LOCALISATION AND MODULATION OF $GABA_B$ AND 5-HT_3 RECEPTORS IN RODENT BRAIN: AN AUTORADIOGRAPHIC STUDY

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ABSTRACT

Autoradiographical visualisation of $GABA_B$ binding sites in sections of rat brain has extended the findings of previous reports in that within the frontal cortex, the distribution of such sites is clearly laminated into four distinct regions. Furthermore, $GABA_B$ binding sites have also been identified in the dorsal vagal complex of the brainstem, especially within the nucleus tractus solitarius (NTS). In parallel with these GABAergic medullary receptors, detailed analysis of the autoradiography of the 5-HT_3 receptor radioligand, [^{3}H]BRL43694 (as well as [^{3}H]zacopride, [^{3}H]GR65630 and [^{3}H]quipazine) has also been investigated within this region. Using histological markers in conjunction with autoradiography, 5-HT_3 binding sites were localised in the area postrema, dorsal motor vagal nucleus and the nucleus of the spinal tract of the trigeminal nerve. However, by far the highest concentration of sites was observed in the NTS.

As the NTS/dorsal vagal complex provides the site of termination for the majority of vagal afferent fibres, 5-HT_3, $GABA_B$ and $GABA_A$ receptor autoradiography was examined 10 days after vagal lesioning achieved via chronic unilateral nodose ganglionectomy. Since all three binding site categories were reduced ipsilaterally in the dorsal vagal complex, this suggested that such sites are likely to be located presynaptically on vagal afferent fibres that terminate in this region.

Conflicting and inconsistent reports regarding $GABA_B$ receptor modulation by chronic antidepressant treatment prompted the use of receptor autoradiography (restricted to the sub-laminal regions of the frontal cortex) in an attempt to resolve this issue. Administration of imipramine (14 days) either orally or via subcutaneously implanted osmotic minipumps failed to increase $GABA_B$ receptor numbers in this region. In contrast, chronic oral and i.p. administration (21 days) of desipramine

significantly up-regulated $GABA_B$ receptors in lamina I of the frontal cortex with a concomitant beta-adrenoceptor down-regulation. Repeated adminstration of amitriptyline, paroxetine or the centrally-active $GABA_B$ receptor antagonist, CGP 35348 was ineffective at modulating $GABA_B$ receptors. Conversely, another putative $GABA_B$ receptor antagonist, designated as Compound X, like desipramine, also increased $GABA_B$ receptor densities in the frontal cortex, but again only in lamina I.

Despite the increase in $GABA_B$ receptor numbers produced by despramine, this drug, like the majority of the above treatment regimes, failed to increase the sensitivities of the $GABA_B$ receptor-modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase activity. However, treatment with Compound X produced an apparent enhancement of the $GABA_B$ receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase.

The phosphonic acid derivative of GABA, 3-aminopropylphosphinic acid (3-APA), has been characterised as a $GABA_B$ receptor agonist. Whilst it is 10 times more potent than (-)-baclofen at displacing [^{3}H]GABA from $GABA_B$ binding sites, it is equipotent with (-)-baclofen at inhibiting forskolin-stimulated adenylyl cyclase. Conversely, whilst (-)-baclofen is a full agonist with respect to the noradrenaline-stimulated system, 3-APA appeared to act as a partial agonist in this response. These contrasting findings may be indices of the possible existence of $GABA_B$ receptor subtypes.

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ABBREVIATIONS

AP	area postrema
BDZ	benzodiazepine
BRL 24924	[(±)-(endo)]-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo-[3.3.1]-non-4-yl)benzamide hydrochloride
BRL 43694	endo-N-(9-methyl-9-azabicyclo[3.3.1]-non-3-yl)-1-methyl-1H-indazole-3-carboxamide hydrochloride; Granisetron
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine 3', 5'-monophosphate
CGP 20712A	2-hydroxy-5-(2-((2-hydroxy-3-(4-((1-methyl-1-4-trifluoromethyl)1H-imidazol-2-yl)-phenoxy)propyl)-amino)ethoxy)- benzamide monoethane sulphonate
CGP 35348	P-(3-aminopropyl)-P-diethoxymethyl-phosphinic acid
CNS	central nervous system
DMNX	dorsal motor vagal nucleus
ECT	electroconvulsive therapy
ENS	enteric nervous system
EPSPs	excitatory postsynaptic potentials
fmol	femtomole
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GDP	guanosine 5'-diphosphate
GDPγS	guanosine 5'-O-(2-thiodiphosphate)
GR38032F	(±)-1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)methyl]4-H-carbazol-4-one
GR65630	3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-

	1H-indol-3-yl)-1-propanone
GR67330	(±)1,2,3,9-tetrahydro-9-methyl-3-[(5-methyl-1H-imidazol-4-yl)methyl]-4H-carbazol-4-one
GTP	guanosine 5′-triphosphate
GTPγS	guanosine 5′-O-(3-thiotriphosphate)
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid
ICI 118,551	erythro-DL-1-(7-methyindan-4-y(oxy)-3-isopropyl-aminobutan-2-ol
ICS 205-930	(3α-tropanyl)-1H-indole-3-carboxylic acid ester
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IPSPs	inhibitory postsynaptic potentials
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen orthophosphate
LY 278584	1-methyl-N-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)1H-indazole-3-carboxamide
MDL 72222	1αH,3α,5αH-tropan-3-yl-3,5,-dichlorobenzoate
mg	milligram
$MgSO_4$	magnesium sulphate
µM	micromolar
NA	noradrenaline
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
$NaH_2PO_4.2H_2O$	sodium dihydrogen orthophosphate 2-hydrate
$Na_2HPO_4.2H_2O$	disodium hydrogen orthophosphate 2-hydrate
NaOH	sodium hydroxide

nM	nanomolar
nSpV	nucleus of the spinal tract of the trigeminal nerve
NTS	nucleus tractus solitarius
p.o.	per os
pmol	picomole
pM	picomolar
r	correlation coefficient
s.c.	subcutaneous
s.e.m.	standard error of the mean
[^{35}S]-t-TBPS	[^{35}S]-t-butylbicyclophosphorothionate
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
3-APA	3-aminopropylphosphinic acid
3-APPA	3-aminopropylphosphonic acid
3-APS	3-aminopropane sulphonic acid
5-HT	5-hydroxytryptamine
8-hydroxy-DPAT	8-hydroxy-2-di-N-propylaminotetralin
9G	4-amino-3-benzo[b]furan-2-yl butanoic acid
9H	4-amino-3-(5-methoxybenzo[b]furan-2-yl) butanoic acid

PUBLICATIONS ARISING FROM THIS THESIS

G.D. Pratt, C. Knott, R. Davey & N.G. Bowery. Characterisation of 3-aminopropyl phosphinic acid (3-APPA) as a $GABA_B$ agonist in rat brain tissue. **Br. J. Pharmac. (1989) 96, 141P.**

G.D. Pratt & N.G. Bowery. The 5-HT_3 receptor ligand, [^{3}H]-BRL 43694, binds to presynaptic sites in the nucleus tractus solitarius of the rat. **Neuropharmac. (1989) 28, 1367-1376.**

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G.D. Pratt & N.G. Bowery. Autoradiographical analysis of $GABA_B$ receptors in rat frontal cortex following chronic antidepressant administration. In: **$GABA_B$ receptors in mammalian function.** eds. Bowery N.G:, Bittiger H. & Olpe H.-R. Wiley. Chichester.

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CHAPTER 1

GENERAL INTRODUCTION

GABA AS A NEUROTRANSMITTER - HISTORICAL PERSPECTIVE

Since the initial discovery of the presence of GABA in the mammalian central nervous system (CNS) (Roberts & Frankel, 1950), research into identifying a role for GABA as a neurotransmitter; the demonstration of GABAergic receptors; the pharmacological manipulation of these receptors together with its therapeutic applications, has been prolific. Indeed, from the high concentrations of GABA found in the brain and spinal cord and its activity at between 20% and 40% of brain synapses (Bloom & Iversen, 1971), it may perhaps be described as the most ubiquitous central neurotransmitter. This is not to say, however, that GABAergic activity is confined to the CNS; GABA is also present (but at lower concentrations) in peripheral neurones and tissues (Erdo et al, 1985) and a role for GABA in intestinal peristalsis has been proposed (Ong & Kerr, 1983).

In order to establish a chemical as a putative neurotransmitter, certain criteria in favour of chemical neurotransmission must be fulfilled (Paton, 1958). Some of the evidence pertaining to GABA in this respect will be briefly reviewed.

Synthesis and distribution

The synthesis and metabolism of GABA function intimately with the pathways of oxidative metabolism via the tricarboxylic acid cycle and a metabolic 'shunt'. GABA is synthesised from the decarboxylation of L-glutamic acid via the pyridoxal phosphate-dependent enzyme, glutamic acid decarboxylase (GAD) (Roberts & Frankel, 1950). This enzyme, located within the cytoplasm of nerve terminals, has been employed as a specific endogenous marker for GABAergic neurones using immunohistochemistry (Roberts, 1979). These neurones are synonymous with those labelled by high affinity uptake (Neale et al, 1983). Within the CNS, the highest levels of GABA are found in the substantia nigra, globus pallidus and the

hypothalamus (Fahn et al, 1976) whilst anatomical studies reveal that it is located on small local circuit interneurones, for example, those innervating the Purkinje cells of the cerebellum and the basket cells of both the hippocampus and cerebellum.

Release

The depolarisation-induced synaptic release of GABA *in vitro* from nerve terminals in cortical synaptosomes (Levy et al, 1973) and slices (Mulder & Snyder, 1974) is calcium-dependent. A number of *in vivo* techniques have been employed to study endogenous GABA release, most recently using brain microdialysis (Tossman et al, 1986). GABA (and glycine) release is inhibited by the convulsant protein, tetanus toxin (Curtis et al, 1973), and enhanced by noradrenaline acting via α_2-adrenoceptors (Pittaluga & Raiteri, 1987). These authors also found that GABA could potentiate [^{3}H]noradrenaline release via $GABA_A$ receptors (see later for definition) and therefore postulated that GABA and noradrenaline function to reciprocally enhance their release.

Uptake, inactivation and metabolism

The primary route for GABA inactivation is via a sodium ion (Na^+)-dependent active neuronal uptake mechanism (Iversen & Neal, 1968) although significant amounts are taken up by glial cells (Iversen & Kelly, 1975). Since GABA uptake is likely to be accompanied by an influx of Na^+ ions, it is possible that GABA may have a depolarising effect on both neurones and glia (Krnjevic, 1984). Detailed Eadie-Hofstee analysis of the kinetics of GABA uptake into rat brain synaptosomes and slices indicates the presence of high, medium and low affinity uptake systems with respective K_m values of 1.1μM, 43μM and 3.9mM. However, since the V_{max} values for the high and medium uptake processes do not significantly differ, this would indicate that such systems are likely to share one

transport protein with two sites for GABA (Wood & Sidhu, 1986). Selective inhibition of either glial or neuronal uptake has been reported; thus, while nipecotic acid is equieffective on both transport systems, the uptake of GABA into glial cells is selectively blocked by β-alanine (Iversen & Kelly, 1975). Conversely, diaminobutyric acid (DABA) and 1S, 3R-cis-3-amino-cyclohexane carboxylic acid (ACHC) are specific substrates for neuronal uptake (Krogsgaard-Larsen & Johnston, 1978).

The purification of a GABA transport protein from rat brain (Radian et al, 1986) provided the foundation for the molecular cloning of its cDNA. The protein sequence of this transporter was expressed by injection of its mRNA (designated as GAT-1) into oocytes. Its kinetic and pharmacological profile resembles that of neuronal transport as opposed to the glial uptake process (Guastella et al (1990).

Accumulated GABA subsequently undergoes catabolic transamination with 2-oxoglutaric acid via the enzyme GABA-aminotransferase (GABA-T) to yield succinic acid semialdehyde. This is further oxidised to succinic acid which then re-enters the tricarboxylic acid cycle, completing the GABA 'shunt'. It is worth noting that the use of GABA-T inhibitors has proved to be therapeutically successful. The GABA-T inhibitor, ethanolamine-o-sulphate, when administered chronically, up-regulates GABA receptors (Sykes et al, 1984) whereas gamma-vinyl GABA (GVG, vigabatrin), is effective orally as an antiepileptic drug (Hammond & Wilder, 1985) as well as in the treatment of tardive dyskinesia (Tamminga et al, 1983).

GABA as an inhibitory neurotransmitter

Application of GABA to central vertebrate neurones mediates a reduction in cell firing, effectively producing synaptic inhibition. The first direct evidence for the role of GABA as an inhibitory neurotransmitter was provided by Krnjevic & Schwartz (1967) who iontophoresed GABA onto cat cortical neurones. This caused the membrane potential to become more

negative (hyperpolarised), lowering the membrane resistance thus increasing its conductance to chloride anions. Comparison of this GABA-mediated hyperpolarisation with chloride-dependent inhibitory postsynaptic potentials (IPSPs) showed that their reversal potentials were alike. Similar responses demonstrated subsequently in the spinal cord, were antagonised by the alkaloid, bicuculline (Curtis et al, 1971) and by the convulsant picrotoxin (Galindo, 1969). These substances appear to function at different sites as demonstrated from studies in rat cuneate nucleus (Simmonds, 1980a) where bicuculline acts as a competitive antagonist, presumably at the GABA receptor, whilst picrotoxin acts non-competitively at a site distinct from the GABA recognition site, probably associated with the chloride ionophore (Ticku et al, 1978).

Whilst considering the mechanisms of action of GABA receptor antagonists it is perhaps now appropriate to define our current understanding of receptors for GABA with particular emphasis on some of their physiological and pharmacological properties.

THE HETEROGENEITY OF GABA RECEPTORS

The advent of radioligand binding techniques in the 1970s has proved invaluable for the characterisation of binding sites for endogenous neurotransmitters on brain synaptic membranes. Indeed, further subclassification of established binding sites with the aid of selective radioligands has meant that 'receptor' heterogeneity is commonplace rather than the exception. This is certainly true in the case of receptors for GABA which have been subdivided into two major categories based on the specificity of GABA agonists and antagonists in both radioligand binding studies as well as functional models.

$GABA_A$ receptors

A comprehensive survey of the pharmacology of $GABA_A$ receptors would be exhaustive and is beyond the scope of this thesis. However, a brief description of the general pharmacological properties and characteristics of these receptors will be given to enable a comparison with $GABA_B$ receptors to be made.

The role of bicuculline as an antagonist of the action of GABA in electrophysiological studies has already been mentioned (Curtis et al, 1971), furthermore, it has also been shown to competitively inhibit the binding of [^{3}H]GABA to synaptic membranes (Zukin et al, 1974). This classical bicuculline-sensitive GABA receptor (defined now as the $GABA_A$ receptor) is responsible for the gating of chloride ion fluxes through an associated chloride channel (ionophore) and there is an abundance of evidence to suggest that the $GABA_A$ receptor-chloride ionophore is a complex protein containing multiple modulatory recognition sites.

Firstly, electrophysiological studies have revealed that benzodiazepines potentiate GABAergic inhibition (Haefely, 1975) by increasing the frequency of chloride channel opening (Study & Barker, 1981a). Since the identification of specific benzodiazepine binding sites (Squires & Braestrup, 1977; Mohler & Okada, 1977), it has also been found that binding to these sites is enhanced in the presence of GABA whilst conversely, GABA binds with greater affinity to $GABA_A$ receptors in the presence of benzodiazepines (Massotti et al, 1981) thus suggesting positive cooperativity between both benzodiazepine and $GABA_A$ receptors. Moreover, the benzodiazepine site appears to be functionally linked to the chloride ionophore (Simmonds, 1980b).

Secondly, barbiturates enhance $GABA_A$ receptor agonist binding to synaptic membranes in a chloride-dependent manner (Willow & Johnston, 1981) as well as allosterically inhibiting the binding of the $GABA_A$ receptor antagonist, [^{3}H]bicuculline. The action of barbiturates at the chloride

ionophore differs from that of benzodiazepines in that they prolong the duration of the opening of the GABA-activated chloride channel in sympathetic ganglion cells (Brown & Constanti, 1978) and in mouse cultured spinal cord neurones (Study & Barker, 1981b).

Thirdly, electrophysiological and biochemical evidence indicates that relationship of the convulsant, picrotoxin, acts at the $GABA_A$ receptor-chloride ionophore have previously been intimated (Galindo, 1969; Ticku et al, 1978). The latter authors employed [^{3}H]α-dihydropicrotoxinin to identify saturable and pharmacologically specific binding sites in brain membranes, however, the ability of barbiturates to inhibit the binding of this ligand led them to speculate that it is this picrotoxin site through which their actions are mediated (Ticku et al, 1980). The high affinity convulsant, [^{35}S]-t-butylbicyclophosphorothionate (TBPS), confers greater specificity with respect to the identification of the picrotoxin/convulsant site (Squires et al, 1983) however, since barbiturates are competitive ligands for the [^{35}S]-TBPS binding site, the precise location of barbiturate action on the $GABA_A$ receptor chloride ionophore remains to be identified (Supavilai & Karobath, 1984).

Fourthly, the anaesthetic steroid, alphaxolone, enhances evoked inhibition in slices of guinea-pig olfactory cortex (Scholfield, 1980). Furthermore, it potentiates the effects of the $GABA_A$ receptor agonist, muscimol in rat cuneate nucleus (Harrison & Simmonds, 1984). These authors also observed that like barbiturates (but unlike benzodiazepines), alphaxolone possesses GABA-mimetic activity (at muscimol potentiating concentrations) which is not antagonised by the benzodiazepine antagonist, flumazenil. From these findings, it would be reasonable to assume a common site of action exists for both barbiturates and steroids. However, such an assumption is not necessarily correct since in mouse cultured spinal neurones, phenobarbitone and alphaxolone act synergistically to potentiate GABA (Cottrell et al, 1987) making separate recognition sites, probable.

Experimental evidence would suggest that recognition sites for GABA, benzodiazepines, barbiturates, convulsants and steroids are to be found on the same receptor complex and the recent purification of the cloned $GABA_A$ receptor complex has significantly advanced this understanding. Initially, Schofield et al (1987) reported the identification and sequences of two distinct cDNA molecules that coded for the α- and β-polypeptide subunits which were believed to be fundamental for the full spectrum of the biological activity of GABA receptors.

