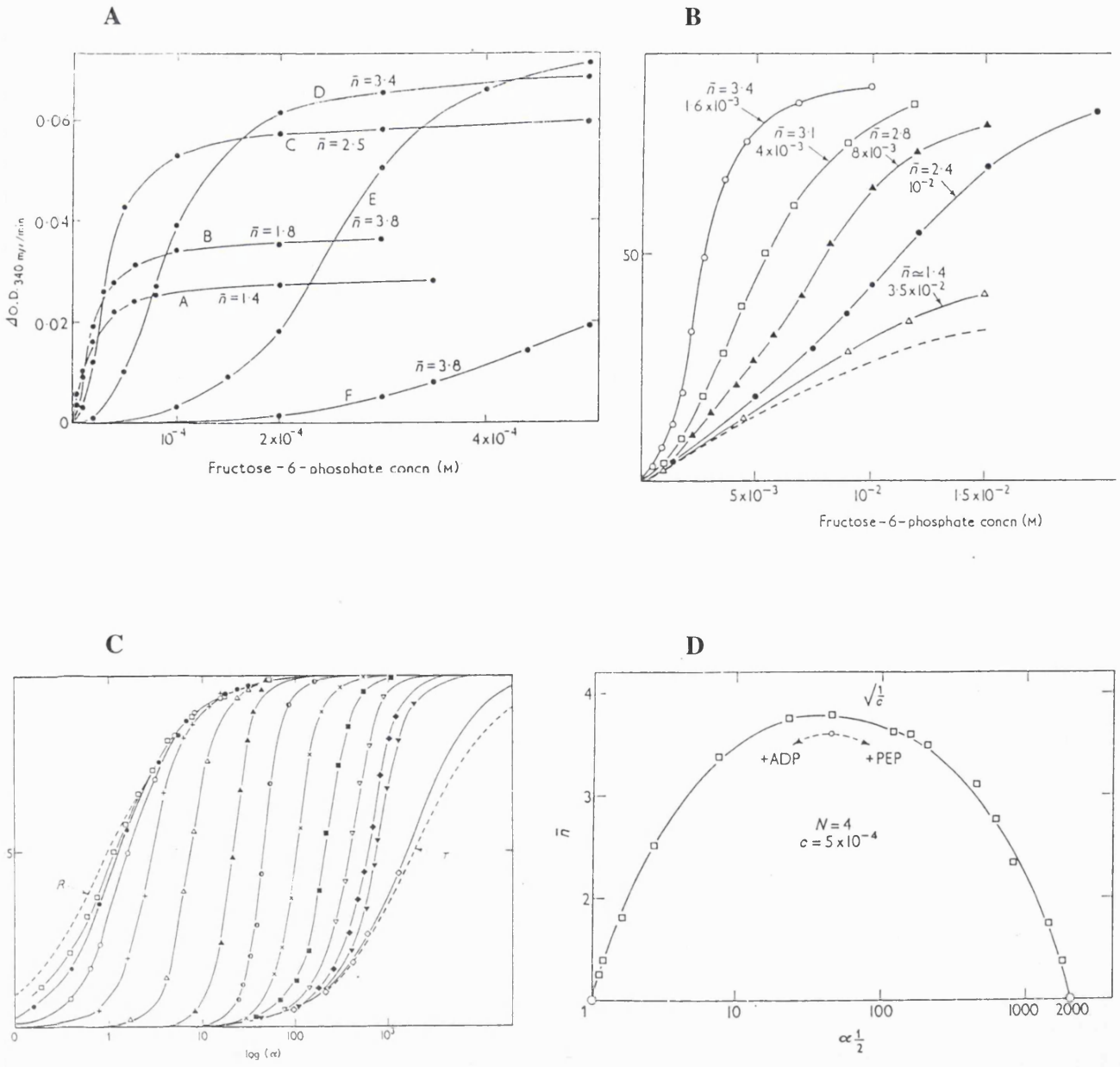


Figure 12.3.3:

General view of phosphofructokinase and the structural changes which occur during the allosteric transition (reproduced from Schirmer & Evans, 1990).

Phosphofructokinase is a tetramer, consisting of four identical subunits; each pair of subunits is rigidly linked together to form a dimer. The active (catalytic) site consists of separate regions for binding of the substrates fructose-6-phosphate ("F6P") and ATP which is converted to the product ADP. The effector site binds the allosteric regulators ADP and phosphoenolpyruvate. The allosteric transition from the T state to the R state consists of a rotation of one dimer relative to the other by 7°. This is shown by the arrow and by the broken lines for the C-D dimer. The ATP binding site remains almost unchanged during the allosteric transition, and this accounts for the lack of cooperativity of ATP which was observed by Blangy et al (1968), expressed as equal affinity of ATP for the T and R states. In contrast, the binding site for fructose-6-phosphate is drastically altered during the allosteric transition: in the R state, the phosphate group of fructose-6-phosphate binds to the positively charged guanidinium groups of Arg162 ("R162") and Arg243 ("R243") of the other dimer; in the T state, the guanidinium group of Arg 162 swings out and is replaced by the negatively charged carboxyl group of a glutamate residue. In this way, the negatively charged phosphate group of fructose-6-phosphate can no longer bind to the enzyme, and this accounts for the large decrease in the affinity of the T state for fructose-6-phosphate.

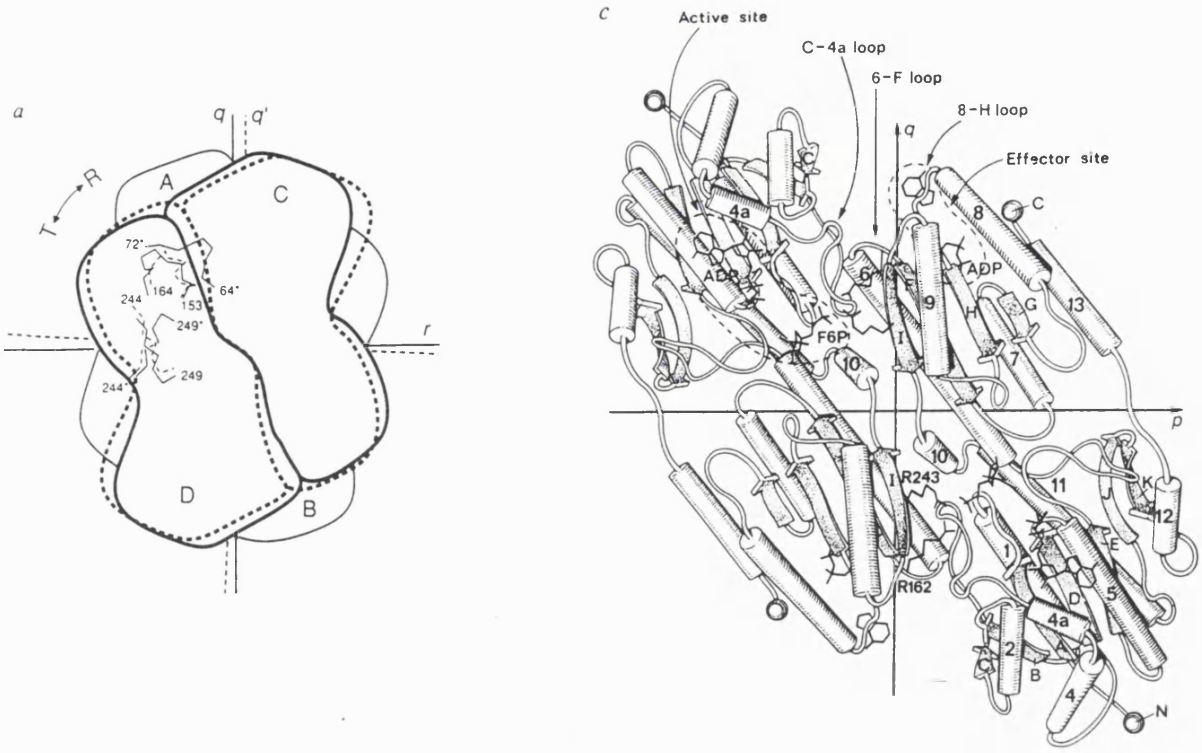


Figure 12.3.4: The haemoglobin oxygen dissociation curves at equilibrium

A. The haemoglobin curve is sigmoidal but the curve for myoglobin is parabolic; myoglobin is a monomer which does not display cooperative binding. From Monod et al, 1963.

B. Oxygen uptake in the presence of allosteric effectors. The data are Hill plots, ie, log substrate concentration *vs* log (fraction bound sites/fraction free sites). The slope of each curve at the midpoint is Hill's coefficient which is a measure of cooperativity. Haemoglobin M Milwaukee remains in the T structure even when it is saturated with oxygen, so there is no cooperativity and the Hill coefficient is 1. Plots for free α and β subunits show no cooperativity as there is no allosteric regulation in these monomers. The extremes of the Hill plots correspond to the effects of the allosteric regulators (ADP and phosphoenolpyruvate) on phosphofructokinase. Reproduced from Imai, 1982.

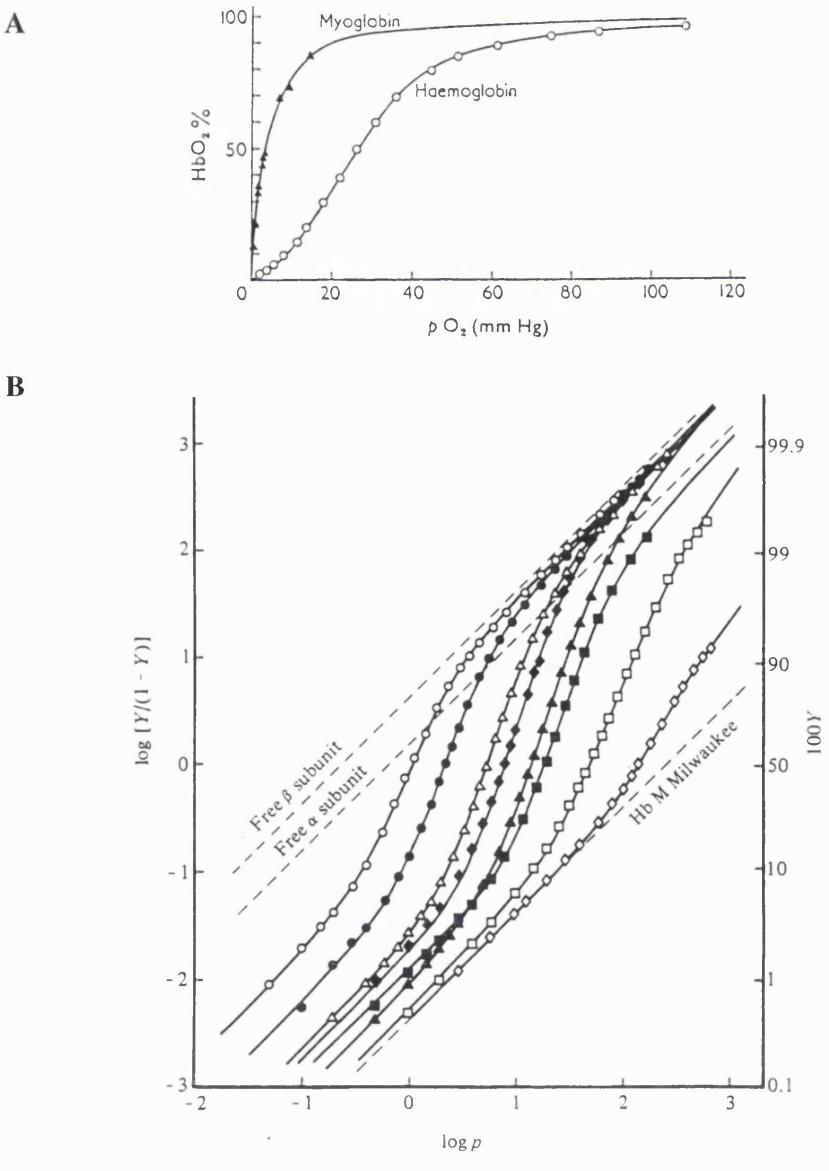


Figure 12.3.5: Examples of allosteric mechanisms in membrane pores

A. The nicotinic receptor consists of 5 subunits which are kinked at their midpoints, so they touch each other; this is the "gate". When acetylcholine binds, the subunits rotate, so the kinks point sideways and the gate is open. The kink in each subunit has a hydrophobic leucine which prevents passage of ions through the closed channel. When open, the parts of the helices which face the channel are occupied by serine and threonine whose polar side chains allow passage of ions through the channel. From Unwin, 1995.

B & C. Potassium channels consist of four subunits which are arranged in the shape of an inverted tepee. The intracellular part contains the channel gate. From Doyle et al, 1998. The helices of the channel rotate, swinging away from the central axis. This widens the the intracellular entrance, allowing exit of potassium ions. From Perozo et al, 1999.

D. Gap junctions consist of six subunits which are arranged in a tilted posture. In the presence of calcium, the rods stand upright; this closes the channel. From Unwin, 1987.