A greater structural complexity is, however, now apparent since multiple subtypes of individual polypeptides plus further subunits, designated as $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1,2}$ and δ, have now been cloned (Schofield, 1989; Olsen & Tobin, 1990; Lüddens & Wisden, 1991 for reviews). The techniques of *in situ* hybridisation and autoradiography of $GABA_A$ and benzodiazepine (BDZ) receptors have enabled the distribution of the four subtypes to be visualised. Firstly, a BDZ-1 receptor population concentrated in the substantia nigra, the molecular layer of the cerebellum and lamina IV of the cerebral cortex has been correlated with the α_1 clone. Secondly, the δ mRNA subtype was located in areas showing high affinity muscimol binding but where BDZ binding was absent. Thirdly, the γ_2 clone appeared to be correlated with high affinity BDZ receptors whilst fourthly, a BDZ-2 receptor population, enriched especially in the hippocampus is likely to represent a variety of polypeptide clones.

$GABA_B$ receptors

The serendipitous discovery of a bicuculline-insensitive receptor (now classified as the $GABA_B$ receptor) arose as a consequence of the search for a model of GABA-mediated presynaptic inhibition in peripheral tissue. It had already been established that within the spinal cord, transmitter release could be inhibited by the action of GABA at a bicuculline-sensitive receptor (Curtis et al, 1971). Moreover, since GABA depolarises neurones of the

superior cervical ganglion as a consequence of increased chloride conductance (Bowery & Brown, 1974; Adams & Brown, 1975) it was hypothesised that if receptors were located on the cell soma, they could also be present at the nerve terminal to function in the control of neurotransmitter release.

Postganglionic processes emanating from the superior cervical ganglion had been well-characterised with respect to the sympathetic innervation of the heart. In particular, Iversen (1967) had demonstrated the transmurally-stimulated release of accumulated [^{3}H]noradrenaline from sympathetic nerve terminals of rat atria and it was this model that was chosen to investigate the effects of GABA. Bowery & Hudson (1979) showed that the evoked release of [^{3}H]noradrenaline was inhibited by GABA (although the maximum inhibition did not exceed 50-60%) and this response was unaffected by bicuculline. Furthermore, the action of GABA was not mimicked by established GABA receptor agonists such as isoguvacine and THIP. Surprisingly, the GABA analogue, β-p-chlorophenyl GABA (baclofen), also inhibited the evoked release of [^{3}H]noradrenaline which was again unaffected in the presence of bicuculline (Bowery et al, 1981). Baclofen had previously been shown to be inactive at bicuculline-sensitive GABA receptors (Curtis et al, 1974; Olpe et al, 1977) and since this inhibition of release was not chloride-dependent (Bowery et al, 1981), it was concluded that baclofen must be acting through a hitherto unidentified GABA receptor. A similar baclofen-induced inhibition of [^{3}H]noradrenaline release from cerebellar slices provided further confirmation of this hypothesis (Bowery et al, 1980).

The initial demonstration of saturable bicuculline-sensitive [^{3}H]GABA binding to synaptic membranes was performed in a sodium-free Tris-citrate buffer (Zukin et al, 1974). If instead, a physiological buffer is used together with nipecotic acid (to prevent the enhancement of GABA uptake by Na^+ ions), an additional [^{3}H]GABA binding component is evident (Bowery et al,

1982), attributable to the presence of Ca^{2+} or Mg^{2+} ions (Hill & Bowery, 1981). This enhanced binding was totally inhibited by baclofen but not by isoguvacine, whereas both binding components were supressed in the presence of GABA (Bowery et al, 1983).

Thus on synaptic membranes, two distinct binding sites for GABA exist and this evidence together with the pharmacologically characterised inhibition of [^{3}H]noradrenaline release, necessitated the introduction of the terms $GABA_A$ to describe the bicuculline-sensitive site and $GABA_B$ for the bicuculline-insensitive, baclofen-sensitive site (Hill & Bowery, 1981).

AUTORADIOGRAPHICAL RECEPTOR LOCALISATION

Perhaps one of the most striking observations differentiating $GABA_A$ and $GABA_B$ receptors has been their distinctive localisation within thin sections of mammalian brain assessed using receptor autoradiography. Palacios et al (1980, 1981) demonstrated that [^{3}H]muscimol bound to high affinity bicuculline-sensitive sites within the granular layer of the cerebellum (now defined as $GABA_A$ sites). By way of contrast, the first autoradiographical visualisation of $GABA_B$ sites in the rat cerebellum showed that unlike $GABA_A$ sites, they predominated in the molecular layer (Wilkin et al, 1981). These authors demonstrated that [^{3}H]GABA could be employed to label both $GABA_A$ and $GABA_B$ sites under appropriate incubation conditions. Thus, binding to $GABA_B$ receptor sites can be achieved in the presence of excess unlabelled isoguvacine (to prevent binding to $GABA_A$ sites) and Ca^{2+} ions, whilst conversely, the removal of Ca^{2+} ions and the replacement of isoguvacine with baclofen preferentially labels $GABA_A$ sites.

Further differences in the distribution of these sites was provided by Bowery et al (1984) with a subsequent detailed quantitative analysis of their

distribution in rat brain (Bowery et al, 1987). The relative densities of $GABA_A$ sites was greater than for $GABA_B$ in the majority of areas examined. However, there were some regions in which binding to $GABA_B$ sites clearly predominated, most notably in the molecular layer of the cerebellum (as previously mentioned), interpeduncular nucleus, superior colliculus, lateral amygdaloid nucleus, lateral posterior thalamic nucleus, globus pallidus, spinal trigeminal tract and the substantia gelatinosa of the spinal cord.

Although receptor autoradiography permits the localisation of binding sites, its resolution at the light microscopic level is not sufficient to allow the distinction between pre- and postsynaptic sites. However, the use of chemical and surgical lesions to denervate nerve terminals has proved successful in the identification of presynaptic $GABA_B$ sites.

The interpeduncular nucleus (IPN) which possesses a high density of $GABA_B$ sites is innervated by the retroflexus of Meynert projecting from the habenula nucleus. Electrolytic lesioning of these afferent fibres reduces $GABA_B$ binding in the IPN by >90% (Price et al, 1984a), indicating that these sites are located presynaptically on the habenula projection.

The anti-spastic activity of baclofen is thought to arise from its action on sensory afferent fibres within the spinal cord (Fox et al, 1978; Davies, 1981). Neonatal administration of capsaicin (Jancso et al, 1977) for a period of 3 months leads to a degeneration of sensory afferent fibres (Faulkner & Growcott, 1980). Such a treatment regime was found to reduce the density of $GABA_B$ binding by 40-50% in the substantia gelatinosa of the dorsal horn of the spinal cord, again suggesting a presynaptic location on these afferent terminals (Price et al, 1984b). In an attempt to eliminate the possibility of presynaptic lesioning on the postsynaptic site, [^{3}H]neurotensin binding was studied in conjunction with [^{3}H]GABA. Ninkovic et al (1981) had concluded that neurotensin binding sites were located postsynaptically since they were unaltered 15 days following rhizotomy of the dorsal horn of the spinal cord, in spite of a 40% reduction in opiate receptor binding. Dorsal rhizotomy

(and capsaicin treatment) produced around a 50% reduction in $GABA_B$ binding in laminae I-IV of the spinal cord whilst [^{3}H]neurotensin binding remained unaltered (Price et al, 1987).

Presynaptic $GABA_B$ sites are also found on terminals in the corpus striatum. Lesioning of the nigrostriatal pathway using 6-hydroxydopamine, reduces $GABA_B$ binding in the corpus striatum (Moratalla & Bowery, 1988) as does surgical ablation of the frontal cortex (Moratalla et al, 1989). Curiously, both $GABA_A$ and [^{3}H]flunitrazepam sites were increased following this latter treatment. An explanation for this is unclear but may reflect changes in postsynaptic sites or on glial cells.

Thus, autoradiographical studies have clearly demonstrated the presence of $GABA_B$ binding sites in rat brain. However, a binding site cannot necessarily be equated with a physiologically active receptor until evidence pertaining to receptor functionality exists. For this, it is necessary to consider the electrophysiological and second messenger systems that are activated in response to $GABA_B$ receptor stimulation.

CHARACTERISTICS OF $GABA_B$ RECEPTOR ACTIVATION

Electrophysiological studies

a) Calcium current modulation by $GABA_B$ receptors

Much of our understanding of the electrophysiology of $GABA_B$ receptor activation originated largely from studies performed in isolated dorsal root ganglia and in hippocampal slices, providing clear evidence that $GABA_B$ receptors are located and function both pre- and postsynaptically.

In early experiments, intravenous administration of baclofen was shown to depress monosynaptic excitatory postsynaptic potentials (EPSPs) in cat spinal motor neurones as a consequence of the stimulation of primary afferent fibres in the dorsal root ganglion (Pierau & Zimmerman, 1973). A

subsequent series of experiments using isolated perfused frog spinal cord supported these findings in that both baclofen and GABA reduced depolarising action potentials and reflex activity recorded in ventral roots following dorsal root stimulation (Davidoff & Sears, 1974). Conversely, action potentials produced by direct stimulation of the ventral roots were reduced by GABA but not by baclofen. It was concluded that baclofen mimicked the presynaptic stimulation but failed to mimic the postsynaptic increased in chloride ion conductance and this suppression of primary afferent depolarisation has been confirmed in a number of reports (Fox et al, 1978; Ault & Evans, 1981).

It had been established by Gallagher et al (1978) that GABA depolarised DRG cells by a bicuculline- and picrotoxin-sensitive increase in chloride ion conductance through a $GABA_A$ receptor-associated ionophore. A secondary action of GABA in cultured embryonic DRG cells involved a reduction in the duration of the calcium- (Ca^{2+}) dependent action potential that was unaffected by bicuculline (Dunlap & Fischbach, 1978). Furthermore, this effect was mimicked by baclofen but not by muscimol (Dunlap, 1981) implicating the existence of $GABA_B$ receptors. Such co-existence of both $GABA_A$ and $GABA_B$ receptors in the same DRG cell has since been confirmed in adult neurones (Desarmenien et al, 1984).

Extensive research into the mechanisms through which calcium currents are reduced by $GABA_B$ receptor activation, using non-hydrolysable forms of GTP and GDP as well as pertussis toxin (PTX), has signified the involvement of GTP-binding (G) proteins. Hydrolysis-resistant GTPγS enhances the baclofen-mediated Ca^{2+} current inhibition (Holz et al, 1986), whilst GDPγS, since it inhibits the binding of GTP to G-proteins, inhibits the $GABA_B$ response (Scott & Dolphin, 1986). Pertussis toxin ADP-ribosylates the α subunit of the $\alpha\beta\gamma$-heterotrimeric G-proteins, G_o and G_i, thus preventing the coupling of GTP with its G-protein (Dolphin, 1987 for review) which is essential for the high affinity binding of GABA and

baclofen to the $GABA_B$ receptor. Incubation of DRG cells in PTX attenuated the inhibitory effects of baclofen on Ca^{2+} currents thus confirming their G-protein coupling (Dolphin & Scott, 1987; Holz et al, 1986).

A precise physiological role for $GABA_B$ receptors on primary afferent fibres still remains to be elucidated and although the inhibition of transmission in these neurones by bicuculline suggests a physiological role for $GABA_A$ receptors (Curtis et al, 1971), few reports exist as to the effects of $GABA_B$ receptor antagonists on these currents. In one recent study by Dolphin & Huston (1990), surprisingly, the $GABA_B$ receptor agonist, 3-aminopropylphosphinic acid (3-APA), was ineffective at mimicking the action of (-)-baclofen. Moreover, the $GABA_B$ receptor antagonist, 2-OH-saclofen was less potent than its phosphonic acid derivative, phaclofen (see later for descriptions) at inhibiting baclofen, despite possessing a 13-fold higher affinity for $GABA_B$ binding sites (Al-Dahan et al, 1990). Clearly, these findings require further clarification.

b) Postsynaptic hippocampal actions

The application of GABA close to the somata of a hippocampal CA1 pyramidal cell causes a marked hyperpolarisation (Andersen et al, 1980; Alger & Nicoll, 1982b) which is blocked by bicuculline (Newberry & Nicoll, 1985). If, GABA is applied to dendritic field of the pyramidal cell, however, a bicuculline-sensitive depolarisation followed by a bicuculline-insensitive hyperpolarisation ensues. Since baclofen mimicked this hyperpolarisation, the effect was attributed to the activation of $GABA_B$ receptors. The underlying mechanism appears to be associated with a selective increase in potassium (K^+) conductance (Newberry & Nicoll, 1984a, 1985; Gahwiler & Brown, 1985; Inoue et al, 1985b) and a more detailed examination of the precise coupling of $GABA_B$ receptors with K^+ channels has been elucidated by Andrade et al (1986). Their experiments showed firstly that the K^+ channels opened by baclofen were also opened by 5-HT and since the

outward currents elicited by these two agonists were non-additive, this implied the presence of a shared common channel. Secondly, the ineffectiveness of intracellular injections of the calcium chelator, EGTA, as well as tetraethylammonium (Adams et al, 1982) to inhibit these currents suggested that the response was not due to a calcium-activated K^+ channel. The hyperpolarising responses to both baclofen and 5-HT were blocked by PTX and since GTPγS mimicked the action of baclofen, this provided strong evidence in favour of a $GABA_B$ receptor-coupling to K^+ channels via a PTX-sensitive G-protein.

The first description of a possible functional role for these $GABA_B$ receptors originated from the observation that phaclofen blocked the dendritic baclofen- (but not 5-HT) induced hyperpolarisation but was without effect on the somatic $GABA_A$ response Dutar & Nicoll, 1988a). Electrophysiological recordings from hippocampal pyramidal cells have revealed the existence of two types of GABAergic inhibitory postsynaptic potentials (IPSPs). Antidromic activation of the axons of CA1 pyramidal cells by stimulation of the alveus results in a fast IPSP peaking at 50ms with a duration of 200-300ms (Alger & Nicoll, 1982a). Since this IPSP is blocked by $GABA_A$ receptor antagonists and altered by changes in membrane chloride gradients, it is thought to arise from a recurrent pathway by the action of GABA on somatic $GABA_A$ receptors. A similar IPSP is observed following low intensity orthodromic stimulation of Schaffer and commissural afferent fibres in the stratum radiatum. However, stronger stimulation produces a slower and later component, peaking at 200ms with a 1 second duration (Newberry & Nicoll, 1984b). This response, termed the late IPSP (or late hyperpolarising potential) is resistant to $GABA_A$ receptor antagonists and arises from an increase in K^+ conductance. Moreover, since this late IPSP is attenuated by pretreatment with PTX and is selectively antagonised by phaclofen, it would appear that this electrically-evoked action shares the properties of activated $GABA_B$ receptors on CA1

pyramidal cell dendrites (Dutar & Nicoll, 1988a, 1988b).

c) Presynaptic hippocampal actions

Baclofen suppresses the EPSP evoked extracellularly in Schaffer collateral/commissural as well as mossy fibre pathways (Lanthorn & Cotman, 1981). This presynaptic action was substantiated using intracellular recording in the Schaffer collateral to CA1 projection but not in the mossy fibre to CA3 synapse (Ault & Nadler, 1982; Inoue et al, 1985a). Presynaptic inhibition of IPSPs by baclofen has also been demonstrated in cultured hippocampal neurones (Harrison et al, 1988).

It could perhaps be argued that the baclofen-induced reduction in the EPSP may be due to postsynaptic K^+ ion conductances or that presynaptic $GABA_B$ receptors are responsible for the inhibition of the late IPSP. However, experiments with PTX indicate that this is not the case. Injection of PTX into the lateral ventricle blocks the postsynaptic $GABA_B$ receptor-mediated events (as previously discussed) whereas the presynaptic EPSP depression is unaffected by this treatment (Dutar & Nicoll, 1988b). Moreover, these authors also reported that phaclofen was relatively inactive at antagonising the presynaptic effects. This immediately prompts the question as to whether or not pre- and postsynaptically located $GABA_B$ receptors are heterogeneous. More detailed analysis of the effects of higher affinity $GABA_B$ receptor antagonists will be required to answer this question.

Second messenger systems

The role of G-proteins in effecting membrane transduction in response to $GABA_B$ receptor activation is becoming increasingly clear. Guanyl nucleotides selectively inhibit $GABA_B$ (but not $GABA_A$) receptor binding via a reduction in the affinity of the agonist for the receptor (Hill et al, 1984). Moreover, incubation of bovine cerebral synaptic membranes

with PTX resulted in a dose and time-dependent reduction in $GABA_B$ binding with a concommitant increase in the ADP-ribosylation of membrane proteins (Asano et al, 1985). It is conceivable that since GTP is essential for the activation of adenylyl cyclase, then GTP-sensitive binding sites may be positively- or negatively-linked to this enzyme (Rodbell, 1980; Neer & Clapham, 1988).

The first demonstration of a negative coupling of $GABA_B$ receptors to adenylyl cyclase was provided by Wojcik & Neff (1984). Using synaptic membranes from a number of brain regions, they showed that although baclofen had no direct stimulatory effect on adenylyl cyclase activity, direct activation of the enzyme with the diterpine, forskolin, (Seamon & Daly, 1984) was inhibited in the presence of baclofen, which in the cerebellum, was attributable to granule (rather than Purkinje) cells. This inhibition by baclofen in synaptic membranes was extended to brain slices (Karbon & Enna, 1985), whilst Hill (1985), demonstrated stereoselectivity for the (-)-enantiomer of baclofen.

In keeping with electrophysiological studies involving PTX, The inhibition of adenylyl cyclase activity is also PTX-sensitive. A 14 hour exposure of cultured cerebellar granule cells to PTX (1µg/ml) attenuated the inhibitory effect of baclofen (Xu & Wojcik, 1986). [^{32}P]ADP-ribosylation studies indicated that 47% of proteins of 41,000 molecular weight (corresponding to α_i and α_o subunits of G_i- and G_o-proteins) had been ADP-ribosylated.