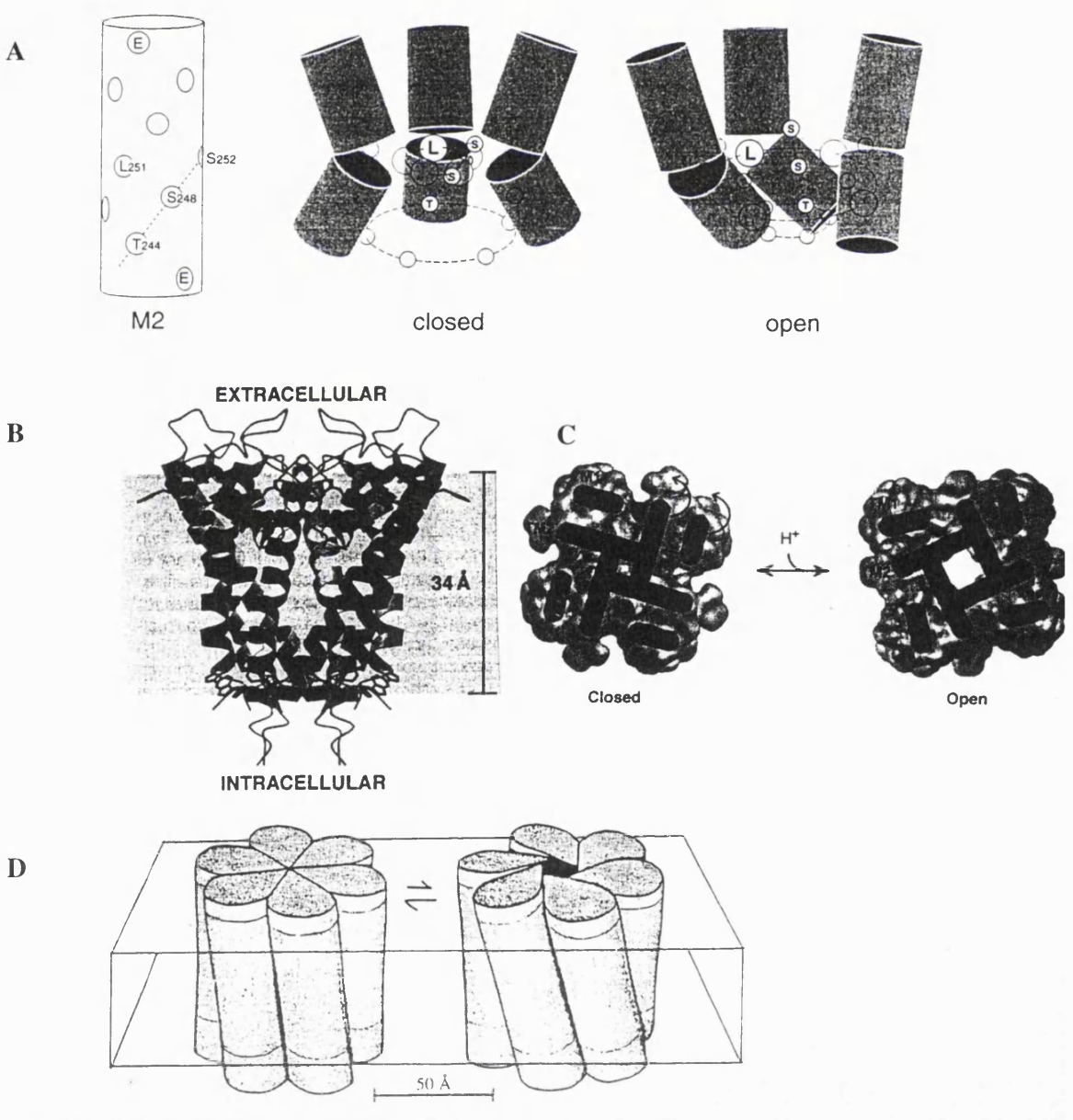
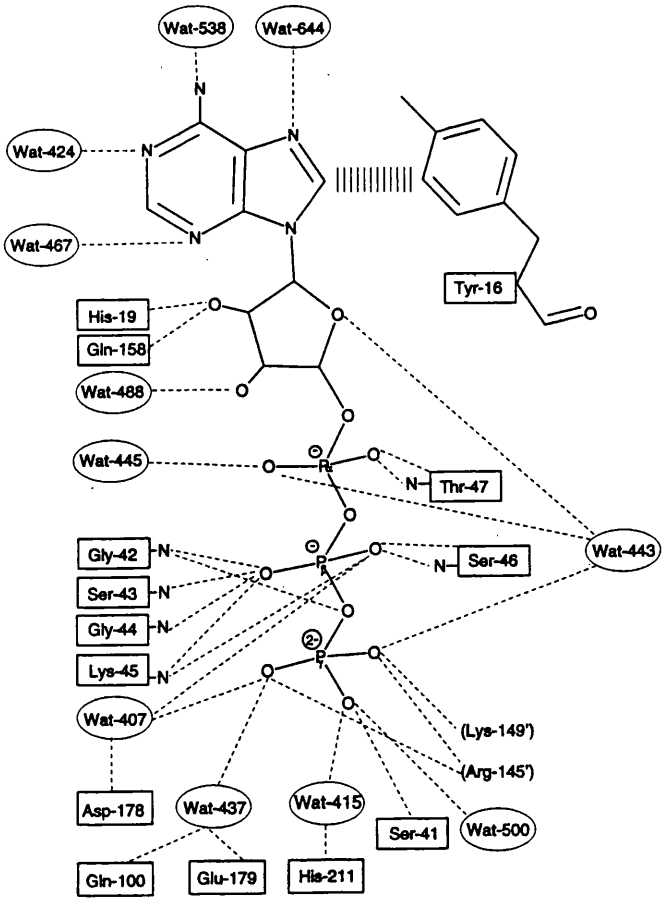


Figure 12.3.6: Allosteric change in an ABC transporter

In the histidine permease of *Salmonella typhimurium,* each ATP-binding domain is in the shape of an "L" which includes the Walker A motif. The adenine base of ATP is stacked against the aromatic group of a tyrosine residue (Tyr-16). The β-phosphate of ATP binds to the Walker A motif and the γ-phosphate forms a hydrogen bond with a serine residue. The γ-phosphate of ATP is linked via a water molecule (Wat-407) to an aspartate (Asp-178) which is highly conserved in ABC transporters. Glu 179 and Gln 100 which are also highly conserved, form hydrogen bonds with another water molecule (Wat-437) which interacts with the γ-phosphate of ATP through a hydrogen bond which is parallel to the bond between the γ-phosphate and the β-oxygen; this is the bond which breaks during the hydrolysis of ATP. Presumably, binding of ATP is followed by a movement in the arms of the "L" conformation, resulting in a break in the bond between the γ-phosphate and the β-oxygen of ATP. Reproduced from Hung et al, 1998.



12.4 TRANSPORT-P AND THE ANTIDEPRESSANT EFFECT

Endogenous depression is a common mental illness which is associated with significant morbidity and mortality. Tricyclic antidepressants and their newer derivatives are the main treatment for this disease. The prevailing view is that these compounds exert their therapeutic effect by inhibiting the pre-synaptic re-uptake of neurotransmitter amines. However, there are objections to a hypothesis which is based purely on inhibition of pre-synaptic re-uptake. Transport-P is a new factor in this field: therapeutic concentrations of antidepressants are active at Transport-P. A new hypothesis is presented, based on the work on Transport-P. This hypothesis resolves the problems with the pre-synaptic re-uptake hypothesis and offers a unitary explanation for hitherto inexplicable observations. If the hypothesis is correct, compounds which act as potent and selective ligands for Transport-P would have a more rapid onset of action and would represent an advance in the treatment of depressive illness.

12.4.1 Depressive illness

Endogenous depression is a mental illness of unknown aetiology, causing a disorder of mood or affect. It is distinguishable from 'exogenous or reactive depression' in which sadness is precipitated by some identifiable external event, such as a loss or bereavement. Endogenous depression is a common disease with significant morbidity and mortality. The point prevalence in Great Britain, Western Europe and the United States is approximately 5-15% and is consistently twice as great in women as in men (Kraepelin, 1921; Weissman & Boyd, 1984; Kessler et al, 1994). The annual incidence is 250-350 cases/100,000 which approximates to 180,000 new cases in Great Britain each year (Gath, 1996). In approximately 10% of patients, depression alternates with mania ('bipolar disorder'). The annual incidence of bipolar disorder is 3-10/100,000 (Weissman & Boyd, 1984; Gath, 1996). Approximately 10% of severely depressed patients die by suicide (Guze & Robins, 1970; Goodwin & Jamison, 1984; Squillace et al, 1984). Many patients who receive modern therapy remain disabled by their illness. In view of its prevalence and potentially serious consequences, depressive illness has become a major target for pharmaceutical development.

In addition to frank psychiatric disease, many individuals possess emotional temperaments which have been described as *manic*, *depressive* or *cyclothymic*; these temperaments are thought to predispose to the development of frank psychiatric disease (Kraepelin, 1921). In any case, the frequency of these personality traits among the general population has led some physicians to extend the prescription of antidepressants to individuals who would not

conventionally be described as suffering from a psychiatric disease.

The current concept of the disease was introduced by Kraepelin (1921) who defined 'manic-depressive insanity' and distinguished it from schizophrenia. Before Kraepelin, the way had been paved by French investigators who had defined a group of mental illnesses by their 'circular' (ie, periodic) nature. However, Kraepelin believed that 'circularity' was not a defining aspect of mental disease. He defined 'manic-depressive insanity' as a unitary syndrome consisting of the clinical symptoms and signs of depression, mania and manic-depression. Kraepelin's views were confirmed independently in the 1950s when it was found that schizophrenia responded to phenothiazines, whereas depression responded to tricyclics; this was consistent with the view that these were separate diseases which were likely to have a neurochemical basis. To a large extent, the unitary view is still accepted, although it is now felt that depressive illness can be divided into two sub-groups which are described as unipolar and bipolar. The distinction of these sub-groups is as follows:

1. In bipolar disease, there are usually recurrent episodes of depression and mania, whereas unipolar disease usually consists of depression alone.
2. A family history is much more common in bipolar than in unipolar disease, suggesting a genetic basis in bipolar disease.
3. Unipolar disease responds to tricyclics whereas bipolar disease responds to lithium. Tricyclics may aggravate the manic phase of depressive disease.