The detection of the inhibition of adenylyl cyclase activity by $GABA_B$ receptor activation can only be achieved following direct stimulation of the enzyme since baclofen does not influence basal cAMP levels (Hill, 1985). Increased cAMP concentrations can also be produced by direct activation of receptors that are positively coupled to adenylyl cyclase via G_s-proteins (Neer & Clapham, 1988). In view of this, increased cAMP levels produced in response to noradrenaline were enhanced by both GABA and baclofen

(Karbon et al, 1984; Karbon & Enna, 1985; Hill, 1985). This effect is not confined solely to noradrenergic receptors coupled to adenylyl cyclase since baclofen also potentiates the stimulatory responses to isoprenaline, adenosine, histamine, and vasoactive intestinal polypeptide (Karbon et al, 1985; Watling & Bristow, 1986). $GABA_B$ receptor involvement in these actions is confirmed by the inability of bicuculline to antagonise the effects of GABA and baclofen (Hill, 1985; Watling & Bristow, 1986). Moreover, in the presence of the Ca^{2+} ion chelator, EGTA, baclofen fails to potentiate the cAMP response to isoprenaline (Karbon et al, 1985; Duman et al, 1986).

$GABA_B$ receptor-mediated augmentation of adenylyl cyclase activity is observed only in brain slices and not synaptic membranes suggesting that a complex transduction mechanism is required to effect this response. The calcium-dependent enzyme, phospholipase A_2 (PLA_2) which catalyses the release of arachidonic acid from membrane phospholipids, may contribute to the $GABA_B$ response. Inhibition of PLA_2 activity by quinacrine and glucocorticoids blocks the baclofen-induced potentiation of cAMP accumulation whilst not affecting the response to isoprenaline alone (Duman et al, 1986).

Thus, baclofen can either enhance or inhibit cAMP formation in the same tissue preparation depending upon the nature of the second messenger stimulus. Interestingly α_2-adrenoceptor activation inhibits adenylyl cyclase in synaptic membranes but potentiates cAMP accumulation in brain slices (Duman et al, 1986). In the same way that subpopulations of α_2-adrenoceptors have been proposed (Bylund & Ray-Prenger, 1989), it is possible that $GABA_B$ receptors too, may be heterogeneous and indeed evidence to support this hypothesis is now emerging.

EVIDENCE FAVOURING POSSIBLE $GABA_B$ RECEPTOR HETEROGENEITY

It is clear from the electrophysiological and adenylyl cyclase studies described in the preceding section that $GABA_B$ receptor activation causes a multiplicity of physiological and pharmacological responses. This immediately poses the question as to whether or not these effects are mediated by homologous receptors or that the $GABA_B$ receptor population is, in fact, heterogeneous. A brief re-assessment of certain $GABA_B$ receptor-coordinated responses, will perhaps lend weight to an argument in favour of the latter hypothesis.

The first non-functional evidence for $GABA_B$ subsites was provided by Hill & Bowery (1981) and Karbon et al (1983). Using [^{3}H]GABA and [^{3}H]baclofen, these authors showed that the binding of these two ligands to $GABA_B$ sites in rat brain synaptic membranes could be resolved into both high and low affinity components. The existence of kinetically distinct $GABA_B$ sites was confirmed by Karbon et al (1983) who found that both the B_{max} and K_D of the low (but not high) affinity site was reduced 12 days following unilateral lesioning of the dorsal noradrenergic bundle.

Secondly, strong evidence in favour of a differential coupling of $GABA_B$ receptors to distinct effector mechanisms is derived from studies with second messenger systems. The inhibition by baclofen of forskolin-stimulated cAMP accumulation suggests a negative coupling of $GABA_B$ receptors to adenylyl cyclase activity (Wojcik & Neff, 1984; Karbon & Enna, 1985) through a PTX-sensitive G-protein (Xu & Wojcik, 1986). By way of contrast, a positive $GABA_B$ receptor-coupling to adenylyl cyclase is exemplified by the baclofen-induced augmentation of beta-adrenoceptor stimulation (Karbon et al, 1984; Hill, 1985). Preliminary studies indicate that this response is not linked to PTX-sensitive G-proteins (Hill et al, 1989), but rather to a possible involvement with membrane phospholipid metabolism (Duman et al, 1986). It is curious that baclofen was unable to potentiate

noradrenaline stimulation in the cerebellum (Karbon et al, 1985) in spite of high densities of $GABA_B$ receptors within this region (Wilkin et al, 1981) which could be a consequence of $GABA_B$ receptor subclasses.

Evidence is beginning to emerge promoting a link between $GABA_B$ receptors and phosphatidyl inositol (PI) turnover. Whilst baclofen inhibits both 5-HT and histamine-induced formation of inositol phosphates in rat brain slices (Godfrey et al, 1988; Crawford & Young, 1988), it also produces a small, but nonetheless significant increase in PI turnover in cultured DRG neurones (Dolphin et al, 1989).

It is firmly established that $GABA_B$ receptors are linked to both Ca^{2+} channels (reducing the Ca^{2+} current) and K^+ channels (increasing K^+ conductance) (Bormann, 1988 for review) and that these effects function independently (Dolphin & Scott, 1986).

The $GABA_B$ receptor-mediated late IPSP in hippocampal CA1 pyramidal cells (Newberry & Nicoll, 1984a) is effected through increased K^+ conductance and blocked by both pretreatment with PTX (Andrade et al, 1986) and by the weak $GABA_B$ receptor antagonist, phaclofen (Dutar & Nicoll, 1988b). Conversely, the presynaptic suppression of EPSPs in pyramidal cell afferent fibres by baclofen, is neither sensitive to PTX nor phaclofen. Subsequent experiments have found that baclofen-induced pre- and postsynaptic depressions in the cat spinal cord are blocked by the more potent $GABA_B$ receptor antagonist, 2-hydroxy-saclofen (Curtis et al, 1988) which also antagonised baclofen-induced suppression of spontaneous paroxysmal discharges developed in rat neocortical slices is antagonised by (Ong et al, 1990). The latter authors observed that the $GABA_B$ receptor agonist, 3-APA, failed to mimic the effect of baclofen (at an equivalent concentration). A higher concentration produced a marked hyperpolarisation (unlike baclofen) and again did not effect the discharge frequency although a reduced amplitude was seen. Co-perfusion of 2-

hydroxy-saclofen with 3-APA returned the discharge amplitude to control levels however, the hyperpolarisation persisted. Thus, although baclofen and 3-APA have potent selectivity for $GABA_B$ sites in binding assays (Dingwall et al, 1987), their functional responses may be mediated through heterogeneous $GABA_B$ receptors.

In support of these differential agonistic actions, the phosphonic acid derivative of GABA, 3-aminopropylphosphonic acid (3-APPA), mimicked the inhibitory action of baclofen on forskolin-stimulated adenylyl cyclase but was without effect on the noradrenergically-stimulated system (Scherer et al, 1988). In fact, further experimentation has revealed that 3-APPA actually antagonises the baclofen-induced potentiation (Karbon et al, 1990).

Although the evidence postulating $GABA_B$ receptor heterogeneity is accumulating, much more data are still required. The potencies of $GABA_B$ receptor agonists and antagonists continue to improve which should hopefully provide a possible pharmacological resolution of $GABA_B$ receptor subtypes.

$GABA_B$ RECEPTOR ANTAGONISTS

Progress into the identification of a physiological role(s) for $GABA_B$ receptors has, over recent years, been hindered due largely to a lack of specific $GABA_B$ receptor antagonists. Whilst baclofen still remains the prototypic $GABA_B$ receptor agonist, the phosphinic acid analogue of GABA (3-APA), has also emerged as a specific $GABA_B$ receptor agonist. Although more potent than baclofen in binding studies (Dingwall et al, 1987) and on the isolated guinea-pig ileum (Hills et al, 1989), 3-APA does not always precisely mimic the actions of baclofen at equivalent concentrations (Ong et al, 1990) which may indicate actions at separate $GABA_B$ receptor subclasses.

A number of compounds have been shown to exhibit weak $GABA_B$ receptor antagonism but this is usually at the expense of significant agonistic activity at $GABA_A$ receptors. Such has been the case with delta-amino-valeric acid (DAVA) (Muhyaddin et al, 1982) and 3-aminopropane sulphonic acid (3-APS, homotaurine) (Giotti et al, 1983b).

The phosphonic acid derivative of GABA, 3-aminopropyl-phosphonic acid (3-APPA), provided the next significant advance in the search for a novel specific $GABA_B$ receptor antagonist. This compound had first been shown to depress spinal neuronal firing (Curtis & Watkins, 1965) prior to the discovery of the GABA antagonistic properties of bicuculline (Curtis et al, 1971). 3-APPA lacked a bicuculline-sensitive depressant action on rat cerebral and cerebellar neurones (Bioulac et al, 1979) and therefore could not be described as a $GABA_A$ receptor agonist. Although further characterisation of 3-APPA has, in fact, established its role as a partial agonist in the periphery (Kerr et al, 1987) and the CNS (Kerr et al, 1989c) it has nevertheless provided a crucial link in the evolution of novel synthetic $GABA_B$ receptor antagonists, the structural formulae of which are shown in Figure 1.0.

In view of this, it is necessary to accredit much of our current understanding to the work of Dr. D.I.B. Kerr and his colleagues who based their chemical programmes around phosphonic and sulphonic acid derivatives of GABA and baclofen. Thus, phaclofen, the phosphonic acid derivative of baclofen, was the first of a new generation of specific $GABA_B$ receptor antagonists to be introduced (Kerr et al, 1987). In addition to reversing the baclofen-induced suppression of the cholinergic twitch response in the guinea-pig ileum, it also blocked the reduction in monosynaptic spinal interneuronal excitation mediated by baclofen, suggesting both peripherally and centrally-mediated actions.

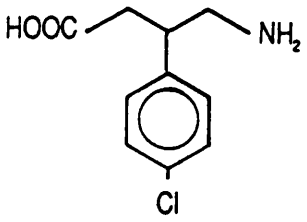
A number of subsequent reports have given credibility to the role of phaclofen as a $GABA_B$ receptor antagonist. In the hippocampus and

Figure 1.0

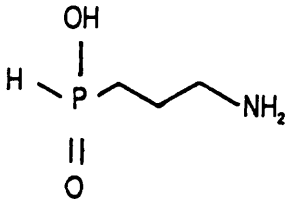
Structural formulae of specific $GABA_B$ receptor agonists and antagonists.

Structures of selective $GABA_B$ ligands.

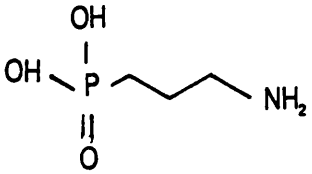
Agonists.



baclofen

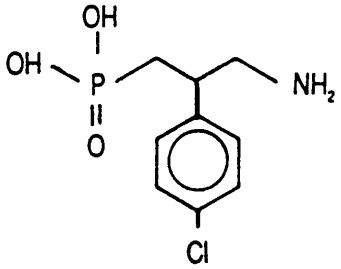


3-aminopropyl phosphinic acid

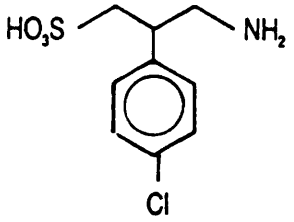


3-aminopropyl phosphonic acid

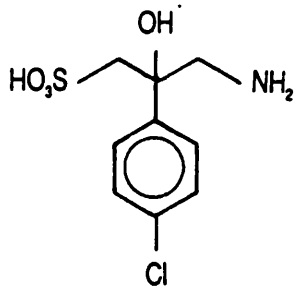
Antagonists.



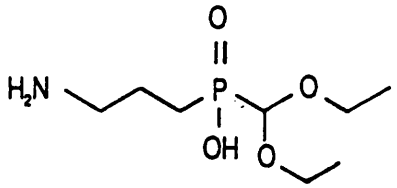
phaclofen



saclofen



2-hydroxysaclofen



CGP 35348

neocortex, late IPSPs are sensitive to phaclofen (Dutar & Nicoll, 1988a, 1988b; Soltesz et al, 1988; Karlsson & Olpe, 1989). Spontaneous paroxysmal discharges are also antagonised by phaclofen in the neocortex (Kerr et al, 1989) and in locus coeruleus neurones (Olpe et al, 1988). Respiratory depression following i.c.v. injections of baclofen into the fourth ventricle decreases phrenic nerve activity which is reversed in the presence of phaclofen (Schmid et al, 1989).

Neurochemical assays have revealed the existence of phaclofen-sensitive autoreceptors in rat cortical synaptosomes (Bonnano et al, 1988). Conversely, Stirling et al (1989) found that K^+-stimulated Ca^{2+} influx, a measure of presynaptic activity, was phaclofen-insensitive. Indeed, phaclofen behaves as a partial agonist in this model and also against baclofen-induced inhibition of forskolin-stimulated adenylyl cyclase (Robinson et al, 1989). With a pA_2 value of approximately 4 and low millimolar concentrations generally required to achieve $GABA_B$ receptor antagonism, phaclofen, although specific in its actions, lacks potency.

The sulphonic acid derivative of GABA, 3-APS, possesses weak $GABA_B$ receptor antagonistic efficacy, however its potent agonistic effects at $GABA_A$ receptors mask its antagonistic actions at $GABA_B$ receptors in many preparations. In contrast, the sulphonic acid derivative of baclofen, 2-hydroxy-saclofen, has a 10-fold greater potency ($pA_2 = 5$) compared to phaclofen in the guinea-pig ileal model and against baclofen-induced suppression of spontaneous discharges in cortical slices (Kerr et al, 1988). $GABA_B$ receptor antagonism by 2-hydroxy-saclofen has been further confirmed by Curtis et al (1988) and Lambert et al (1989) and also its potency at displacing [^{3}H]baclofen binding has been determined (Drew et al, 1990; Al-Dahan et al, 1990).

Saclofen, the sulphonic acid analogue of baclofen, is twice as potent as 2-hydroxy-saclofen ($pA_2 = 5.3$) at reducing baclofen-induced suppression of guinea-pig ileal contractions (Kerr et al, 1989a) but is equipotent at

displacing [^{3}H]baclofen binding (Drew et al, 1990).

Two benzofuran analogues of baclofen, unrelated to the phosphonic and sulphonic acid derivatives already described, were synthesised by Berthelot et al (1987). The unsubstituted analogue, 9G, and the 5-methoxy analogue, 9H, inhibited [^{3}H]baclofen binding with respective IC_{50} values some 6.5 times and 21 times lower than that reported for phaclofen (118μM) by Drew et al (1990). Despite this apparently increased potency, both compounds showed virtually identical pA_2 values to phaclofen in the guinea-pig ileum model and antagonised the effects of baclofen in isolated neocortical slices to similar extents (Kerr et al, 1989b). Baclofen-induced inhibition of motoneurone firing in the cat spinal cord was also reversed by 9H (Beattie et al, 1989).

The most significant recent advance in the pharmacology of $GABA_B$ receptor antagonists has been the introduction of a substituted linear phospho-amino acid, CGP 35348. Following i.p. or i.v. administration, it appears that unlike phaclofen or 2-hydroxy-saclofen, this compound is able to cross the blood-brain barrier. Its potency ranges from 10-30 times that of phaclofen in *in vitro* models, but its comparative potency *in vivo* is much greater (Olpe et al, 1990b). The ability of CGP 35348 to facilitate long-term potentiation following tetanic stimulation of the hippocampal Schaffer collateral/commissural pathway (Olpe et al, 1990a), suggests that a centrally acting $GABA_B$ receptor antagonist may have therapeutic potential in improving cognition.

It is clear that a number of both centrally- and peripherally-mediated effects can be attributed to $GABA_B$ receptor activation (see Bowery, 1989). Furthermore, it is theoretically conceivable that a $GABA_B$ receptor antagonist should be able to discern as to whether or not such effects are of physiological significance, especially with regard to therapeutic potential. In light of this, two such actions warrant particular attention.

Firstly, since intestinal $GABA_B$ receptors are innervated by myenteric

neurones to reduce gastric motility (Ong & Kerr, 1983), an antagonist of such an action would be expected to exert the opposite effect. Secondly, a centrally active $GABA_B$ receptor antagonist should reverse $GABA_B$ receptor-mediated inhibition of monoamine release, thereby increasing intrasynaptic concentrations which may be expected to have an antidepressant effect More detailed accounts of the roles of $GABA_B$ receptors in depression and gastric motility shall now follow.

ASPECTS OF THE PHARMACOLOGY OF ANTIDEPRESSANT DRUGS

Upon consideration of the structural diversity of clinically efficacious antidepressants, it is hardly surprising that their therapeutic actions arise from a plethora of complex biochemical mechanisms. Since the introduction of imipramine in 1957, much research has focussed upon attempting to find a common biochemical denominator from which a neurochemical inbalance would trigger the disease. The clinical observation that reserpine, which depletes monoamines, could also give rise to depression suggested that the disease may be a consequence of a monoamine deficit. Evidence to support this was provided by the fact that synaptic levels of the monoamines noradrenaline (NA) and 5-hydroxytryptamine (5-HT) are increased by tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) by the prevention of either their uptake or breakdown, respectively. Thus the monoamine hypothesis of depression was proposed (Schildkraut, 1965).

Many of the antidepressants clinically prescribed are either selective uptake inhibitors of NA e.g. desipramine, maprotiline and viloxazine; 5-HT e.g. citalopram, paroxetine and fluoxetine; or non-selective for both monoamines e.g. amitriptyline and imipramine. Mianserin is considered to be an atypical antidepressant since it does not possess a tricyclic structure and its ability to inhibit monoamine uptake is negligible. It is, however, an

antagonist at presynaptic α_2 receptors and functions to inhibit the negative feedback mechanisms that control the release of NA thus enhancing noradrenergic activity by a process that is independent of uptake inhibition (Marshall, 1983).

These neurochemical modulations appear to occur pre-synaptically and within a few hours of drug administration yet signs of a clinical improvement in depressed patients are not usually seen until a period of 14-21 days from the onset of all antidepressant therapies has elapsed. This would suggest that events secondary to those occurring presynaptically may be responsible for their therapeutic actions with postsynaptic receptor modulation likely to be most relevant.