In the absence of objective laboratory tests based on disease aetiology, affective disorders are defined on the basis of clinical features. The clinical features of depressive illness fall into three categories:

1. Disordered affect: depression is characterised by low mood, impaired capacity for enjoyment, fatigue, social withdrawal, helplessness, passivity, hypochondria and suicidal ideas. The opposite features are seen in mania, which is characterised by euphoria, grandiose ideas and hyperactivity which is usually ineffectual.
2. Disordered hypothalamic function, such as disturbances of circadian rhythms, autonomic function, sleep pattern, appetite, reproductive function and secretion of pituitary hormones;
3. In severe cases, there may be psychotic features such as hallucinations and delusions which are concerned with guilt, worthlessness and persecution. Some patients become stuporous and completely unresponsive to external stimuli (Kraepelin, 1921). Early treatment may prevent the appearance of psychotic features (Klerman, 1984).

Most depressive episodes last three to nine months but some persist for years. Relapse is common, especially in bipolar disease (Kraepelin, 1921; Gath, 1996). As patients age, the frequency and the duration of both unipolar and bipolar relapses usually increases, so disease-free intervals become shorter (Kraepelin, 1921; Goodwin & Jamison, 1984). Further, many patients suffer a gradual decline in personal and social performance, because of the illness itself and its social consequences. In many patients, depressive episodes are precipitated by stressful events and individuals who cannot cope with stress appear to be more liable to developing depressive illness (Kraepelin, 1921). The role of stress has attracted much interest but its significance is unclear as the onset of many physical and mental illnesses can be shown statistically to have been associated with stressful events.

12.4.2 The biogenic amine hypothesis of depressive illness

The aetiology of endogenous depression is unknown. Genetic factors are probably more important in the aetiology of bipolar disorder than in depression alone, as patients with bipolar disorder have a far greater frequency of positive family history than patients with depression alone. In families afflicted with bipolar disease, the risk of depressive illness is 100% in monozygotic twins and approximately 30% in first degree relatives of index patients (MacKinnon et al, 1997). However, no genetic mutations have been linked with certainty to the disease phenotype. Depression is a feature of some endocrine diseases including Cushing's syndrome, hypothyroidism and hypercalcaemia. Depression may also be an adverse effect of some medications such as reserpine, clonidine, progestogens and glucocorticoids. However, in the vast majority of depressed patients, the aetiology of the disease is unknown. The hypothesis which has attracted the greatest attention is the biogenic amine hypothesis of affective disorders. According to this hypothesis, depression is due to deficiency of the action of serotonin or noradrenaline at post-synaptic receptors in the brain (Schildkraut, 1965; Coppen, 1967). The evidence for this hypothesis is:

1. Depression may follow administration of drugs which are known to deplete brain noradrenaline and serotonin (eg, reserpine);
2. Depression can be treated with drugs which are known to increase extracellular concentrations of noradrenaline and serotonin (eg, monoamine oxidase inhibitors and tricyclic compounds).

The region of the brain which is responsible for endogenous depression is unknown, but the

hypothalamus is likely to be involved in this disease:

1. Derangements of hypothalamic function are prominent among the clinical features of endogenous depression (see above).

2. The human hypothalamus receives the densest innervation of both noradrenergic and serotonergic neurones, the cell bodies of which are located in the brain stem (Bertler, 1961; Nobin & Bjorklund, 1973; Pearson et al, 1979).

3. The hypothalamus contains the greatest density of antidepressant binding sites in the human brain (Rehavi et al, 1980; Langer et al, 1981).

12.4.3 The pre-synaptic re-uptake hypothesis

Tricyclic compounds and their newer derivatives are the main pharmacological treatment for depressive disease. They are used in the treatment of both unipolar and bipolar disease; in the latter, they may be combined with lithium, in order to prevent tricyclic-induced exacerbation of mania (Potter et al, 1991; Baldessarini, 1996). These compounds are also used as long term prophylaxis, to prevent recurrence of depressive episodes (Montgomery, 1994). The mechanism of the therapeutic effect of these compounds is unknown, but they are believed to act by inhibiting the pre-synaptic re-uptake of neurotransmitter amines in the brain (Glowinski & Axelrod, 1964; Giros et al, 1994; Barker et al, 1994). Pre-synaptic nerve terminals possess two sequential uptake processes for neurotransmitter amines; transporter molecules which are located in the plasma membrane of pre-synaptic nerve terminals accumulate molecules of neurotransmitter from the extracellular synaptic space into the cytoplasm; a second set of transporters which are located in the membranes of neurosecretory vesicles then accumulate the amines from the cytoplasm into the interior of the vesicles. These uptake processes terminate the actions of the neurotransmitter amines by reducing their concentrations in the extracellular synaptic space (Iversen, 1967; Axelrod, 1971; Lester et al, 1994; Liu & Edwards, 1997). According to the pre-synaptic re-uptake hypothesis, antidepressants exert their therapeutic effect by blocking the pre-synaptic plasma membrane transporters for noradrenaline and serotonin, resulting in an increase in synaptic concentrations of these neurotransmitter amines and restoration of their excitatory post-synaptic action (Glowinski & Axelrod, 1964; Schildkraut, 1965; Coppen, 1967). However, there are problems with this hypothesis:

1. Inhibition of pre-synaptic re-uptake is immediate but the therapeutic antidepressant effect is delayed for 2-6 weeks (Oswald et al, 1972). This applies both to the tricyclic

antidepressants and to newer compounds, such as the serotonin-selective re-uptake inhibitors (SSRIs; Potter et al, 1991; Leonard, 1993). These findings indicate that inhibition of pre-synaptic amine re-uptake is insufficient for appearance of the therapeutic effect, and that other factors which have a slower time course must be involved. The delay is a problem as these patients become non-compliant and are prone to suicide risk.

2. There is no correlation between potency at inhibition of pre-synaptic re-uptake and clinical therapeutic potency (Morris & Beck, 1974; Leonard, 1993). Some antidepressants are highly potent in inhibiting the re-uptake of noradrenaline or serotonin whereas others are weak and non-selective; however, the clinical potency of all the antidepressants is very similar and they are all used in similar clinical doses, usually ranging from 20 to 200 mg/day (Leonard, 1993; Baldessarini, 1996; British National Formulary, 1998). This applies both to the tricyclic compounds and to the SSRIs (Leonard, 1993). For example, trimipramine is an analogue of imipramine which possesses a single branched methyl group in the side chain (Figure 12.4.1). This simple substitution makes trimipramine 39 fold less potent than imipramine at inhibition of the uptake of noradrenaline, and 60 fold less potent at inhibition of the uptake of serotonin (Richelson & Pfenning, 1984). If these two compounds exert their therapeutic effects by inhibiting the pre-synaptic re-uptake of these amines, one would expect imipramine to be administered in much smaller doses than trimipramine. However, these two compounds are administered in identical clinical doses (50-200 mg/day; British National Formulary, 1998). Paroxetine and fluoxetine are classified as SSRIs. Paroxetine is 19 fold more potent than fluoxetine in inhibiting the uptake of serotonin (Bolden-Watson & Richelson, 1993). If these compounds exerted their therapeutic effects by inhibiting the pre-synaptic re-uptake of serotonin, paroxetine would be expected to be effective in smaller doses than fluoxetine; yet these two compounds are administered in identical doses clinically (20-60 mg/day; British National Formulary, 1998; Figure 12.4.2). This lack of correlation between potency at inhibition of pre-synaptic re-uptake and clinical therapeutic potency suggests that the antidepressant effect may be mediated by some unidentified action of these compounds, at which they have similar potencies.

3. Some compounds which inhibit pre-synaptic re-uptake have no antidepressant activity. Such compounds include cocaine and amphetamines (Hare et al, 1962; Overall et al, 1962; Post et al, 1974). These compounds increase the state of arousal in normal subjects and in depressed patients but they are not useful in treating depression; they have no antidepressant

effect and they make depressed patients feel worse (Hare et al, 1962; Overall et al, 1962; Post et al, 1974). Further, some potent SSRIs have no anti-depressant activity (Ghose et al, 1977). Cocaine inhibits the pre-synaptic plasma membrane transporters for serotonin, noradrenaline and dopamine whereas amphetamines are more potent in inhibiting uptake of noradrenaline and dopamine than serotonin (Giros et al, 1994; Barker et al, 1994). Inhibition of the re-uptake of dopamine is unlikely to account for absence of an antidepressant effect; compounds such as bupropion also inhibit dopamine re-uptake but are effective antidepressants (Ascher et al, 1995). These observations indicate that inhibition of pre-synaptic re-uptake is insufficient for appearance of the antidepressant effect.

4. "Atypical antidepressants": bupropion, trazodone, nefazodone, trimipramine and mianserin are clinically effective antidepressants which have weak effects on the pre-synaptic re-uptake of noradrenaline and serotonin, in comparison to the tricyclics and SSRIs (Richelson & Pfenning, 1984; Bolden-Watson & Richelson, 1993). Trimipramine and mianserin are structurally related to the tricyclics whereas the others are a heterogeneous group. Trazodone and nefazodone are of particular interest: these two compounds are much more potent at antagonising post-synaptic adrenergic and serotonergic receptors than at inhibiting the pre-synaptic re-uptake of noradrenaline and serotonin (Figure 12.4.3; Bolden-Watson & Richelson, 1993; Cusack et al, 1994); their overall effect is therefore to block noradrenergic and serotonergic neurotransmission. Mianserin is another interesting compound as it is a very poor inhibitor of the pre-synaptic re-uptake of serotonin (Richelson & Pfenning, 1984). It was thought to act as an antagonist of pre-synaptic α_2 adrenoceptors which inhibit noradrenaline release; however, mianserin is in fact more potent as an antagonist of post-synaptic α_1 adrenoceptors (Richelson & Nelson, 1984; Figure 12.4.4), so its overall effect is to block noradrenergic neurotransmission. The mechanism of the therapeutic action of the atypical antidepressants is unknown (Baldessarini, 1996). Thus, inhibition of pre-synaptic re-uptake is not a pre-requisite for the antidepressant effect of such compounds.

Clearly, inhibition of the pre-synaptic re-uptake of serotonin and noradrenaline does not provide an adequate explanation for the therapeutic action of antidepressant compounds. Other factors are likely to be involved.

12.4.4 The new hypothesis:

Tricyclic antidepressants and related compounds are internalised by Transport-P in post-synaptic neurones, where they accumulate in acidified intracellular vesicles. The normal function of these acidified vesicles is to degrade internalised post-synaptic receptors. Because of their basic amine groups, the antidepressants tend to neutralise the acidity of the vesicles. The rise of vesicular pH slows the rate of degradation of post-synaptic receptors. The increase in availability of post-synaptic receptors makes post-synaptic neurones more responsive to the excitatory effects of the neurotransmitter amines and improves the clinical features of endogenous depression.

Neurotransmitters down-regulate their post-synaptic receptors, so the density of post-synaptic receptors should be considered in relation to the concentration of the neurotransmitter in the extracellular synaptic space. Thus, chronic administration of antidepressants which inhibit the pre-synaptic re-uptake of noradrenaline causes a consistent reduction in the density of post-synaptic β adrenoceptors (Vetulani & Sulser, 1975; Banerjee et al, 1977). However, the density of post-synaptic α_1 adrenoceptors is either increased or unaffected by chronic administration of antidepressants (Menkes et al, 1983a; Vetulani et al, 1984; Maj et al, 1985; Stockmeier et al, 1987; Nowak & Przegalinski, 1988a & b; Nowak, 1989). Unchanged density of post-synaptic α_1 adrenoceptors despite the increase in neurotransmitter concentration can be regarded as a relative increase in the availability of post-synaptic receptors. This increase in relative availability of α_1 adrenoceptors may be due to the fact that they are colocalised with Transport-P in post-synaptic neurones (Section 11.1). Antidepressants also progressively increase the responsiveness of post-synaptic serotonergic receptors by an unknown mechanism (reviewed by Blier & DeMontigny, 1994). The interaction of Transport-P with the serotonergic system has not been investigated, but it is possible that Transport-P may modulate post-synaptic serotonergic receptors in a manner which is analogous to its proposed effect on α_1 adrenoceptors.

Tricyclic antidepressants and their derivatives are clearly effective in increasing extracellular concentrations of the neurotransmitter amines by inhibition of pre-synaptic re-uptake, and this increase in the availability of neurotransmitters is likely to contribute to the activation of post-synaptic neurones. However, in view of the objections described above, this inhibition

of pre-synaptic re-uptake is unlikely to be the primary mechanism of the therapeutic action of these compounds. In any case, if the aim of therapy is to increase the activation of post-synaptic neurones, then inhibition of pre-synaptic re-uptake is not the best therapeutic strategy. This is because increasing the extracellular concentration of neurotransmitters is followed by receptor down-regulation and reduced responsiveness of post-synaptic neurones. Thus, chronic administration of antidepressants reduces the sensitivity of post-synaptic neurones to the stimulant effect of noradrenaline on the production of c-AMP, due to down-regulation of post-synaptic β adrenoceptors (Vetulani & Sulser, 1975; Banerjee et al, 1977). In contrast, compounds which are internalised by Transport-P can be expected to increase the sensitivity of post-synaptic neurones to a given concentration of neurotransmitter, due to reduced degradation of post-synaptic receptors. A mechanism which acts via Transport-P can be expected to have a synergistic effect with inhibition of pre-synaptic re-uptake, resulting in more sustained activation of post-synaptic neurones.

A further problem which is associated with compounds which inhibit pre-synaptic re-uptake is that the increase in synaptic neurotransmitter concentrations activates pre-synaptic autoreceptors which inhibit the discharge rate of pre-synaptic neurones, resulting in reduced synthesis and release of neurotransmitter (Langer, 1977; Charney et al, 1981; Langer & Lehmann, 1988; Blier & DeMontigny, 1994; Romero et al, 1996). During chronic treatment with antidepressants, these inhibitory pre-synaptic autoreceptors gradually become desensitised, resulting in a partial recovery in the rate of synthesis and release of neurotransmitter (Crews & Smith, 1978; Svensson & Usdin, 1978; Spyraki & Fibiger, 1980; McMillen et al, 1980; Smith et al, 1981; Pilc & Vetulani, 1982; Kreiss & Lucki, 1995). Nevertheless, these inhibitory autoreceptor effects are clearly undesirable, as they diminish the activation of post-synaptic neurones. In contrast, compounds which act selectively on Transport-P can be expected to have no inhibitory effect on the release of neurotransmitters, and this should result in more effective activation of post-synaptic receptors.