Functional changes in beta-adrenoceptors following chronic antidepressant administration

Chronic administration of the NA uptake inhibitor, desipramine, has been shown to down-regulate the number of beta-adrenoceptor binding sites in rat brain membranes (Banerjee et al, 1977; Bergstrom & Kellar, 1979). Furthermore, the sensitivity of NA-sensitive adenylyl cyclase is also reduced by the prolonged administration of TCAs and MAOIs (Vetulani & Sulser, 1975). Resolution of beta-adrenoceptors into $beta_1$ and $beta_2$ subtypes prompted the question as to which subtype was influenced by the activity of antidepressants. Evidence implicating the selective down-regulation of $beta_1$-adrenoceptors has been provided following chronic desipramine treatment (Minneman et al, 1979) and more recently with a variety of antidepressants (Heal et al, 1989). Thus, it appears that prolongation of the presence of NA in the synaptic cleft functions to down-regulate beta-adrenoceptors as a consequence of persistent receptor occupation by the endogenous agonist.

However, this phenomenon cannot be generalised to all inhibitors of NA uptake since maprotiline is ineffective at reducing either the B_{max} or the

sensitivity of the beta-adrenoceptor-linked adenylyl cyclase system. (Barbaccia et al, 1986). Similar inactivity has been observed with the specific 5-HT uptake inhibitor, citalopram (Garcha et al, 1985) and whereas mianserin and zimeldine decrease cAMP production, they do not reduce beta-adrenoceptor number (Mishra et al, 1980).

These studies may therefore question (but not preclude) the therapeutic relevance of beta-adrenoceptor down-regulation in isolation, perhaps suggesting a greater neurochemical mechanistic complexity. Interestingly, and in support of this, if 5-HT neurones are lesioned with 5,7-dihydroxytryptamine, then desipramine is unable to down-regulate beta-adrenoceptors (Brunello et al, 1982; Janowsky et al, 1982). These findings were substantiated with a further study by Nimgaonkar et al (1985), although inhibition of tyrosine hydroxylase activity by treatment with para-chlorophenylalanine (pCPA) was without effect on desipramine-induced reduction in beta-adrenoceptors. Thus, it seems that the integrity of the serotonergic neurones rather than 5-HT itself that is a prerequisite for beta-adrenoceptor modulation.

Functional changes in serotonergic receptors following chronic antidepressant administration

Considerable biochemical and behavioural evidence exists as to the modulation of central 5-HT_2 receptor binding by antidepressants. These receptors, possessing higher affinity for 5-HT receptor antagonists rather than agonists (Peroutka & Snyder, 1979), are down-regulated by chronically administered antidepressants (Peroutka & Snyder, 1980; Kellar et al, 1981; Goodwin et al, 1984). Moreover, these changes are also reflected in a functional model of 5-HT_2 receptor activation, the mouse head-twitch (Goodwin et al, 1984). It is interesting that repeated electroconvulsive therapy (ECT), effective in major depression), actually up-regulates the 5-HT_2 receptor population in rat frontal cortex (Kellar et al, 1981; Vetulani et

al, 1981; Green et al, 1983) in addition to increasing 5-HT_2 receptor functionality in the mouse head-twitch model (Goodwin et al, 1984). These surprising findings may be indicative of the possible involvement of heterogeneous 5-HT receptors and although it is the 5-HT_2 receptor that is traditionally associated with antidepressant actions, mention should also be made of the 5-HT_{1A} receptor subtype. Autoradiographical analysis following amitriptyline administration for 21 days has shown that the binding of [^{3}H]8-hydroxy-DPAT to postsynaptic 5-HT_{1A} sites in the hippocampus is increased whereas in the raphe nucleus (the location presynaptic 5-HT_{1A} somatodendritic autoreceptors) binding is unaltered (Welner et al, 1989). By way of contrast, the selective 5-HT uptake inhibitor, fluoxetine and the 5-HT_{1A} agonist, gepirone, reduced 5-HT_{1A} binding in the raphe nucleus but not in the hippocampus. These findings support the proposal from electrophysiological data that enhanced serotonergic activity may be affected by either an enhancement of postsynaptic hippocampal neurotransmission by TCAs (de Montigny & Aghajanian, 1978) or by desensitisation of the 5-HT autoreceptor following prolonged treatment with selective 5-HT uptake inhibitors (Chaput et al, 1986) or gepirone (Blier & de Montigny, 1987).

The experimental findings discussed so far provide clear evidence that monoamine uptake inhibitors relieve depression by mechanisms apparently unrelated to monoamine uptake inhibition. Although beta-adrenoceptor and 5-HT_2 down-regulation is common to many antidepressants, a drug such as maprotiline fails to produce any modulation in spite of inhibiting NA uptake and exhibiting clinical efficacy. Thus, sufficient scope exists for the identification of a possible neurotransmitter that may be a prerequisite to the mechanisms of action of diverse antidepressants. Over recent years there has been an accumulation of preclinical and clinical evidence to suggest that GABA could be such a candidate.

The involvement of GABAergic mechanisms in depression

a) Clinical investigations

Studies involving depressed patients have revealed lower GABA concentrations compared to control subjects in the cerebrospinal fluid (CSF) (Gold et al, 1980; Gerner & Hare, 1981) and also in the plasma (Berrettini et al, 1982; Petty & Sherman, 1984). Since synaptic GABA concentration and GAD activity are generally unaltered following either acute or prolonged antidepressant administration or ECT (Pilc & Lloyd, 1984; Green & Vincent, 1987), the causal mechanisms from which low GABA levels arise are, at present, unidentified. Perhaps one must therefore look beyond the synapse to GABA receptors in order to identify possible modulations by antidepressants.

Progabide and fengabine were the first GABA-mimetic drugs demonstrated to exert antidepressant activity in depressed patients (Morselli et al, 1986; Musch & Garreau, 1986). There is, however, a relative paucity of clinical studies in which selective $GABA_A$ or $GABA_B$ receptor agonists have been examined as putative antidepressants. The selective $GABA_A$ receptor agonist, THIP, has anxiolytic rather than antidepressant activity (Hoehn-Saric, 1983) and while the anxiolytic efficacy of benzodiazepines acting through the $GABA_A$/benzodiazepine/chloride ionophore complex is unquestioned, clinical trials with triazolobenzodiazepines such as alprazolam have shown some antidepressant activity (Feighner et al, 1983).

Although there are no published clinical trials of the selective $GABA_B$ receptor agonist, baclofen, in depression, some behavioural and biochemical evidence does exist as to the involvement of GABA in the actions of standard antidepressants mediated particularly through $GABA_B$ receptors.

b) The role of GABA in animal models of depression

The search for an animal model truly predictive of the human depressive condition has been exhaustive and relatively unsuccessful. *In*

vivo and *in vitro* models such as reserpine-induced ptosis and hypothermia, yohimbine toxicity or the inhibition of monoamine uptake have been relevant to such compounds that increase synaptic monoamine concentrations but not to GABA-mimetics. Two models that do, however, parallel some aspects of human depression are the learned helplessness model and the olfactory bulbectomised rat (see Willner, 1984 for review).

The behavioural deficits arising from inescapable shock in the learned helplessness model are reversed by classical antidepressants and GABA-mimetics (Sherman et al, 1982) in addition to intracerebral injections of GABA to the frontal cortex or hippocampus (Petty & Sherman, 1981). Furthermore, these authors also reported a 20% reduction in K^+-stimulated GABA release from hippocampal slices of 'helpless' animals (Sherman & Petty, 1982).

Behavioural manifestations of olfactory bulbectomy such as muricide and open field hyperactivity are reversed by baclofen whereas the ensuing passive avoidance is reversed by progabide, fengabine and also muscimol suggesting both $GABA_A$ and $GABA_B$ receptor involvement (Lloyd et al, 1983; Sanger et al, 1986). Moreover, $GABA_B$ receptors are reduced in the frontal cortex following olfactory bulbectomy. (Lloyd & Pichat, 1986). It therefore follows that if depression is associated with a $GABA_B$ receptor deficit, then one might expect $GABA_B$ receptors to be increased following repeated antidepressant administration.

c) $GABA_B$ receptor-modulation following chronic antidepressant administration

Pilc & Lloyd (1984) presented the first study demonstrating increased $GABA_B$ receptor numbers in membranes of rat frontal cortex after repeated administration of a number of antidepressants. These findings were further substantiated with representatives from each major class of antidepressant as well as with GABA-mimetics and ECT (Lloyd et al, 1985) and also in

mouse cortical membranes using imipramine (Suzdak & Gianutsos, 1986).

However, not all reports are in agreement with these findings (Szekely et al, 1987; Cross & Horton, 1987, 1988) especially since $GABA_B$ receptors are not altered in drug-free suicide victims (Cross et al, 1988) (these findings will be more fully discussed in Chapter 5).

Nonetheless, increased $GABA_B$ receptor functionality with respect to baclofen-induced nociception is reduced by chronic desipramine (Borsini et al, 1986) whereas baclofen-induced hypothermia is enhanced by repeated administered antidepressants or ECT (Gray et al, 1987). Furthermore, these authors have also shown using similar treatments an enhanced $GABA_B$ receptor-mediated inhibition of 5-HT release (Gray & Green, 1987). Additional evidence supporting a link between increased $GABA_B$ receptor functionality and monoaminergic systems arises from the fact that chronic imipramine treatment enhances the $GABA_B$ receptor-mediated potentiation of NA-stimulated adenylyl cyclase activity (Suzdak & Gianutsos, 1986).

From these studies, $GABA_B$ receptors appear to have some involvement in the etiology of depression although the apparent controversies arising from different research groups clearly require resolution.

GASTRIC MOTILITY AND $GABA_B$ RECEPTORS

Gastrointestinal actions of GABA were first identified by Hobbiger (1958a, 1958b) who found that in addition to contractory and relaxatory effects, GABA also suppressed the excitatory actions of acetylcholine and 5-HT. Apart from a few publications inthe early 1960s, research into the role of GABA in intestinal motility then entered a latent phase, to be rejuvenated upon the isolation of GABA and GAD in the myenteric plexus of guinea-pig taenia coli (Jessen et al, 1979) and the re-establishment of gastrointestinal

GABAergic inhibition (Krantis et al, 1980).

One of the most striking characteristics of the enteric nervous system (ENS) is its ability to function independently from central influence. The inherent myogenicity of the gut is achieved by the sensory neurones of the myenteric (Auerbach's) plexus lying between the outer longitudinal and the inner circular muscle of which it innervates. The submucosal (Meissner's) plexus is found beneath the circular muscle and supplies the glands and villi of the inner mucosa. Both plexuses uninterruptedly ramify the entire length of the gastrointestinal tract.

Unlike the skeletal neuromuscular junction, where a single axon terminates on a specific effector muscle, in the ENS, transmitters are not released at discrete junctions. Axons of the myenteric plexus enter the circular muscle and are organised into axons bundles swelling periodically to form varicosities. These are believed to be the location of a prejunctional element where transmitters are released either onto the surrounding muscle or onto parallel axons from which the release of additional transmitters is modulated (Jessen et al, 1987 for review).

Since the application of GABA onto guinea-pig intestinal muscle produces no direct contractile response (Krantis et al, 1980), it appears that GABA receptors are likely to be found on myenteric neurones rather than on gut muscle. Myenteric neurones have been subdivided into type I(S), which show fast excitatory (and inhibitory) postsynaptic potentials whilst type II(AH) neurones are characterised by an after hyperpolarisation following a rapid action potential. Pharmacological investigations have revealed that GABA elicits transient bicuculline-sensitive, cholinergically-mediated contractions of the guinea-pig ileum (Krantis & Kerr, 1981; Kaplita et al, 1982; Giotti et al, 1983a) through an action at $GABA_A$ receptors, whereas baclofen was without effect on this unstimulated preparation.

Conversely, in transmurally-stimulated tissue, GABA produced a rapid twitch, enhanced from baseline contractions, that was bicuculline-

sensitive. This was followed by a reduction in the repeated twitch height that was unaffected by bicuculline. The $GABA_A$ selective receptor agonist, 3-APS, mimicked the first phase but did not produce a subsequent reduction in twitch height. Conversely, baclofen mimicked this depression of the electrically-evoked twitch indicating an involvement of $GABA_B$ receptors, to which desensitisation occurred within three minutes (Kaplita et al, 1982; Ong & Kerr, 1983). Thus in the guinea-pig ileum, both stimulation and inhibition of evoked cholinergic contractions can be mediated through $GABA_A$ and $GABA_B$ receptors, respectively.

In light of these findings, Cherubini & North (1984a) found that $GABA_A$ receptors were responsible for the depolarisation of type II(AH) neurones following iontophoresed or pressure-applied GABA. Furthermore, presynaptic $GABA_B$ receptors are present on cholinergic terminals which mediate fast EPSPs in type I(S) neurones, whereas slow EPSPs, probably produced in response to released 5-HT or substance P, are controlled by $GABA_B$ receptors on both neuronal subtypes (Cherubini & North 1984b).

The study of Ong & Kerr (1983) was further extended to examine the motility of the distal colon. The involvement of both $GABA_A$ and $GABA_B$ receptors in peristaltic movements was inferred firstly from the fact that picrotoxinin (and desensitisation to 3-APS) slowed faecal pellet expulsion ($GABA_A$ receptor-mediated event). Secondly, baclofen and GABA caused picrotoxinin-insensitive depression of cholinergic contractions indicating an additional $GABA_B$ receptor component. The combined antagonism of $GABA_A$ receptors plus desensitisation to baclofen, slowed the pellet expulsion rate to the same extent as desensitisation of both receptors by GABA alone, providing further confirmation of a multi-GABA receptor involvement.

Although GABAergic neurones do not appear to directly influence gastric motility, prejunctional inhibition of transmitter output by the action of GABA at both receptor subtypes does provide a likely physiological role

for this neurotransmitter in the ENS. This has already been exemplified with the modulation of acetylcholine release, however, there is an accumulation of evidence implicating an interaction between 5-HT and GABAergic neurones.

5-HT produces a slow EPSP in myenteric neurones (Wood & Mayer, 1978) and like GABA, initiates cholinergic contractions. These responses to GABA were resistant to nicotinic antagonists and desensitising doses of 5-HT blocked the contractile responses to GABA. This led Tonini et al (1983) to hypothesise that GABA stimulated the release of 5-HT from an interneurone, which in turn, stimulated cholinergic neurones to elicit a contraction. This conclusion was contradicted by Ong & Kerr (1985), since GABA responses persisted following desensitisation with the serotonergic partial agonist, quipazine, thus negating the concept of 5-HT acting as a mediator of GABA-induced ileal contractions.

A recent report by Shirakawa et al (1989) possibly arbitrates a solution to this controversy since these authors found that 5-HT exerts a dual action on the release of GABA from guinea-pig myenteric neurones. Firstly, 5-HT-induced increases in [^{3}H]GABA release were blocked by the 5-HT_3 receptor antagonist, ICS 205-930 (see later for definition), whilst inhibition of evoked [^{3}H]GABA release by 5-HT was mimicked by the 5-HT_{1A} receptor agonist, 8-hydroxy-DPAT. This reciprocal co-modulation between 5-HT and GABA, highlights one aspect of the functional complexity of the ENS. It would be of equal importance to consider the possible interactions between GABAergic neurones and peptide neuroeffectors as well as the non-adrenergic, non-cholinergic (NANC) system.

Although enteric neurotransmission is obviously far from simple, it is clearly established that GABA inhibits gastric motility by actions on peripheral $GABA_A$ and $GABA_B$ receptors. However, a central action which in contrast, enhances gastric motility has also been described (Andrews &

Wood, 1986; Fargeas et al, 1988). Baclofen (administered i.v. or s.c.) dose-dependently increased gastric motility (and secretion) in the rat. These effects were abolished by both atropine, signifying the involvement of intramural cholinergic nerves and by abdominal and cervical vagotomy, suggesting that centrally-mediated stimulation of $GABA_B$ receptors on vagal efferent fibres to the stomach is responsible for such actions.

THE EVOLUTION OF 5-HT_3 RECEPTOR ANTAGONISTS FROM GASTRIC MOTILITY STIMULANTS

The heterogeneity of 5-HT receptors is far from being a recent concept, for in 1957, the classic paper of Gaddum and Picarelli proposed that 5-HT contracted the guinea-pig ileum by actions at two pharmacologically distinct sites. Firstly, stimulation of receptors on smooth muscle could be blocked by dibenzyline (phenoxybenzamine) and secondly, by an indirect 5-HT-induced contractile response (mediated by the release of acetylcholine from the myenteric plexus) that was antagonised by morphine. Thus, from these two respective sites of action originated the 'D' and 'M' 5-HT receptor classification. Interestingly, these authors confirmed the findings of Rocha de Silva et al (1953) and Gaddum & Hameed (1954), that the direct contractile response to nicotine as well as the indirectly mediated contraction to 5-HT acting at D-receptors could be blocked by cocaine.

The development of radioligand binding techniques plus the synthesis of selective 5-HT receptor agonists and antagonists, classified 5-HT actions at either 5-HT_1 or 5-HT_2 sites (the latter being synonymous with Gaddum's D-receptor). However, since 5-HT-sensitive M-receptors were resistant to the effects of selective 5-HT_1 or 5-HT_2 receptor antagonists, it was necessary to describe a third site, the 5-HT_3 receptor, which is responsible for

mediating many of the excitatory actions of 5-HT in the periphery (Bradley et al, 1986).

Low doses of 5-HT potentiate electrically-evoked cholinergically-mediated contractions of the guinea-pig ileum by an action likely to be presynaptic in the myenteric plexus (Sanger, 1985a, 1987a). This action is also mimicked by the benzamide, metoclopramide (Sanger, 1987b), which is in wide clinical use as a stimulant of upper gut motility and as an antiemetic (Gralla, 1983). The mechanism behind the gastrokinetic action of metoclopramide and related structures has been the subject of intensive research over recent years. Since it is an antagonist at dopamine D_2 receptors (Jenner & Marsden, 1979), it was postulated that increased gastric motility may arise from blockade of these receptors in the gastrointestinal tract (Valenzuela, 1976). However, the inability of the gastrokinetic and antiemetic agent, domperidone (predominantly a D_2 receptor antagonist) to enhance electrically-evoked cholinergic contractions (unlike metoclopramide) in either rat or human stomach (McClelland, 1987; Sanger, 1985b), suggested that D_2 receptors were unlikely to be involved in these actions.

The stimulatory action of metoclopramide appears to be due to an increase in acetylcholine release from postganglionic myenteric neurones (Kilbinger et al, 1982; Sanger 1987b). Since the effects of metoclopramide and its related benzamide, BRL 24924 were antagonised by high concentrations of 5-HT (but not by the 5-HT_{1A} receptor agonist, 8-hydroxy-DPAT) this led Sanger (1987a, 1987b) to propose that these two benzamides increased cholinergic activity by a hitherto unidentified myenteric 5-HT receptor. BRL 24924 has negligible affinity for D_2 receptors and is a potent stimulant of rat (Cooper et al, 1986) isolated gastrointestinal motility. However, evidence from electrophysiological studies suggests that the action of BRL 24924 is more complex than initially perceived.

The slow depolarising action of low concentrations of 5-HT (Wood &

Mayer, 1978; Surprenant & Crist, 1988) on type II/AH neurones in the guinea-pig jejunum can be blocked by BRL 24924 (Mawe et al, 1986). The receptor responsible for this action was designated a 5-HT_{1P} receptor and it was suggested that the compound could act by antagonising the 5-HT-mediated activation of neurones that are inhibitory to cholinergic function. Saturable 5-HT_{1P} binding sites to the selective agonist, [^{3}H]5-hydroxy-indalpine, were subsequently demonstrated in enteric membranes (Branchek et al, 1988). Interestingly, the 5-HT_3 receptor antagonist, zacopride, mimics the slow response to 5-HT, presumably by acting at the 5-HT_{1P} receptor (Wade et al, 1989), however, the 5-HT_3 receptor agonist 2-methyl-5-HT, mimics the fast 5-HT response which is in turn blocked by the 5-HT_3 receptor antagonist, ICS 205-930. Thus it seems that whilst the slow response to 5-HT is mediated by the 5-HT_{1P} receptor, it is 5-HT_3 receptors that appear to be involved with the fast response. Since BRL 24924 also has 5-HT_3 antagonistic properties (Sanger, 1987a) it would perhaps now be appropriate to discuss more fully the concept of 5-HT_3 receptor antagonism.

5-HT_3 receptor antagonists

The ability of cocaine to block the contractile response to 5-HT on the guinea-pig ileum, prompted Fozard and his colleagues (1977) to re-assess these actions in isolated tissues. It was concluded that cocaine blocked both 5-HT-evoked tachycardia in rabbit isolated heart and bradycardia (the Bezold-Jarisch reflex) by an action at 'M' (5-HT_3) receptors. Furthermore, 5-HT-evoked tachycardia was also antagonised by metoclopramide (Fozard & Mobarok-Ali, 1978) Despite specific actions at 5-HT_3 receptors, neither cocaine nor metoclopramide are selective, solely for these receptors, and with pA_2 values of around 6, sufficient scope existed for developing more potent 5-HT_3 receptor-selective antagonists.

Synthesis of the first specific 5-HT_3 receptor antagonist was based on

the structure of cocaine and from a series of substituted benzoic acid esters of tropine, emerged MDL 72222. This compound had potent activity in the rabbit heart (pA_2 = 9.3) and Bezold-Jarisch models, although only high concentrations antagonised 5-HT-evoked contractions in the guinea-pig ileum Fozard et al, 1984). Concurrently with research at Merrell Dow, medicinal chemists at Sandoz based their chemical programme on the indoleamine structure of 5-HT itself and by incorporating a tropine ring system developed ICS 205-930. Again, this 5-HT_3 receptor antagonist was potent in the rabbit heart model (pA_2 = 10.6), the vagus nerve and guinea-pig ileum (Richardson et al, 1985).

In pursuit of these first 5-HT_3 receptor antagonists a number of subsequent compounds have evolved, most notably, GR38032F (Butler et al, 1988), BRL 43694 (Sanger & Nelson, 1989) and zacopride (Smith et al, 1988) which possess potent antagonistic activity in a number of peripheral (and central, see next section) 5-HT_3 receptor models.

Many of the peripheral physiological responses to 5-HT arise as a consequence of the activation of 5-HT_3 receptors that are heterogeneously distributed in a number of neuronal preparations (Richardson & Engel, 1986, for review). In the autonomic nervous system, 5-HT_3 receptors are present both on postganglionic parasympathetic and sympathetic fibres to the heart and on sympathetic fibres innervating the superior cervical ganglion (SVG) for which in membranes of the latter structure, recognition sites for the ligand, [^{3}H]ICS 205-930, have also been identified (Hoyer et al, 1989). The presence of 5-HT_3 (M) receptors in the enteric nervous system has been long established, however, they have recently been characterised on partially purified enteric neuronal membranes using [^{3}H]zacopride (Gordon et al, 1990).

The blockade of both the Bezold-Jarisch reflex and the nociception arising from 5-HT-induced subcutaneous blisters by ICS 205-930

(Richardson et al, 1985) indicate that 5-HT_3 receptors are located on sensory afferent neurones. Such sites have been confirmed in vagus nerve membranes (Kilpatrick et al, 1989; Hoyer et al, 1989) and visualised autoradiographically, in nodose ganglion cell somata (the cell bodies of the vagus nerve) by the latter authors who proposed that 5-HT_3 receptors are presynaptically located in the vagal system. Such a hypothesis could be of significance in considering a possible mechanism underlying the antiemetic actions of 5-HT_3 receptor antagonists which will be reviewed in the next section.

5-HT_3 RECEPTORS AND THE CENTRAL NERVOUS SYSTEM

The existence of 5-HT_3 binding sites in mammalian brain was first demonstrated by Kilpatrick et al (1987). Using the ligand [^{3}H]GR65630, specific, high affinity (K_D = 0.23nM) 5-HT_3 binding sites in rat brain synaptic membranes were found throughout the cerebral cortex (especially the entorhinal cortex) with lower numbers in areas associated with the limbic system. This finding has been substantiated by a number of reports using a variety of 5-HT_3 receptor ligands; [^{3}H]zacopride (Barnes et al, 1988b; Pinkus et al, 1989), [^{3}H]quipazine (Peroutka & Hamik, 1988), [^{3}H]quaternised ICS 205-930 (Watling et al, 1988), [^{3}H]BRL 43694 (Nelson & Thomas, 1989), [^{3}H]LY 278584 (Wong et al, 1989) and [^{3}H]GR67730 (Kilpatrick et al, 1990). 5-HT_3 recognition sites have also been identified in membranes of human hippocampus and amygdala (Barnes et al, 1988d, 1989a) in addition to the human cerebral cortex (Kilpatrick et al, 1989).

Characterisation of a novel receptor based on receptor binding data should not be accepted in isolation but rather with the support of functional receptor-mediated events. Whilst 5-HT_3 sites in the periphery are unquestionably, functional receptors, there has been considerable interest

in attempting to relate the behavioural and functional effects of 5-HT_3 receptor antagonism to prospective central binding sites.

The anxiolytic potential of 5-HT_3 receptor antagonists has been demonstrated in a number of rodent and primate models of anxiety. Social interaction between two rats is manifest by a series of behavioural paradigms such as grooming, smelling and climbing/mounting which are suppressed in a brightly lit, unfamiliar environment. Treatment with the 5-HT_3 receptor antagonist, GR38032F reverses the effects of such aversive stimuli (Jones et al, 1988) and also the aggressive behaviours of marmosets and cynomolgus monkeys. Similar effects have also been observed with BRL 43694 (Piper et al, 1988).

It appears that 5-HT_3 receptor antagonists may also have potential in anti-psychotic therapy and one model that is predictive of the psychotic condition is the hyperlocomotor activity produced by the administration of dopamine receptor agonists to midbrain regions. In light of this, GR38032F reverses the hyperactivity produced in response to infusions of dopamine and amphetamine into the nucleus accumbens of rats and marmosets (Costall et al, 1987a). Neurochemical evidence to support these dopaminergic-related behavioural findings was provided by Blandina et al, (1989) who demonstrated that the 5-HT-induced release of endogenous dopamine from superfused rat striatal slices could be mimicked by the 5-HT_3 receptor agonist, 2-methyl-5-HT, and antagonised by ICS 205-930. Furthermore, Imperato & Angelucci (1989) using *in vivo* microdialysis, found that ICS 205-930 antagonised morphine-induced dopamine release from the nucleus accumbens through an action in the ventral tegmental area (the location of the cell bodies of mesolimbic dopaminergic neurones). This suppression of the action of morphine has suggested that 5-HT_3 receptor antagonists could have potential applications in the treatment of addiction. To this end, both ICS 205-930 and MDL 72222 reverse the rewarding effects of acute morphine and nicotine in the place-preference conditioning test

(Carboni et al, 1988, 1989) in addition to blocking the effects of alcohol and benzodiazepine withdrawal (Oakley et al, 1989a, 1989b). Potassium-stimulated [^{3}H]acetylcholine release in rat entorhinal cortex is inhibited by 2-methyl-5-HT and 5-HT (but only in the presence of the 5-HT_2/5-HT_{1C} receptor antagonist, ritanserin) and this inhibition is reversed by GR38032F and zacopride. In the absence of ritanserin, however, 2-methyl-5-HT in the presence of GR38032F or zacopride potentiated potassium-stimulated [^{3}H]acetylcholine release (Barnes et al, 1989b). Thus, 5-HT_3 receptor-agonism reduces release whilst 5-HT_3 receptor antagonism promotes release either by preventing the action of the agonist or by permitting a facilitatory agonist to act at a ritanserin-sensitive site.

To date, electrophysiological studies of 5-HT_3 receptors have been slow to emerge. Using single cell recording, 2-methyl-5-HT applied to medial prefrontal cortical cells, suppressed cell firing, and this effect was antagonised by BRL 43694 and ICS 205-930 (Ashby et al, 1989). The precise ionic nature of the response to central 5-HT_3 receptor activation has not yet been elucidated although research by Derkach et al (1989) has proposed that in the guinea-pig submucous plexus, 5-HT_3 receptors are ligand-gated cation channels. Using the neuroblastoma cell line N1E-115, voltage clamp studies have demonstrated that 5-HT-induced depolarisation responses are blocked by MDL 72222 and ICS 205-930 (Neijt et al, 1986). This action of 5-HT is believed to mediate the opening of a 5-HT_3 receptor-coupled channel through which monovalent cations such as Na^+ and K^+ are conducted (Lambert et al, 1989).

Perhaps one of the most exciting actions of 5-HT_3 receptor antagonists with respect to therapeutic potential has been the discovery of their potent anti-emetic action. Cancer patients undergoing irradiation or certain cytotoxic chemotherapeutic regimes such as cisplatin, often experience nausea and vomiting as concomitant side effects. Until recently, this has been treated with such antiemetics as domperidone and metoclopramide,

dexamethasone and synthetic cannabinoids either alone or in combination. However, since these drugs produce side effects of their own, the introduction of 5-HT_3 receptor antagonists, (Cunningham et al, 1987; Leibundgut & Lancranjan, 1987; Carmichael et al, 1988; Seinen et al, 1989), appeared to offer a superior alternative to conventional therapy in view of their lack of untoward side effects; indeed GR38032F is already marketed for this indication as 'Ondansetron'.

Emesis induced by cisplatin in the ferret has proved to be an invaluable model for the screening of antiemetic drugs and especially for 5-HT_3 receptor antagonists. Miner et al (1986) showed that BRL 24924 was a potent antagonist of cisplatin-induced emetic episodes (in comparison with metoclopramide and domperidone) as well as those induced by doxorubicin and cyclophosphamide (Miner & Sanger, 1986). Further confirmation of the antiemetic efficacy of 5-HT_3 receptor antagonists was provided by Costall et al (1986, 1987b) using ICS 205-930 and zacopride. At this stage, precisely why cisplatin causes emesis was not understood. The role of abdominal vagal afferent neurones in conveying sensory information regarding gastric contents and contractility (Andrews & Wood, 1986) led Miner & Sanger (1986) to propose that since these fibres could be activated by 5-HT, cytotoxic agents may stimulate the release of this neuroactive agent to subsequently activate 5-HT_3 receptors. One possible central reception site for this information could be the area postrema.

The importance of the area postrema as the location of the chemoreceptor trigger zone is widely accepted (Borison & Wang, 1953). It is often referred to as one of the circumventricular organs since it is a discrete region located at the caudal end of the fourth ventricle lying outside the blood brain barrier (Leslie, 1986). How the area postrema functions in relation to emetic stimuli is unclear but it is known that certain emetic agents, including dopamine, activate specific neurones within it (Carpenter et al, 1983). Since this structure is innervated by vagal afferent

fibres, the area postrema could provide a possible site for the action of 5-HT_3 receptor antagonists (Andrews & Hawthorn, 1987).

Whole body irradiation of the ferret results in severe retching and vomiting 30 minutes later which lasts for approximately 90 minutes. Abdominal vagotomy virtually abolishes emetic episodes in the initial 30 minute period but is ineffective on the ensuing 60 minute epoch. The effect of a small dose ($1mgkg^{-1}$) of BRL 24924 is synonymous with that of vagotomy whilst a higher dose ($5mgkg^{-1}$) abolishes vomiting over the entire 90 minute period (Andrews & Hawthorn ,1987). Thus it seems that two possible sites of action could be involved in mediating the emetic response; the abdominal vagus nerve is a likely site of action, however, a central extra-abdominal location such as the area postrema cannot be excluded.

This hypothesis was investigated by Higgins et al (1988) who found that low (microgram) doses of GR38032F, GR65630 and MDL 72222 injected directly into the area postrema of the ferret protected against emesis induced by intraperitoneally administered cisplatin. These findings indicated that 5-HT_3 receptors are likely to be located within the area postrema itself, and indeed, saturable 5-HT_3 binding sites have been found on area postrema membranes of a number of species (Barnes et al, 1988c; Kilpatrick et al, 1989) in addition to membranes of vagus nerve (Kilpatrick et al, 1989; Hoyer et al, 1989). However, in order to determine the precise central location of 5-HT_3 binding sites, several research groups have turned to the technique of receptor autoradiography.

The first autoradiographical study of 5-HT_3 receptor localisation in mouse and human brain, using the ligand [^{3}H]ICS 205-930 (Waeber et al, 1988, 1989), showed that these sites are distributed heterogeneously. However, high densities predominate in the nuclei of certain medullary regions, especially of the solitary tract, the spinal tract of the trigeminal nerve, the vagus nerve in addition to the substantia gelatinosa of the spinal cord. Whilst lower densities were observed in the hippocampus and

amygdala by far the most concentrated location of 5-HT_3 sites in the human brain was reported to be the area postrema. This concurred with membrane binding studies and provided a strong link between the established relevance of the area postrema as being the likely site for the co-ordination of the emetic reflex and the discrete injection studies of Higgins et al (1988). Moreover, Kilpatrick et al (1988), showed that in the rat hindbrain, the binding of [^{3}H]GR65630 was attributable to the area postrema but not to any other surrounding nuclei. The apparent differential targetting of the two radioligands for specific brainstem nuclei has therefore prompted the question as to the possible existence of discretely located 5-HT_3 receptor subtypes which will provide one aspect of the aims of this thesis.

AIMS OF THE THESIS

Having confirmed the presence of $GABA_B$ (and $GABA_A$) receptor binding sites both in synaptic membranes as well as autoradiographically, these two methodological approaches will provide the basis for the characterisation of $GABA_B$ receptor agonists. The relative potencies of baclofen (enantiomers and racemic mixture) in addition to the phosphinic acid derivative of GABA, 3-aminopropylphosphinic acid (3-APA) to displace the binding of [^{3}H]GABA from $GABA_B$ binding sites will be assessed. Furthermore, structure activity relationships of the $GABA_B$ receptor antagonists, phaclofen, saclofen, 2-hydroxy-saclofen and CGP 35348 will also be determined in membrane binding assays for both receptor subtypes.

Autoradiographical localisation of beta-adrenoceptors using the radioligand, (-)-[^{125}I]iodopindolol under conditions to specifically label β_1- and β_2-adrenoceptor subtypes will be evaluated. Particular reference would be made to the relative distribution of β_1-adrenoceptors and $GABA_B$ receptors within the laminar regions of the frontal cortex. This will have

particular significance in assessing the influence of prolonged antidepressant administration on these receptor sites (see below).

As already mentioned in the preceding section, a detailed autoradiographical analysis of the distribution of [^{3}H]BRL 43694 binding sites, especially in those rat hindbrain regions associated with the dorsal vagal complex will be described. This would be performed in conjunction with other 5-HT$_3$ receptor radioligands, namely, [^{3}H]GR65630, [^{3}H]zacopride and [^{3}H]quipazine with a view to identifying the possible locations of 5-HT$_3$ receptor subtypes. Since the dorsal vagal complex provides the site for the primary integration of the majority of vagal sensory information, the binding of [^{3}H]BRL 43694 would be examined after lesioning of the vagus nerve (via removal of the nodose ganglion) with a view to providing an association of 5-HT$_3$ receptor sites with either a pre- or postsynaptic neuronal location.

A separate chapter is devoted to an attempt to resolve the discrepancies in existing literature reports regarding the possible modulation of GABA$_B$ receptors, observed in membrane preparations of rat frontal cortex, following repeated antidepressant administration. Autoradiographical examination of GABA$_B$ receptor sites within the laminar regions of the frontal cortex would be investigated in parallel with β_1-adrenoceptors, after treatment with a variety of antidepressants and also the GABA$_B$ receptor antagonist, CGP 35348.

The final chapter describes the characteristics of GABA$_B$ receptor modulation of forskolin- and noradrenaline-activated adenylyl cyclase transduction mechanisms, with a view evaluating the action of 3-APA on these systems in conjunction with (-)-baclofen. The sensitivities of such adenylyl cyclase-coupled GABA$_B$ receptors would also be examined in tissue from rats treated either with antidepressants or GABA$_B$ receptor antagonists.