The objections to the pre-synaptic re-uptake hypothesis in its present form can be resolved in the light of the Transport-P hypothesis:

1. The delay in the antidepressant effect is because available antidepressants do not activate Transport-P (Section 11.2). They accumulate in relatively small amounts in post-synaptic neurones, and it takes several weeks for them to exert a significant effect on the pH of the

acidified vesicles. Novel compounds which selectively activate Transport-P should accumulate more rapidly in post-synaptic neurones and their therapeutic effect should be more rapid. In collaboration with Professor CR Ganellin FRS, we are synthesising and testing novel compounds which are intended to activate Transport-P selectively. If the hypothesis is correct, such compounds would represent a significant advance, as they should improve compliance and may be particularly valuable in view of the risk of suicide in sub-optimally treated patients.

2. Absence of a correlation between clinical potency and potency at inhibition of pre-synaptic re-uptake is because inhibition of pre-synaptic re-uptake is not the primary site of the therapeutic action of antidepressants. It is proposed that their primary therapeutic effect is exerted on Transport-P in post-synaptic neurones. The antidepressants are almost equipotent at Transport-P (Table 11.2.1); for example, imipramine is equipotent to trimipramine and paroxetine is equipotent to fluoxetine (Figures 12.4.1 and 12.4.2). This is because all available antidepressants consist of a basic amine and a carbon skeleton which consists either of a condensed aromatic structure of 18-20 carbons, or of an aromatic structure with phenolic halogens (Figure 12.4.5). These are the chemical properties of the Group B compounds which are accumulated by Transport-P (Section 11.5). This explains why the antidepressants are equipotent at Transport-P. If the hypothesis is correct, this may explain why the antidepressants are equipotent clinically.

3. Cocaine and amphetamine: these compounds have poor affinity for Transport-P, in comparison to antidepressants:

Compound	*Relative potency at Transport-P*
Imipramine	1.00
Amphetamine	0.18
Cocaine	0.09

Amphetamine has poor affinity for Transport-P because its skeleton consists of only 9 carbons (Figure 11.5.4); compounds which have the greatest affinity for Transport-P possess 18-20 carbons in a condensed cyclic structure (Section 11.5.3 and Table 11.5.3). Cocaine has poor affinity for Transport-P because it possesses two carboxyl groups (Figure 11.4.3); these carboxyl groups reduce affinity for Transport-P (Section 11.5.1.1). These observations explain the poor affinities of amphetamine and cocaine for Transport-P. If the

hypothesis is correct, this may also explain why these compounds are not antidepressants.

4. "Atypical antidepressants": the chemical structures of these compounds conform to those of Group B ligands of Transport-P; they all possess hydrophobic phenyl groups and a basic amine which are separated by an alkyl side chain (Figures 12.4.3 and 12.4.5). All the atypical antidepressants which have been tested are ligands for Transport-P (Table 11.2.1); they can be expected to exert their therapeutic effect on the acidified vesicles as described above. Their poor effect on pre-synaptic re-uptake is not relevant, as it is proposed that this is not the primary site of action of the antidepressant effect.

The new hypothesis may explain the following observations:

1. Why are antidepressants concentrated in the brain?

Antidepressants block pre-synaptic transporters but they are not accumulated by pre-synaptic nerve terminals. Thus:

A. Sympathomimetic amines such as tyramine enter pre-synaptic nerve terminals via the pre-synaptic plasma membrane and vesicular transporters and they release accumulated neurotransmitter amines by exchange via the pre-synaptic transporters (Iversen, 1967; Raiteri et al, 1977; Johnson et al, 1982; Bonisch & Rodrigues-Pereira, 1983). In contrast, antidepressants bind to pre-synaptic nerve terminals but they do not release accumulated neurotransmitter amines (Paton, 1973; Raiteri et al, 1979; Bonisch & Rodrigues-Pereira, 1983).

B. [^{3}H]Imipramine which is bound to pre-synaptic nerve terminals cannot be released by tyramine which releases accumulated neurotransmitter amines via pre-synaptic transporters (Langer et al, 1982).

C. [^{3}H]Imipramine cannot be released by electrical stimulation which releases neurotransmitter amines from their vesicular storage sites in pre-synaptic nerve terminals (Langer et al, 1982).

D. [^{3}H]Imipramine cannot be released by calcium-dependent depolarisation which releases neurotransmitter amines from their vesicular storage sites in pre-synaptic nerve terminals (Langer et al, 1982).

E. Uptake of [^{3}H]5-HT via the serotonin transporter requires an electrochemical gradient of sodium ions. In contrast, the association of [^{3}H]Imipramine with the serotonin transporter does not require any electrochemical gradient, suggesting that [^{3}H]Imipramine binds to the

transporter but does not enter the cells (Talvenheimo et al, 1979).

Antidepressants are therefore described as "dead end" inhibitors of pre-synaptic re-uptake. However, following peripheral administration, the concentrations of antidepressants in the brain far exceed their concentrations in plasma (Kafoe et al, 1976; Bickel et al, 1983; DeVane & Jarecke, 1992). Atypical antidepressants such as mianserin and bupropion which are poor inhibitors of the pre-synaptic re-uptake of serotonin and noradrenaline are also highly concentrated in the brain, confirming that accumulation in the brain is unrelated to inhibition of pre-synaptic re-uptake (Kafoe et al, 1976; Schroeder, 1983). The accumulation of antidepressants in the brain could not be explained by the lipophilic nature of these compounds, as brain concentrations far exceeded their concentrations in adipose tissue (Bickel et al, 1983). It was not possible to explain the concentration of antidepressants in the brain on the basis of existing knowledge (DeVane & Jarecke, 1992). Accumulation of antidepressants in post-synaptic neurones via Transport-P may explain the above observations.

2. Why do antidepressants have no phenolic hydroxyl groups?

Phenolic hydroxyl groups increase the affinity of ligands for the pre-synaptic re-uptake of noradrenaline and serotonin (Horn, 1973a & b). If antidepressants exert their therapeutic effects by inhibiting pre-synaptic re-uptake, one would expect at least some clinically effective antidepressants to have phenolic hydroxyl groups, in order to increase their affinity for their target site. However, compounds with phenolic hydroxyl groups have not been developed as antidepressants. Indeed, analogues of tricyclic antidepressants which possess phenolic hydroxyl groups are known to inhibit pre-synaptic re-uptake, to cross the blood brain barrier and appear in human cerebrospinal fluid (reviewed in DeVane & Jarecke, 1992); however, these compounds have not been developed as antidepressants, presumably because they have no antidepressant activity. Phenolic hydroxyl groups reduce affinity for Transport-P (Section 11.5.1.3 and Figure 11.5.6), which is consistent with the hypothesis that antidepressants exert a therapeutic effect via Transport-P.

3. Why are α_1 adrenoceptors not down-regulated by chronic administration of antidepressants?

In rats, chronic administration of antidepressants which inhibit the pre-synaptic re-uptake of

noradrenaline reduces the density and responsiveness of β adrenoceptors , due to the increase in the extracellular synaptic concentration of noradrenaline (Vetulani & Sulser, 1975; Banerjee et al, 1977; Wolfe et al, 1978; Schweitzer et al, 1979; Hall et al, 1984; Mogilnicka et al, 1987). However, the density of post-synaptic α_1 adrenoceptors is either increased or unaffected by chronic administration of antidepressants (Menkes et al, 1983a; Vetulani et al, 1984; Maj et al, 1985; Klimek et al, 1985; Stockmeier et al, 1987; Mogilnicka et al, 1987; Nowak & Przegalinski, 1988a & b; Nowak, 1989; Martin et al, 1989). It was not possible to explain this difference on the basis of the pre-synaptic re-uptake hypothesis (Charney et al, 1981; Maj et al, 1984). Some antidepressants act as α_1 adrenergic antagonists, but this is not the explanation for the increase in α_1 adrenoceptor density, because there was a parallel increase in the functional responsiveness of the adrenergic receptors to agonist stimulation, indicating that the receptors were not blocked by administration of antidepressants in the rats (Menkes & Aghajanian, 1981; Menkes et al, 1983b; Plaznik et al, 1984; Plaznik & Kostowski, 1985; Mogilnicka et al, 1987; Maj et al, 1989; Bijak, 1989; Przegalinski et al, 1991). Further, antidepressants which are weak α_1 antagonists (eg., citalopram and bupropion) had the same enhancing effect on α_1 adrenoceptor density as antidepressants which are much more potent α_1 antagonists (eg, mianserin and amitriptyline; Richelson & Nelson, 1984; Maj et al, 1985; Klimek et al, 1985; Nowak & Przegalinski, 1988a). This indicates that the increase in density of α_1 adrenoceptors is unrelated to α_1 adrenergic blocking activity.