CHAPTER 2

GENERAL METHODOLOGY

AUTORADIOGRAPHICAL PROCEDURES

Tissue preparation

A perfusing solution of 0.1% paraformaldehyde in 0.01M phosphate-buffered saline (0.9%) adjusted to pH 7.4 with 0.2M hydrochloric acid was formulated as follows. To 20.2ml $Na_2PO_4.2H_2O$ was added 4.75ml $NaH_2PO_4.2H_2O$ plus 5ml 10% paraformaldehyde and diluted to a final volume of 500ml with 0.9% saline solution.

Male Wistar rats (180-250g) were anaesthetised with sodium pentobarbitone (Nembutal, 40mgkg^{-1} i.p.) and perfused-fixed with 250ml of the above solution via intra-cardiac administration through the left ventricle. Brains were carefully removed and mounted onto a cork slice with 'Tissue Tek' (Miles Scientific), frozen in iso-pentane cooled to approximately -40°C in liquid nitrogen and stored at -80°C until required.

Either parasagittally-, coronally- or horizontally-orientated sections (10µm) were cut at -20°C using a 'Frigostat' cryostat (Reichert-Jung Ltd.) and thaw-mounted onto glass microscope slides to be stored at -20°C until use.

Localisation of $GABA_A$ and $GABA_B$ binding sites

Frozen brain sections were thawed for 45 minutes and pre-incubated at room temperature for 60 minutes in 200ml Tris-HCl buffer (50mM; pH 7.4) containing 2.5mM $CaCl_2$. After thorough drying under ambient conditions, sections were incubated individually for 20 minutes with 150µl buffer containing [^{3}H]GABA (50nM) with or without test drugs. Selectivity for $GABA_B$ sites was achieved in the presence of 40µM isoguvacine (to block $GABA_A$ binding sites), whilst $GABA_A$ sites were labelled in the presence of 100µM (-)-baclofen (blocking $GABA_B$ sites). Non-specific binding to these respective sites was defined by either (-)-baclofen (100µM) or isoguvacine (100µM).

Following incubation, excess radiolabel was aspirated from the section which then received 2 x 3 second washes in room temperature buffer. Sections were allowed to dry under a stream of cold air prior to their apposition to tritium-sensitive 'Hyperfilm' (Amersham, U.K. Ltd.) for between 3 and 4 weeks to generate autoradiograms.

a) Saturation analysis

Derivation of the kinetic parameters K_D and B_{max} was achieved either by varying the [^{3}H]GABA concentration over the following range; 400, 200, 100, 50, 25nM. Alternately, by retaining a fixed radioligand concentration and increasing the concentration of non-radioactive GABA (20, 50, 250, 950 and 3000nM). This latter method enabled the determination of the amount of radiolabel bound at a given concentration of non-radioactive ligand.

b) Analysis of binding data

Based on the assumption that both labelled and unlabelled GABA bind to $GABA_B$ sites (in the presence of excess isoguvacine) with equal affinities, it is possible to calculate the B_{max} and K_D of these binding sites using a fixed [^{3}H]GABA concentration in the presence of increasing concentrations of unlabelled GABA. This approach yields a binding displacement curve from which the specific [^{3}H]GABA binding (B_0) is represented by the difference between total (in the absence of unlabelled GABA) and the non-specific binding. The IC_{50} value (the concentration of GABA that displaced 50% of the specific radioligand binding) can then be used to derive the kinetic binding parameters (DeBlasi et al, 1989).

According to the equation of Cheng & Prussoff (1973),

$$IC_{50} = K_C (1 + [L]/K_H)$$

where L is the ligand concentration whilst K_C and K_H denote the

dissociation constants of unlabelled and labelled ligand, respectively. If it is assumed that only a small percentage of the total radioligand added binds to the receptor then, free radioligand equals total radioligand. Also, if K_C and K_H are identical then, K_D equals K_C and K_H. Therefore,

$$\begin{aligned} IC_{50} &= K_D\,(1 + [L]/K_D) \\ &= K_D + [L] \qquad \text{so that} \qquad (A) \\ K_D &= IC_{50} - [L] \end{aligned}$$

The B_{max} value is derived by rearranging the following equation which denotes the law of mass action

$$B_0 = \frac{B_{max}\,[L]}{[L] + K_D}$$

$$B_{max} = B_0\,(K_D + [L]/[L]$$

Substituting from equation (A),

$$B_{max} = B_0\,IC_{50}/[L]$$

Such an approach, however, can only be undertaken if the following conditions are satisfied.

(a) labelled and unlabelled ligands have equal affinities for the binding sites.

(b) only one class of binding site exists.

(c) there is no cooperativity between sites.

(d) only a small proportion of the total radioligand added is bound to the receptor/binding site.

Localisation of beta-adrenoceptor binding sites

Frozen brain sections were thawed for 45 minutes and then incubated for 60 minutes at room temperature in a Tris-HCl/saline buffer (20mM Trizma base; 135mM NaCl; pH 7.4) containing (-)-[^{125}I]iodopindolol (150pM). Beta-adrenoceptor binding was resolved into $beta_1$- and $beta_2$-adrenoceptor subtypes in the presence of either the $beta_2$-adrenoceptor antagonist, ICI 118,551 (50nM) or the $beta_1$-adrenoceptor antagonist, CGP 20712A (100nM), respectively. Non-specific binding was defined using (-)-isoprenaline (200µM).

Following incubation, sections were washed for 2 x 5 minute periods in buffer at 4°C, given a rapid 3 second rinse in ice-cold distilled water to remove buffer salts and then quickly dried in a stream of cold air. Sections were apposed to 'Hyperfilm' for between 24 and 48 hours to generate autoradiograms with calibrated brain paste standards made according to the following method of Clark & Hall (1986). Briefly, whole brains from 6 rats were homogenised in 2ml distilled water plus 6 drops of silicone oil (to prevent the tissue from foaming) using a Polytron homogeniser (setting 5 for 30 seconds). Ten aliquots of tissue were then weighed into Eppendorf microfuge tubes with decreasing concentrations of (-)-[^{125}I]iodopindolol (40µl) over a 20-fold dilution range from the stock solution of radioactivity. Tubes were vortexed for 10 minutes to ensure thorough mixing prior to centrifugation for 3 minutes at 7000g to remove any trapped air and then snapped-frozen in liquid nitrogen. Frozen paste standards were mounted onto microtome chucks and sectioned (10µm) prior to thaw-mounting onto subbed gelatinised slides.

Localisation of 5-HT_3 binding sites

For 5-HT_3 receptor autoradiography, sections were mounted onto slides subbed with a solution of 0.1% gelatine containing 0.05% chromic potassium sulphate. Frozen brain sections were thawed for 45 minutes prior

to a 60 minute pre-incubation at room temperature in 200ml HEPES buffer (5mM), adjusted to pH 7.4 with 40% potassium hydroxide. Sections were then thoroughly dried in air before the addition of 150µl 5mM HEPES buffer containing [^{3}H]BRL 43694 (10nM). Non-specific binding was defined in the presence of GR38032F (100µM). Incubation was allowed to proceed for 30 minutes at room temperature after which the radiolabelled solution was quickly aspirated and the sections rinsed in ice-cold buffer (2 x 3 seconds). Having been dried in a stream of cold air, bound sections were apposed to 'Hyperfilm' for 4 weeks.

The above 5-HT$_3$ receptor binding protocol was also employed for the ligands; [^{3}H]zacopride, [^{3}H]GR65630 and [^{3}H]quipazine using the same concentrations of radioligand.

Autoradiographical analysis

After the required duration of exposure had elapsed, tritium-sensitive 'Hyperfilm' was developed in Kodak D-19 developer for between 30 and 45 seconds, fixed for 5 minutes in a 1 + 4 dilution of 'Unifix' (Kodak) at room temperature and washed for at least 10 minutes in running tap water before thorough drying.

Optical densities of radioligand binding were measured using a 'Quantimet 970' image analysis system (Cambridge Instruments, U.K., Ltd.) against calibrated [^{3}H]micro-scales (Amersham International Ltd.) The radioactive tissue equivalent values of these standards in relation to intact brain grey matter ranged from 1.3 - 33.0 nCi/mg tissue. In the case of (-)-[^{125}I]iodopindolol calibrated brain paste standards were made according to the method of Clark & Hall (1986) described earlier. Optical density values were converted to fmol/mg tissue using the simple relationship:-

$$1\text{nCi} = 1/\text{specific activity} \times 1000 \text{ fmol}$$

MEMBRANE BINDING PROCEDURES

Preparation of whole brain synaptic membranes

This procedure was based on the method of Bowery et al (1983) for which all centrifugation steps were performed at 4°C. Freshly dissected brains from male Wistar rats were homogenised in 15 volumes (approximately 25ml/brain) ice-cold 0.32M sucrose solution using a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 1000g for 10 minutes, the resulting P_1 nuclear pellet discarded and the supernatant re-centrifuged at 20,000g for 20 minutes forming a P_2 pellet. This was lysed by re-suspension in 20ml ice-cold distilled water followed by centrifugation at 8000g. The 'buffy coat' was retrieved by washing with the supernatant and the combined suspension was then centrifuged for a further 20 minutes at 48,000g. The resultant crude synaptic membranes were either washed in distilled water and recentrifuged prior to resuspension in 50mM Tris-HCl buffer (pH 7.4) for further washing and usage, or, were stored at -20°C for a minimum of 24 hours.

Determination of $GABA_A$ and $GABA_B$ binding sites

Frozen and thawed membranes were resuspended in 25ml Tris-HCl buffer (50mM; pH 7.4). containing 2.5mM $CaCl_2$ and allowed to stand for 45 minutes at ambient temperature prior to centrifugation at 9000g for 10 minutes. This washing procedure was repeated a further 3 times with 15 minute incubation periods between each centrifugation. The final pellet was resuspended in 35ml buffer together with either isoguvacine (40µM) or (-)-baclofen (100µM) to resolve binding to $GABA_B$ and $GABA_A$ sites, respectively.

For the binding assay, 800µl of the membrane suspension (0.5 - 1mg protein) were added to Eppendorf microfuge tubes containing 100µl of either (-)-baclofen (100µM) or isoguvacine (100µM) to define non-specific

binding to $GABA_B$ and $GABA_A$ sites, respectively. Alternatively, these agonists were replaced with an equivalent volume of test compound.

The incubation was initiated by the addition of 100µl [^{3}H]GABA (50nM) giving a final radioactive concentration of 5nM (in an assay volume of 1ml) and allowed 10 minutes to reach equlibrium at room temperature. Bound radiolabel was separated from free, by centrifugation at 7000g in an Eppendorf bench centrifuge for 5 minutes. The resulting supernatant was aspirated from the pellet which was solubilised overnight with 100µl 'Soluene 350' (Hewlett-Packard Ltd). This was subsequently neutralised with 0.4ml HCl (0.2M) and bound radioactivity measured by inverting each tube into 10ml 'Optiphase Safe' scintillation fluid (LKB Scintillation Products) and counted by and LKB 1219 'Rackbeta' scintillation counter.

Expression of specific radioligand binding

The amount of radiolabel specifically bound was taken as the difference between total and non-specific binding and expressed either as a function of the tissue concentration (autoradiography) or normalised to a percentage of total binding thus enabling the construction of displacement/inhibition curves to various compounds.

ADENYLYL CYCLASE STUDIES

Tissue preparation and adenylyl cyclase activation

This protocol was based essentially on the methods of Hill (1985) and Watling & Bristow (1986).

Freshly removed brains from stunned and decapitated male Wistar rats (180-250g) were dissected on ice and cross-chopped slices of cerebral cortex (250µm x 250µm) prepared using a McIlwain tissue chopper. Slices were gently dispersed by means of a Pasteur pipette in 10ml Krebs-Ringer

bicarbonate (KRB) buffer of the following composition (mM); NaCl (118), KCl (4.7), $CaCl_2$ (2.5), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25), glucose (11) and ascorbic acid (0.005%). They were then transferred to a larger volume of KRB (100ml/brain), continually gassed with O_2:CO_2 (95%:5%) to maintain the pH at 7.4 and washed for 90 minutes (with one buffer change after 30 minutes).

Following this, excess KRB buffer was aspirated and 50µl aliquots of the resulting tissue suspension were added to flat-bottomed tubes containing fresh KRB with or without test drugs diluted in buffer (such as (-)-baclofen). Tubes containing slices were then pre-incubated for 10 minutes at 37°C prior to the initiation of adenylyl cyclase activity by the addition of either forskolin (10µM) or noradrenaline (100µM) in 50µl aliquots to give a final assay volume of 500µl. In experiments assessing the effects of $GABA_B$ receptor antagonists, these compounds were preincubated for 5 minutes at 37°C prior to the addition of (-)-baclofen for a further 10 minutes.

The reaction was allowed to proceed for 10 minutes and terminated in a boiling water-bath (3 minutes). Having allowed the slices to settle to the bottom of the tube, 50µl aliquots of the supernatant were transferred to Eppendorf microfuge tubes to be assayed for cAMP content by radioimmunoassay.

Determination of cAMP concentrations

(a) Preparation of cAMP binding protein

This protocol was based on the method of Brown et al (1972). Cortices from 20 bovine adrenal glands were dissected on ice and homogenised in 1.5 volumes of ice-cold 50mM Tris buffer (pH 7.4) (containing 0.25M sucrose, 25mM KCl and 5mM $MgCl_2$) using a Waring blendor. The homogenate was centrifuged at 2000g for 5 minutes and the resulting supernatant recentrifuged at 5000g for 15 minutes. The ensuing secondary

supernatant was dispensed into 0.5ml aliquots and stored at -20°C until use.

(b) cAMP radioimmunoassay

Dilutions in water to give a nine point cAMP standard curve over the range of 0.05 - 15 pmol cAMP were made and 50µl aliquots added to microfuge tubes in duplicate. cAMP binding protein was diluted (1 + 40) with 50mM Tris-HCl 'cAMP buffer' (pH 7.4) containing 2mM theophylline from which 100µl were added to each tube to be followed by a further 150µl cAMP buffer containing [^{3}H]cAMP (1nM). Total [^{3}H]cAMP bound was determined in the absence of unlabelled cAMP whereas a blank value in the absence of cAMP binding protein was also made which denoted non-charcoal adsorbed [^{3}H]cAMP. Assay tubes were thoroughly vortexed and placed on ice for at least 90 minutes.

A 0.4% suspension of charcoal was made in 20ml ice-cold cAMP buffer into which had been dissolved 400mg bovine serum albumin (A-4503; Sigma Chemical Co. Ltd.). Bound radiolabel was separated from free by the addition of a 100µl aliquot of this charcoal suspension into each tube followed by vortexing and centrifugation for 3 minutes at 7000g in an Eppendorf bench centrifuge. 250µl of the resulting supernatant were added to a vial containing 10ml 'Optiphase Safe' scintillation fluid which was then counted by an LKB 'Rackbeta' scintillation counter. Unknown cAMP concentrations were derived from the standard curve and expressed in terms of the amount formed in 10 minutes per milligram of protein.

PROTEIN CONCENTRATION DETERMINATION

Estimates of the protein concentrations of brain tissue were made using the method of Bradford (1976). Coomassie Brilliant Blue (Bradford) reagent (G-250; Aldrich Chemical Co. Ltd.) was made by dissolving 100mg

G-250 in 50ml 95% ethanol. To this solution was added 100ml 85% (w/v) phosphoric acid and diluted further with distilled water to a final volume of 1 litre. The reagent was continually stirred overnight, filtered and stored at 4°C until use.

Aliquots of tissue (50µl) were diluted with 1.95ml NaOH (1M) and incubated at 60°C for 20 minutes to solubilise the tissue. Dilutions of 0.5mg/ml bovine serum albumin (fraction V) to give an 8-point concentration standard curve over the range of 10 - 50µg protein were made up to 100µl with 0.15M NaCl. 50µl aliquots of the 'unknown' samples were assayed; to each tube was added 1ml Bradford reagent which was vortexed after standing for 15 minutes, its optical density read spectrophotometrically at 595nm using a Hewlett-Packard spectrophotometer. Unknown protein concentrations were then determined from the standard curve.

RADIOLIGANDS, DRUGS AND CHEMICALS

Sources of radioligands

[2,8-^{3}H]adenosine 3',5'-cyclic phosphate ammonium salt (specific activity = 34.2 Ci/mmol) (New England Nuclear Research Products).

[methyl-^{3}H]AHR 11190 hydrochloride (zacopride) (specific activity = 78.9 Ci/mmol) (Amersham International plc).

4-amino-n-[2,3-^{3}H]butyric acid (GABA) (specific activity = 92.5 Ci/mmol) (Amersham International plc).

[^{3}H]BRL 43694 (specific activity = 21 & 61 Ci/mmol) (SmithKline Beecham Pharmaceuticals plc).

[^{3}H]GR 65630B (specific activity = 85 Ci/mmol) (Glaxo Group Research plc).

(-)-[^{125}I]iodopindolol (specific activity = 2200 Ci/mmol) (New England Nuclear Research Products).

[piperazinyl-^{3}H]quipazine (specific activity = 55 Ci/mmol) (New England Nuclear Research Products).

Sources of drugs and chemicals

Baclofen isomers, CGP 35348 (kindly supplied by Ciba-Geigy Ltd. Basel, Switzerland).

Isoguvacine (Cambridge Research Biochemicals).

Zacopride, ICS 205-930 (kindly supplied by Wyeth Research Laboratories).

BRL 43694, GR 38032F, 8-hydroxy-DPAT, methysergide, metoclopramide, quipazine, CGP 20712A & ICI 118,551 (kindly supplied by SmithKline Beecham Pharmaceuticals).

General laboratory reagents were of analytical grade and were obtained from either BDH Chemicals Ltd. or the Sigma Chemical Co. Ltd.

CHAPTER 3

LOCALISATON OF CENTRAL GABA RECEPTORS, BETA-ADRENOCEPTORS AND 5-HT_3 RECEPTORS, USING RECEPTOR AUTORADIOGRAPHY

INTRODUCTION

The pioneering work of Young & Kuhar (1979) localising central opiate receptors, established receptor autoradiography as a powerful technique which has subsequently enabled the identification of central (and peripheral) binding sites for a plethora of neurotransmitter classes. A substantial refinement from radioligand binding to synaptic membranes since it confers the advantage of preserving the morphology intact, thus enabling the localisation of discrete binding sites.

This chapter will first seek to confirm the autoradiography of central $GABA_A$ and $GABA_B$ receptor sites reported by Bowery et al (1987) in addition to beta-adrenoceptors (Rainbow et al, 1984; Ordway et al, 1988). These findings will act as a foundation for the subsequent studies described in this thesis.

Secondly, a detailed analysis of the localisation of 5-HT_3 sites in the rat hindbrain will be described with the aid of the 5-HT_3 receptor radioligands, [^{3}H]BRL 43694, [^{3}H]zacopride, [^{3}H]GR65630 and [^{3}H]quipazine.

RESULTS

Autoradiographical localisation of GABA receptors

The autoradiographical distribution of $GABA_A$ and $GABA_B$ receptor sites in coronal sections of rat brain may is illustrated in Figure 3.0 (A-L). Although a comparatively uniform density of binding was apparent across the wide majority of regions, there were nevertheless a number of structures in which the presence of one GABA receptor subtype clearly predominated over the other. With regard to $GABA_A$ sites of the telencephalic region, these were noted especially in the internal granular and external plexiform layers of the olfactory bulb in addition to the frontal

cortex (Figure 3.0; A,B). Although densities of both receptor subtypes were low in the basal ganglia the medial forebrain bundle was labelled preferentially under conditions in which $GABA_A$ receptor sites are detected (Figure 3.0; C). This was also evident in the ventroposterolateral thalamic nucleus (Figure 3.0; E) and the lateral mammillary body (Figure 3.0; I). Perhaps the most striking contrast between the distribution of both GABA receptor subtypes was observed in the cerebellum. High densities of $GABA_A$ sites were a feature of the granular layer with correspondingly lower densities within the molecular layer (Figure 3.0; K), Conversely, $GABA_B$ receptor autoradiography assumed the opposite characteristics of $GABA_A$ sites in that the highest densities of these baclofen-sensitive sites were found in the molecular layer (Figure 3.0; L). A preponderance of $GABA_B$ binding sites was also noted in a number of diencephalic structures, especially the ventroposteromedial and the lateral posterior thalamic nuclei in addition to the nucleus of the medial habenula (Figure 3.0; F).

Within the mesencephalon, lower densities of predominantly $GABA_B$ sites were detected in the fascicular retroflexus of Meynert (Figure 3.0; H). This afferent habenular projection innervates the interpeduncular nucleus which was also selectively labelled with $GABA_B$ sites (Figure 3.0; J). Finally, mapping of central GABA receptors revealed heavy labelling of the superior colliculus with $GABA_B$ sites, especially in the dorsally located superficial grey layer (Figure 3.0; H, J).

Characterisation of $GABA_B$ receptor agonists and antagonists in brain tissue

Pharmacological characterisation of $GABA_B$ receptor binding sites labelled by [^{3}H]GABA (5nM) under conditions preventing any binding to $GABA_A$ sites was performed in whole brain synaptic membranes with the racemate of baclofen or its (-)- and (+)-enantiomers. Displacement profiles (Figure 3.1A) revealed that (-)-baclofen potently inhibited [^{3}H]GABA

binding to $GABA_B$ sites (IC_{50} = 33.9 ± 4.9nM, n=3; Table 3.0). Stereoselectivity of this binding was clearly evident since (+)-baclofen was some 350 times weaker than the (-)-enantiomer (IC_{50} = 12,000 ± 500nM, n=3; Table 3.0) whilst the racemic mixture of baclofen was only some 6 times less potent than (-)-baclofen (IC_{50} = 196 ± 50nM, n=3; Table 3.0).

In parallel with the baclofen assay, the ability of the phosphinic acid derivative of GABA, 3-aminopropylphosphinic acid (3-APA) to inhibit [^{3}H]GABA binding from $GABA_B$ receptor sites was also examined. This $GABA_B$ receptor agonist was 10 times more potent than (-)-baclofen at $GABA_B$ receptor sites (IC_{50} = 3.4 ± 0.6nM, n=3; Table 3.0). Moreover, the selectivity of 3-APA for $GABA_B$ receptors was also demonstrated autoradiographically in para-sagittal sections of rat brain (Figure 3.2).

A comparison of the abilities of phaclofen, saclofen, 2-hydroxy-saclofen and CGP 35348 to interact with $GABA_B$ receptor binding sites showed that whilst these compounds were selective for these sites (IC_{50} values for displacing [^{3}H]GABA from $GABA_A$ sites were greater than 100μM), their potencies were much lower than the $GABA_B$ receptor agonists previously described (Figure 3.3; Table 3.0). Nonetheless, structure activity relationships showed that phaclofen was competitive for $GABA_B$ receptor binding (IC_{50} = 241 ± 34.0μM, n=3). By way of contrast, with an IC_{50} value of 18.8 ± 0.8μM (n=4), saclofen was some 13 times more potent than phaclofen. The centrally-active $GABA_B$ receptor antagonist, CGP 35348, possessed a similar displacing potency to saclofen (IC_{50} = 12.5 ± 1.0μM; n=2) whilst 2-hydroxy-saclofen displayed the highest affinity for $GABA_B$ receptor binding sites (IC_{50} = 3.5 ± 0.8μM; n=3).

Characterisation of (-)-[^{125}I]iodopindolol binding to beta-adrenoceptors in rat cerebral cortex

With the aid of horizontally-orientated slices of rat frontoparietal cortex, displacement curves for the inhibition of (-)-[^{125}I]iodopindolol (IPIN)

binding by isoprenaline, the $beta_1$-adrenoceptor antagonist, CGP 20712A, and the $beta_2$-adrenoceptor antagonist, ICI 118,551, were constructed (Figure 3.4). Fitting of the data to either one site or two site models was achieved with the Graph-Pad 'Inplot' computer package and the possible improvement of the fit to a two site model was examined using a partial F-test (DeLean et al, 1978).

Isoprenaline (a non-selective beta-adrenoceptor agonist) inhibited the binding of IPIN with an IC_{50} value of 59.4 $\pm$ 1.1nM (n=3; Table 3.1) with a fit of its displacement profile significantly in favour of a one site model, consistent with a single affinity component. By way of contrast, statistical analysis showed that both CGP 20712A and ICI 118,551 best fitted a biphasic model. The IC_{50} values for CGP 20712A at these two sites were 4.6 $\pm$ 0.3nM and 11110 $\pm$ 730nM (n=3; Table 3.3), reflecting their respective affinities at the $beta_1$- and $beta_2$-adrenoceptor subtypes, of which the former accounted for 82.3% of the total beta-adrenoceptor population. Conversely, in the presence of ICI 118,551, IC_{50} values for the displacement of IPIN binding equal to 6.9 $\pm$ 0.4nM and 282 $\pm$ 14nM (n=3; Table 3.1) were indices of the affinity of this antagonist at $beta_2$- and $beta_1$-adrenoceptors, respectively, for which in the cortex, the former site contributed to only 30.1% of the total radioligand binding.

Autoradiographic representation of the IPIN (150pM) binding in para-sagittal and coronal sections of rat brain is shown in Figure 3.5. Binding was resolved into $beta_1$- and $beta_2$-adrenoceptor subtypes in the presence of 50nM ICI 118,551 and 100nM CGP 20712A, respectively. $Beta_1$-adrenoceptors predominated in the cerebral cortex, hippocampus (CA1 region), caudate putamen together with the ventroposteromedial and the gelatinosus thalamic nuclei (Figure 3.5; E,F). Selective labelling of $beta_2$-adrenoceptors was evident in the molecular layer of the cerebellum, the optic tract and nucleus, the parietal cortex (lamina IV), the centromedial thalamic nucleus,

the olfactory tubercle and the globus pallidus (Figure 3.5; G,H).

Colocalisation of $GABA_B$ and beta-adrenoceptor binding sites in rat frontal cortex

A detailed examination of the comparative distributions of $GABA_B$ and beta-adrenoceptors was performed in the rat frontal cortex. This was established as a basis for subsequent studies on their distribution following chronic antidepressant administration (see Chapter 5).

Binding of [^{3}H]GABA (50nM) to $GABA_B$ sites within the frontal cortex revealed a clear non-uniform distribution over four distinct sub-laminar regions (Figure 3.6). These laminae were designated as I, II-III, V and VI. The greatest concentration of $GABA_B$ sites was found in the laminae II-III 145.0 $\pm$ 6.8 fmol/mg tissue (n=5) with lower amounts in laminae I, V and VI (Table 3.2). The binding of IPIN (150pM) also demonstrated a clear laminarity of beta-adrenoceptor site distribution. In this instance, only three laminar regions could be differentiated since there was no visibly precise demarcation between laminae V and VI in either total or resolved $beta_1$-adrenoceptor populations (Figure 3.6) (designated as laminae I, II-III and V-VI). The preponderance of sites, like $GABA_B$ sites, was evident in laminae II-III (1.62 $\pm$ 0.09 fmol/mg tissue, n=5) with lower amounts in the two surrounding regions. Furthermore, $beta_1$-adrenoceptors in this sub-laminar region accounted for 67% of the total beta-adrenoceptor population (Table 3.2).

Localisation of [^{3}H]BRL 43694 binding sites in rat hindbrain

Autoradiographical visualisation of [^{3}H]BRL 43694 (10nM) binding to sequential sections of rat hindbrain is demonstrated in Figures 3.7 and 3.8. Although non-specific binding was relatively high, densitometric analysis revealed that the greatest concentration of specific binding (usually in excess of 75%) was located in the nucleus tractus solitarius (NTS) and

amounted to 631 ± 9.0 fmol/mg tissue. Histological examination of the coronal orientation of the brain (Figure 3.7) with the aid of cresyl fast violet staining, showed that this region originated discretely in more caudal sections as the medially-situated commissural NTS (cNTS). Moving in a caudal to rostral direction, the solitary tracts and their associated nuclei became more pronounced, projecting mediolaterally from the cNTS. The area postrema (AP) appeared just rostral to the obex, positioned dorsally, relative to the cNTS. Where the AP terminated at the caudal end of the fourth ventricle, the NTS was seen to bifurcate and project bilaterally, immediately adjacent to the fourth ventricle.

This characteristic neuroanatomical identification of the NTS was also apparent with respect to sections cut from the horizontal orientation of the brain (Figure 3.8). Again, stained sections revealed that this region originated in dorsal sections immediately adjacent to the fourth ventricle. Moving in a dorsal to ventral direction, these discrete 'wing-like' projections surrounded the AP, becoming more pronounced to converge at the cNTS, subjacent to the AP. Specific binding was also evident in this latter region but represented only 20% of that seen in the NTS (130 ± 32.3 fmol/mg tissue; n=5). An even lower concentration of 5-HT_3 recognition sites was found in the entorhinal cortex (107 ± 13.8 fmol/mg tissue).

Localisation of [^{3}H]zacopride, [^{3}H]GR65630 and [^{3}H]quipazine binding sites in rat hindbrain

From the autoradiograms of 5-HT_3 receptor radioligand binding to sequential coronally-cut sections of rat hindbrain shown in Figure 3.9, it was immediately apparent that in common with [^{3}H]BRL 43694 receptor autoradiography, [^{3}H]zacopride, [^{3}H]GR65630 and [^{3}H]quipazine all identified high densities of 5-HT_3 recognition sites throughout the caudal to rostral extent of the NTS. Interestingly, although there was a gradation of the numbers of these sites in the NTS identified by the four radioligands

at a concentration of 10nM, there was however, no apparent discrimination in the AP where similar numbers of binding sites were observed. At a lower concentration (1nM), differential binding densities in the AP were evident (Table 3.3).

With the exception of [^{3}H]zacopride, at a concentration of 1nM which enabled quantifiable 5-HT$_3$ receptor site binding in the dorsal motor nucleus of the vagus nerve (DMNX) and the nucleus of the spinal tract of the trigeminal nerve (nSpV) (Figure 3.10), the labelling of such regions by either [^{3}H]GR65630 or [^{3}H]quipazine at an equivalent concentration was not evident. The labelling of these nuclei by [^{3}H]BRL 43694 (1nM) in 5mM HEPES (Figure 3.9) and by [^{3}H]GR65630 (0.2nM) in 50mM HEPES (Figure 3.11) although visibly detectable, was not delineated clearly enough to allow accurate quantitation. In contrast to [^{3}H]BRL 43694, [^{3}H]zacopride or [^{3}H]GR65630, [^{3}H]quipazine failed to identify 5-HT$_3$ sites within these two nuclei; the apparent high lipophilicity of this radioligand may have masked their possible identification.

When incubated in either 5mM or 50mM HEPES buffer, at respective concentrations of 10nM and 0.2nM, the autoradiographical distribution of specific [^{3}H]GR65630 binding was qualitatively similar under both conditions (Figure 3.11). Binding again predominated throughout the NTS although in the presence of the higher buffer concentration, non-specific binding was markedly reduced.

Saturation analysis of [^{3}H]BRL 43694 binding in the NTS

The kinetic parameters K_D and B_{max} for [^{3}H]BRL 43694 binding to the NTS of rat brain were derived by saturation and Scatchard analyses. Binding in the presence of 50mM and 5mM HEPES buffer was compared using a radioligand concentration range of 0.375 - 3nM (5 concentrations). In both instances, specific binding in this hindbrain region was saturable (Figure 3.12A) and for which Scatchard analysis (Figure 3.12B) revealed no

significant differences in B_{max} values (502.6 ± 69.5 and 530.0 ± 50.1 fmol/mg tissue, n=3; 50mM and 5mM buffer, respectively). However, reducing the concentration of HEPES buffer from 50mM to 5mM significantly increased the affinity of the ligand by approximately 2.5-fold, denoted by respective K_D values of 0.92 ± 0.1nM and 0.38 ± 0.04nM (n=3).

Drug inhibition profiles for the displacement of [^{3}H]BRL 43694 binding in the NTS

Binding sites labelled by [^{3}H]BRL 43694 in the NTS were characterised by autoradiographical competition experiments using a variety of 5-HT_3 receptor-selective or non-selective drugs. At a concentration of 1nM, the density [^{3}H]BRL 43694 sites within the NTS was high (423.8 ± 16.9 fmol/mg tissue; n=9) with specific binding generally in excess of 80% of the total.

The 5-HT_3 receptor antagonists, zacopride, quipazine, ICS 205-930, BRL 43694, GR38032F and MDL 72222, all potently displaced [^{3}H]BRL 43694 binding with IC_{50} values in the low nanomolar range (Figure 3.13; Table 3.4; n=3). 5-HT and the 5-HT_3 receptor agonist, 2-methyl-5-HT possessed moderate displacing ability with respective IC_{50} values of 134 ± 1.0nM and 325 ± 13.2nM. Conversely, the non-selective 5-HT_3 receptor antagonist, metoclopramide, was much weaker in its ability to displace [^{3}H]BRL 43694 binding (IC_{50} = 747 ± 78.9nM). With IC_{50} values greater than 10,000nM, the 5-HT_{1A} receptor agonist, 8-OH-DPAT and the 5-HT_2 receptor antagonist, methysergide displayed little affinity for the 5-HT_3 site, indicating that within the NTS, [^{3}H]BRL 43694 identified a homologous population of recognition sites.

Colocalisation of $GABA_B$ and 5-HT_3 receptor sites in rat hindbrain

A direct comparison of 5-HT_3 and $GABA_B$ recognition sites in the dorsal vagal complex is illustrated by the autoradiograms shown in Figure 3.14. As has been previously shown, [^{3}H]BRL 43694 (1nM) binding sites

predominated in the NTS whilst immediately subjacent to this nucleus, lower densities were observed in the DMNX and the nSpV. Although no specific 5-HT_3 receptor sites were detected in the cerebellum, concentrated labelling of $GABA_B$ binding sites within the molecular layer of this structure was clearly evident. Additionally, $GABA_B$ sites observed in the dorsal vagal complex were attributed to both the NTS and the DMNX. Since it was impossible to delineate these two nuclei, quantitation of their binding densities was taken collectively (114.7 $\pm$ 18.7 fmol/mg tissue; n=2).

Figure 3.0 opposite: page 89

[^{3}H]GABA (50nM) autoradiography in coronal sections of rat brain. The selective labelling of $GABA_A$ sites was achieved in the presence of (-)-baclofen (100μM), whilst $GABA_B$ sites were visualised in the presence of isoguvacine (40μM) to selectively block $GABA_A$ sites. White areas depict the highest concentrations of radioligand binding.

Regions in which the densities of $GABA_A$ sites were clearly higher than those for $GABA_B$ were (A) the frontal cortex (FrCtx) and external plexiform layer of the olfactory bulb (EPl); (C) the medial forebrain bundle (mfb); (E) ventroposterolateral thalamic nucleus (VPL); (I) lateral mammillary body (LM) and (K) the granular layer of the cerebellum (Gran).

Conversely, $GABA_B$ sites predominated in (F) the ventroposteromedial (VPM) and lateral posterior (LP) thalamic nuclei and also the medial habenula (MHb); (H) the superficial grey layer of the superior colliculus (SuG), fascicular retroflexus of Meynert (fr) and the lateral amygdaloid nucleus (La); (J) the interpeduncular nucleus (IPN) and (L) the molecular layer of the cerebellum (Mol).

IGr (internal granular layer of the olfactory bulb), CPu (caudate putamen), LG (lateral geniculate thalamic nucleus), MGD (dorsomedial geniculate thalamic nucleus), MGV (ventromedial geniculate thalamic nucleus), MG (medial geniculate thalamic nucleus).

Autoradiograms were taken from tissue sections positioned approximately 4.2mm anterior to bregma (A,B) in addition to the following coordinates posterior to bregma: 0.26mm (C,D); 3.80mm (E,F); 5.30mm (G,H); 5.80mm (I,J) and 10.52mm (K,L).