α_1 Adrenoceptors are colocalised in post-synaptic neurones with Transport-P (Section 11.1). According to the new hypothesis, accumulation of antidepressants via Transport-P prevents receptor down-regulation. This may explain why α_1 adrenoceptors are not down-regulated following chronic administration of antidepressants, unlike β adrenoceptors .

4. Why do antidepressants cause hyponatraemia?

The following advice appears in the British National Formulary (1998): "Hyponatraemia

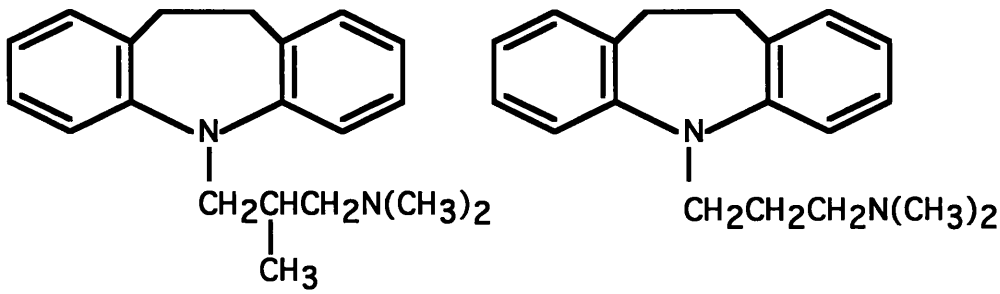
(usually in the elderly and possibly due to inappropriate secretion of antidiuretic hormone) has been associated with all types of antidepressants and should be considered in all patients who develop drowsiness, confusion or convulsions while taking an antidepressant". Chronic mild hyponatraemia is often asymptomatic but plasma sodium concentrations in the range 110-120 mmol/l may be associated with personality changes and confusion; severe hyponatraemia (sodium less than 110 mmol/l) may cause convulsions, coma and death (Baylis, 1996). The mechanism of antidepressant-induced hyponatraemia is unknown. Transport-P exists in vasopressin neurones (Al-Damluji et al, 1993); it seems possible that accumulation of antidepressants in vasopressin neurones may alter intracellular osmolality, causing inappropriate secretion of vasopressin and consequent hyponatraemia.

12.4.5 Conclusion:

The mechanism of action of antidepressants is unknown and the treatment of depressive illness remains unsatisfactory. Despite the availability of many antidepressants, many patients remain disabled by their disease. Newer antidepressants have fewer unwanted effects than the original tricyclic compounds, but they are still based on the pre-synaptic re-uptake hypothesis which was formulated thirty-five years ago. Transport-P is an antidepressant-sensitive uptake process for amines in post-synaptic neurones. It is possible that antidepressants may exert a therapeutic action on Transport-P, possibly in synergy with blockade of pre-synaptic re-uptake. If this hypothesis is correct, then compounds which act as selective and potent ligands for Transport-P would represent a novel approach which may have utility in the treatment of depressive illness.

Figure 12.4.1:

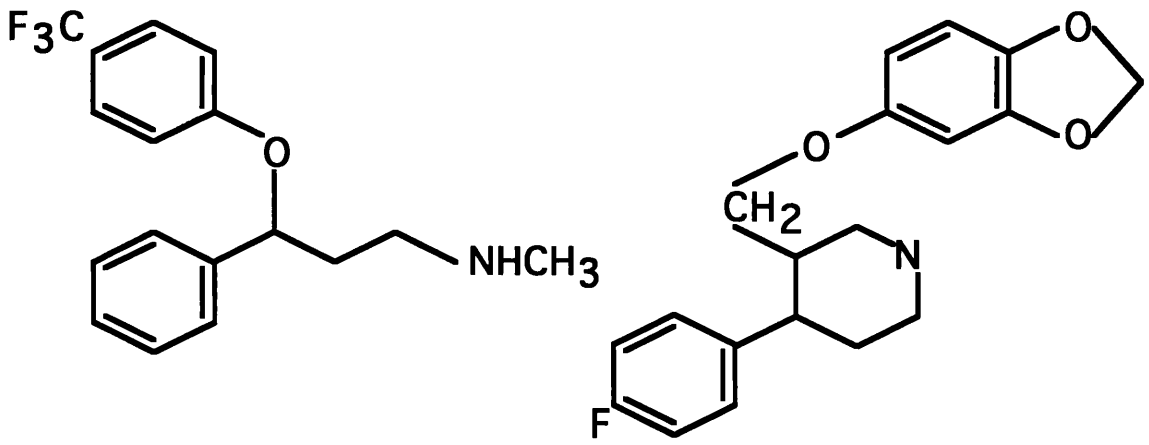
Trimipramine differs from imipramine by a single branched methyl group in the side chain. This simple substitution makes trimipramine 39 fold less potent than imipramine at inhibition of the uptake of noradrenaline, and 60 fold less potent at inhibition of the uptake of serotonin (Richelson & Pfenning, 1984). If these two compounds exert their therapeutic effects by inhibiting the pre-synaptic re-uptake of these amines, one would expect imipramine to be administered in much smaller doses than trimipramine. However, these two compounds are administered in identical clinical doses (British National Formulary, 1998). This suggests that these antidepressants may exert their therapeutic effects at some unknown site, at which they have equipotent effects.



	Trimipramine ***(Surmontil)***	**Imipramine** ***(Tofranil)***
Inhibition of noradrenaline uptake (K_i)	**510 nM**	**13 nM (39 fold as potent)**
Inhibition of serotonin uptake (K_i)	**2500 nM**	**42 nM (60 fold as potent)**
Transport-P (IC_{50})	**270 nM**	**460 nM**
Tablets	**10 mg & 25 mg**	**10 mg & 25 mg**
Clinical dose	**50-300 mg/day**	**50-300 mg/day**

Figure 12.4.2:

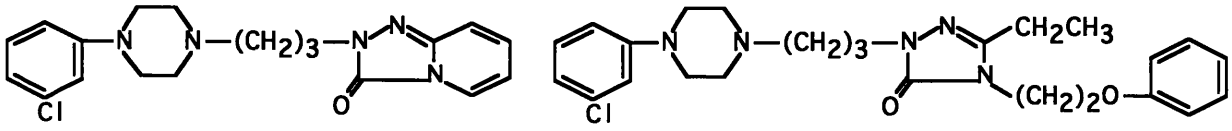
Paroxetine and fluoxetine are classified as serotonin-selective re-uptake inhibitors (SSRIs). Paroxetine is 19 fold more potent than fluoxetine in inhibiting the uptake of serotonin (Bolden-Watson & Richelson, 1993). If these compounds exerted their therapeutic effects by inhibiting the pre-synaptic re-uptake of serotonin, paroxetine would be expected to be effective in smaller doses than fluoxetine; yet these two compounds are administered in identical doses clinically (British National Formulary, 1998). Although there may be differences in pharmacokinetics, the lack of correlation between potency at inhibition of pre-synaptic re-uptake and clinical therapeutic potency suggests that the antidepressant effect may be mediated by some unidentified action of these compounds, at which they have similar potencies.



	Fluoxetine *(Prozac)*	**Paroxetine *(Seroxat)***
Inhibition of serotonin uptake (K_i)	**14 nM**	**0.73 nM (19 fold as potent)**
Transport-P (IC_{50})	**1.0 uM**	**1.3 uM**
Tablets	**20 mg**	**20 mg**
Clinical dose	**20-60 mg/day**	**20-60 mg/day**

Figure 12.4.3:

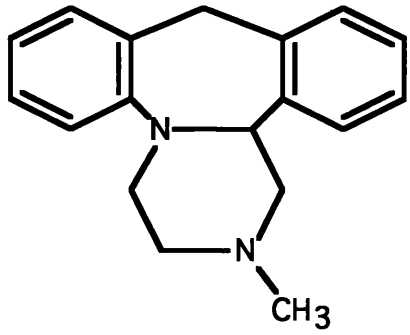
Trazodone and nefazodone are "atypical antidepressants". These two compounds are much more potent at antagonising post-synaptic adrenergic and serotonergic receptors than at inhibiting the pre-synaptic re-uptake of noradrenaline and serotonin (Bolden-Watson & Richelson, 1993; Cusack et al, 1994); their overall effect is therefore to block noradrenergic and serotonergic neurotransmission.



	Trazodone	Nefazodone
Inhibition of noradrenaline uptake (K_i)	**9500 nM**	**570 nM**
Antagonism of alpha-1 adrenergic receptors (K_i)	**42 nM**	**48 nM**
Inhibition of serotonin uptake (K_i)	**490 nM**	**137 nM**
Antagonism of 5-HT_{1A} serotonin receptors (K_i)	**96 nM**	**80 nM**
Antagonism of 5-HT_2 serotonin receptors (K_i)	**25 nM**	**26 nM**

Figure 12.4.4:

Mianserin is an "atypical antidepressant". It is a very poor inhibitor of the pre-synaptic re-uptake of serotonin (Richelson & Pfenning, 1984). It was thought to act as an antagonist of pre-synaptic α_2 adrenoceptors which inhibit noradrenaline release; however, mianserin is in fact more potent as an antagonist of post-synaptic α_1 adrenoceptors (Richelson & Nelson, 1984), so its overall effect is to block noradrenergic neurotransmission. The mechanism of the therapeutic action of the atypical antidepressants is unknown

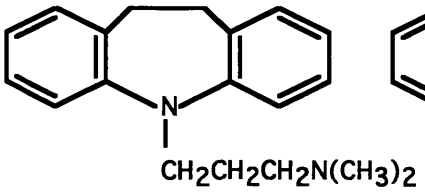


Antagonism of alpha-2 adrenergic receptors (K_i)	**73 nM**
Antagonism of alpha-1 adrenergic receptors (K_i)	**34 nM**
Inhibition of noradrenaline uptake (K_i)	**42 nM**
Inhibition of serotonin uptake (K_i)	**2300 nM**

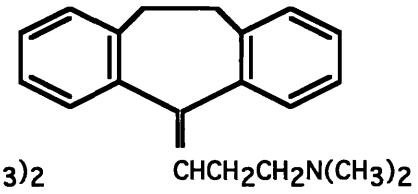
Figure 12.4.5:

Chemical structures of some commonly used antidepressant compounds.

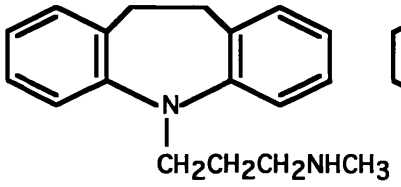
Tricyclic antidepressants



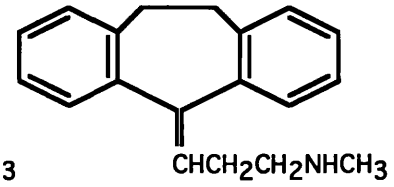
Imipramine



Amitriptyline

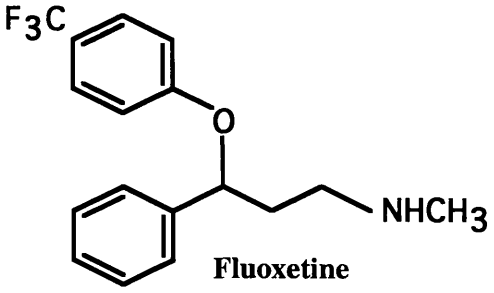


Desipramine

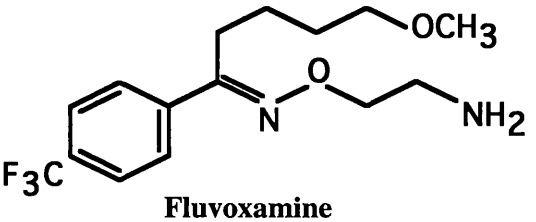


Nortriptyline

Serotonin-selective re-uptake inhibitors

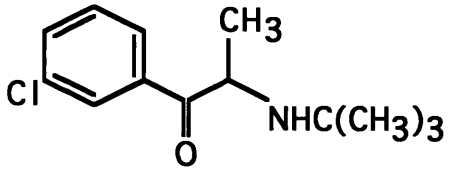


Fluoxetine

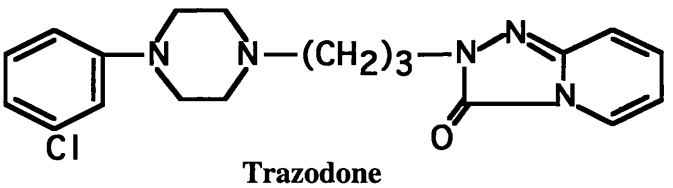


Fluvoxamine

Atypical antidepressants



Bupropion



Trazodone

13. ORIGINAL ARTICLES ARISING FROM THE THESIS

1. Al-Damluji, S. & Kopin, I.J. (1996) Functional properties of the uptake of amines in immortalised peptidergic neurones (transport-P). British Journal of Pharmacology, 1996, 117, 111-118.

2. Al-Damluji, S. & Kopin, I.J. (1996) Binding and competitive inhibition of amine uptake at post-synaptic neurones (transport-P) by tricyclic antidepressants. British Journal of Pharmacology 117, 811-816.

3. Al-Damluji, S., Porter, D., Krsmanovic, L.Z., Knutson, J.R.& Kopin, I.J. (1997) Visual detection of transport-P in peptidergic neurones. British Journal of Pharmacology 120, 876-882.

4. Al-Damluji, S. & Kopin, I.J. (1998) Structural properties of phenylethylamine derivatives which inhibit transport-P in peptidergic neurones. British Journal of Pharmacology 124, 693-702.

5. Al-Damluji, S., Shen, W.B., White, S. & Barnard E.A. (2001) α_{1B} adrenergic receptors in gonadotrophin-releasing hormone neurones: relation to Transport-P. British Journal of Pharmacology 132, 336-344.

6. Al-Damluji, S. & Shen, W.B. (2001) Release of amines from acidified stores following accumulation by Transport-P. British Journal of Pharmacology 132, 851-860.

14. PATENT APPLICATIONS ARISING FROM THE THESIS

1. United Kingdom Patent application 9812624.6

   Title: Method for identifying antidepressant compounds

   Year: 1998

   Assigned inventor: Saad Al-Damluji

2. International Patent application PCT/GB99/01859

   Title: Method of identifying antidepressant compounds

   Year: 1999

   Assigned inventor: Saad Al-Damluji

3. United States Patent application 09/330,460

   Title: Method of identifying antidepressant compounds

   Year: 1999

   Assigned inventor: Saad Al-Damluji

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