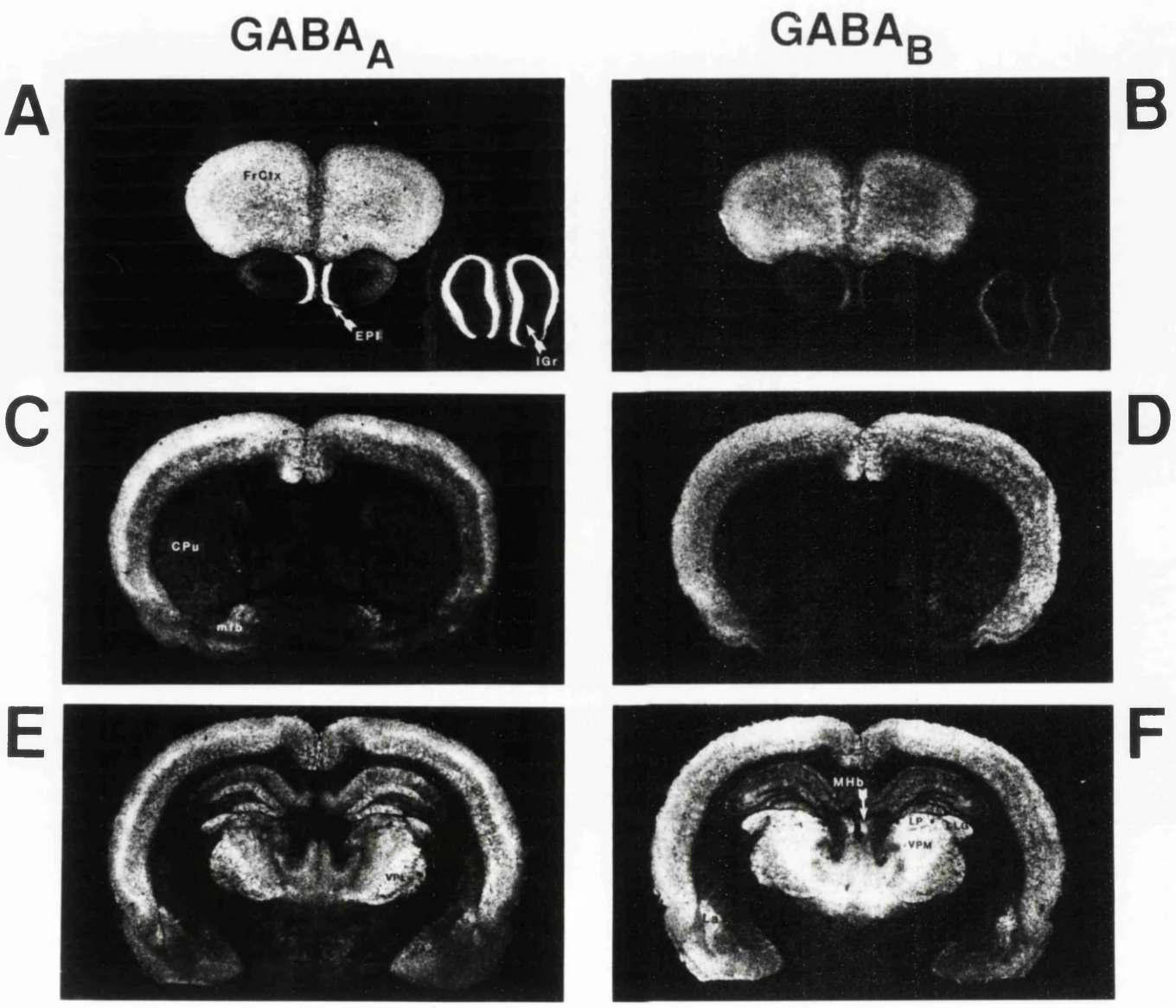

GABA$_A$
GABA$_B$
A
B
C
D
E
F
FrCtx
EPl
IGr
CPu
mfb
MHb
LP
VPM

Figure 3.0 contd. opposite: page 91

[^{3}H]GABA (50nM) autoradiography in coronal sections of rat brain.

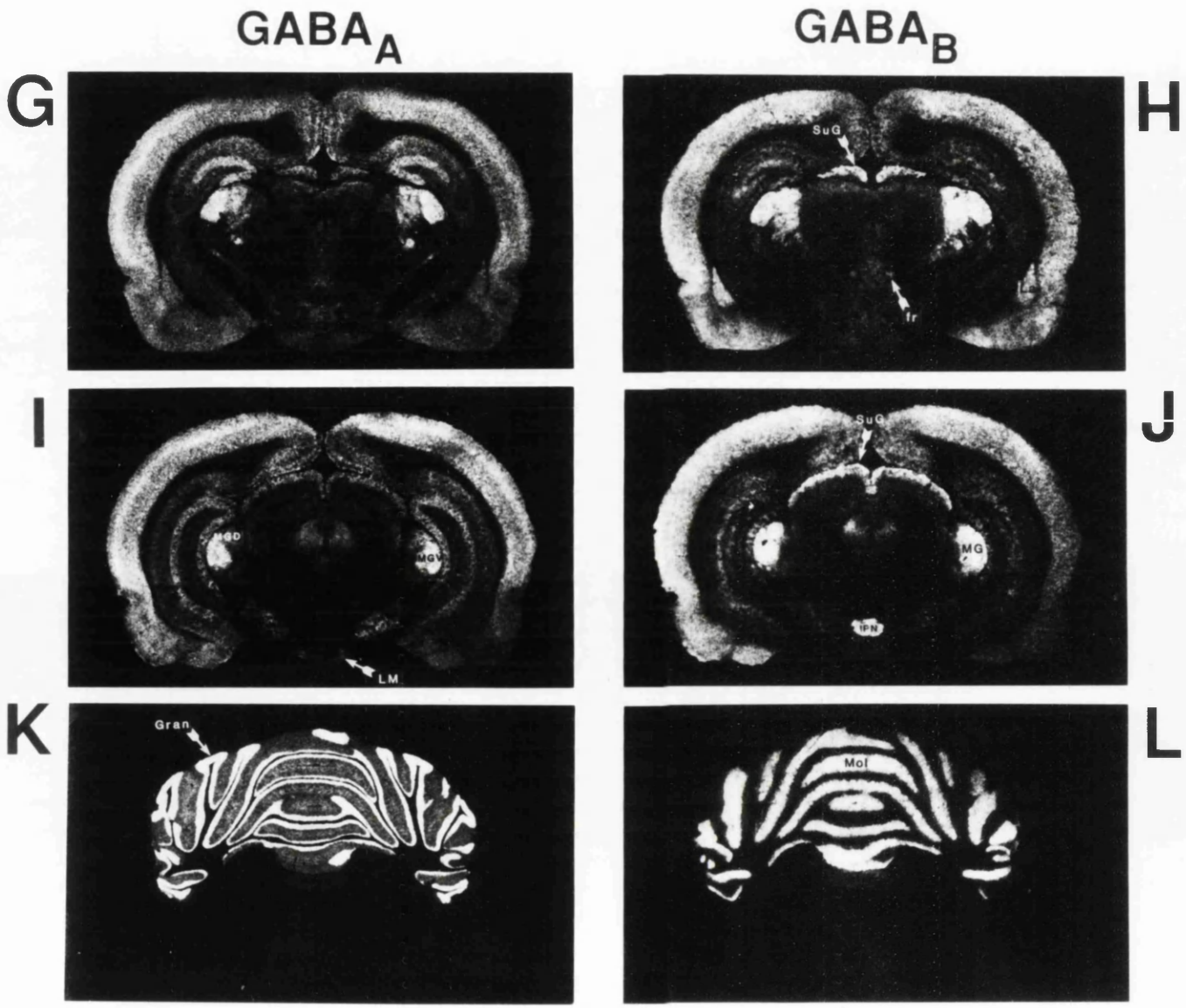

GABA$_A$
GABA$_B$
G
H
I
J
K
L
SuG
fr
MGD
MGV
LM
SuG
MG
IPN
Gran
Mol

Figure 3.1

Pharmacological characterisation of $GABA_B$ receptor agonists in whole brain crude synaptic membranes. (A) The abilities of baclofen (racemic mixture and enantiomers) and 3-aminopropylphosphinic acid (3-APA) to displace [^{3}H]GABA (5nM) binding from $GABA_B$ receptor binding sites (in the presence of 40µM isoguvacine) were assessed. 3-APA (●) displayed the greatest displacing potency (IC_{50} = 3.39nM) followed by (-)-baclofen (■), (±)-baclofen (▲) and (+)-baclofen (♦), with respective IC_{50} values of 33.9nM, 195.7nM and 12,000nM. (B) With IC_{50} values of 1055µM and 73µM, respectively, 3-APA (●) was approximately 14 times weaker than isoguvacine (▼) at displacing the binding of [^{3}H]GABA from $GABA_A$ sites (in the presence of 100µM (-)-baclofen). Data shown represent the mean ± s.e.m. of between two and six experiments for which triplicate determinations were made (see also Table 3.0).

ordinate: [^{3}H]GABA bound (% specific)
abscissa: concentration (nM) of displacing ligand

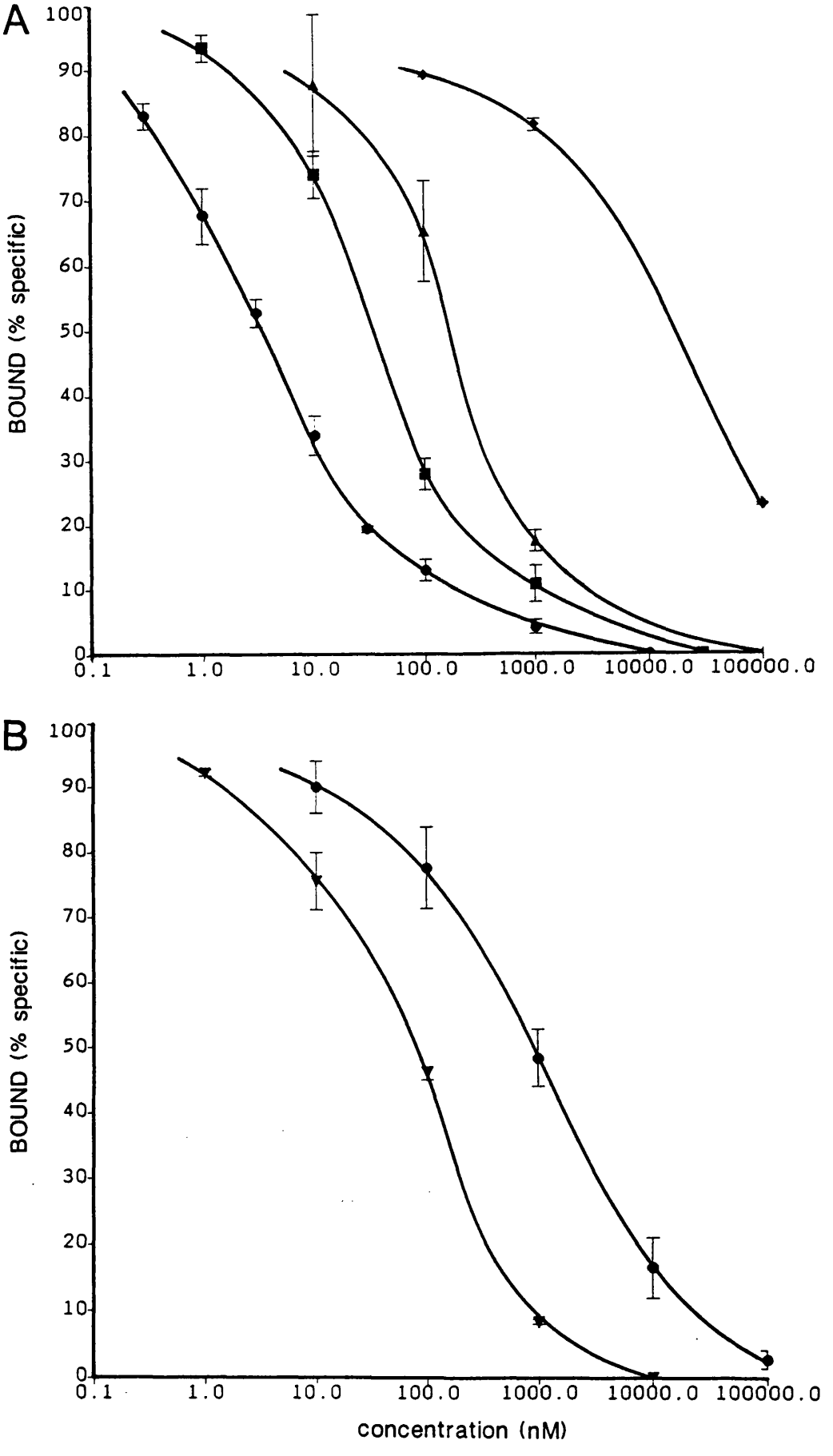

A
B
BOUND (% specific)
100
90
80
70
60
50
40
30
20
10
0
0.1
1.0
10.0
100.0
1000.0
10000.0
100000.0
concentration (nM)

Figure 3.2 opposite: page 95

Autoradiographical characterisation of 3-aminopropylphosphinic acid (3-APA) as a $GABA_B$ receptor agonist. [^{3}H]GABA (50nM) was used to selectively label either $GABA_A$ binding sites (in the presence of 100μM (-)-baclofen) or $GABA_B$ binding sites (in the presence of 40μM isoguvacine) in para-sagittal sections of rat brain. The ability of 3-APA to displace this radioligand was assessed by comparing the IC_{50} values for these respective sites. With an IC_{50} value of 2.38 $\pm$ 0.40nM (n=3), 3-APA demonstrated a 1000-fold greater selectivity for $GABA_B$ as opposed to $GABA_A$ binding sites (IC_{50} = 2142 $\pm$ 363μM; n=3) (see also Table 3.0). The experiment was repeated three times with triplicate determinations being performed in each case.

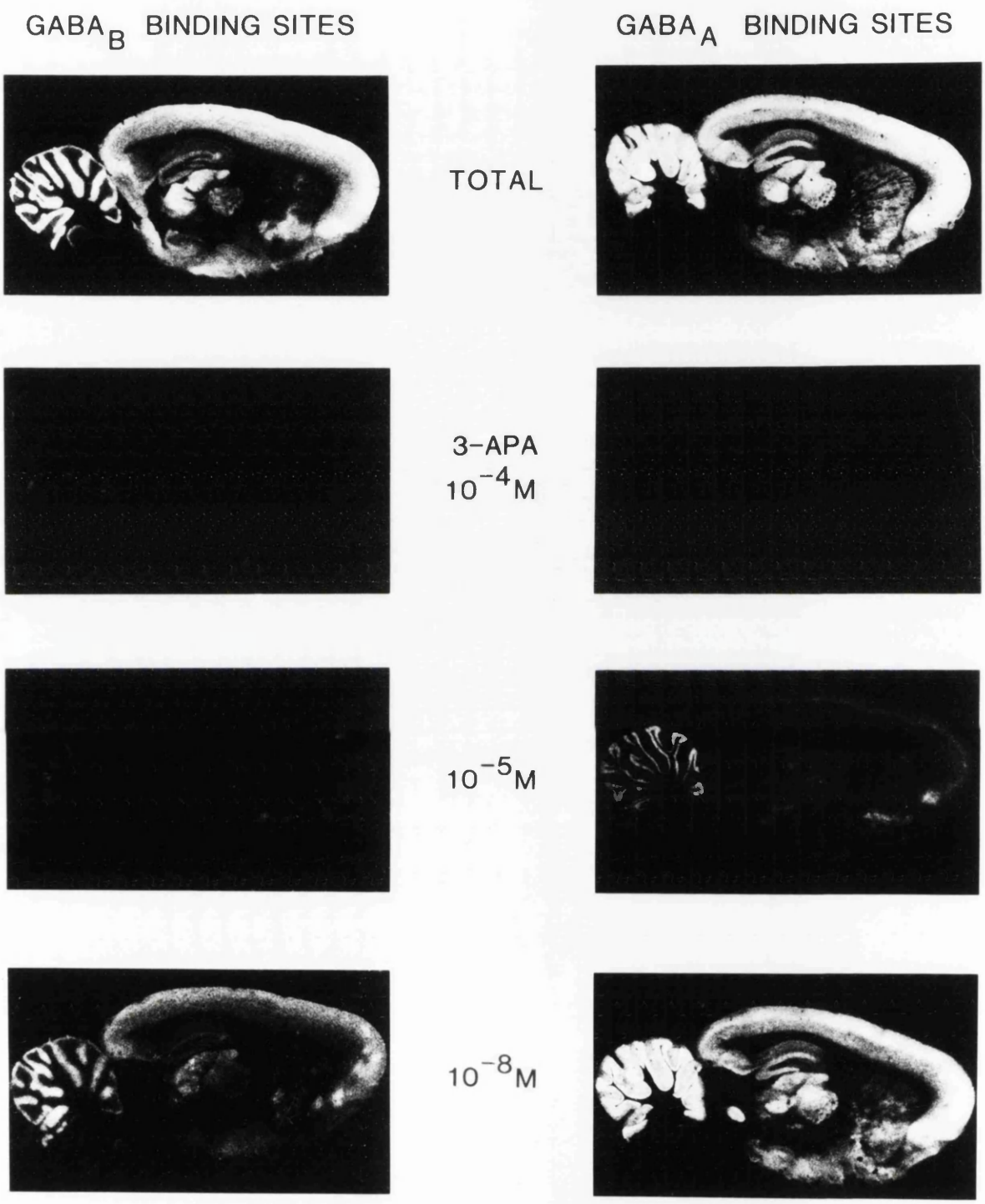

GABA$_B$ BINDING SITES
GABA$_A$ BINDING SITES
TOTAL
3-APA
10^{-4}M
10^{-5}M
10^{-8}M

Figure 3.3

Pharmacological characterisation of $GABA_B$ receptor antagonists in whole brain crude synaptic membranes. The abilities of 2-hydroxy-saclofen (♦), CGP 35348 (▲), saclofen (■) and phaclofen (●) to displace the binding of [^{3}H]GABA (5nM) from $GABA_B$ receptor binding sites (in the presence of 40μM isoguvacine) were assessed. With respective IC_{50} values of 3.5μM, 12.5μM, 18.8μM and 240.7μM the following structure activity potency relationship was obtained: 2-hydroxy-saclofen > CGP 35348 > saclofen > phaclofen. IC_{50} values for the displacement of [^{3}H]GABA from $GABA_A$ receptor binding sites were in excess of 100μM (1mM in the case of phaclofen). Data shown represent the mean of between two and four experiments for which triplicate determinations were made in each case (see also Table 3.0).

ordinate: [^{3}H]GABA bound (% specific)
abscissa: concentration (μM) of displacing ligand

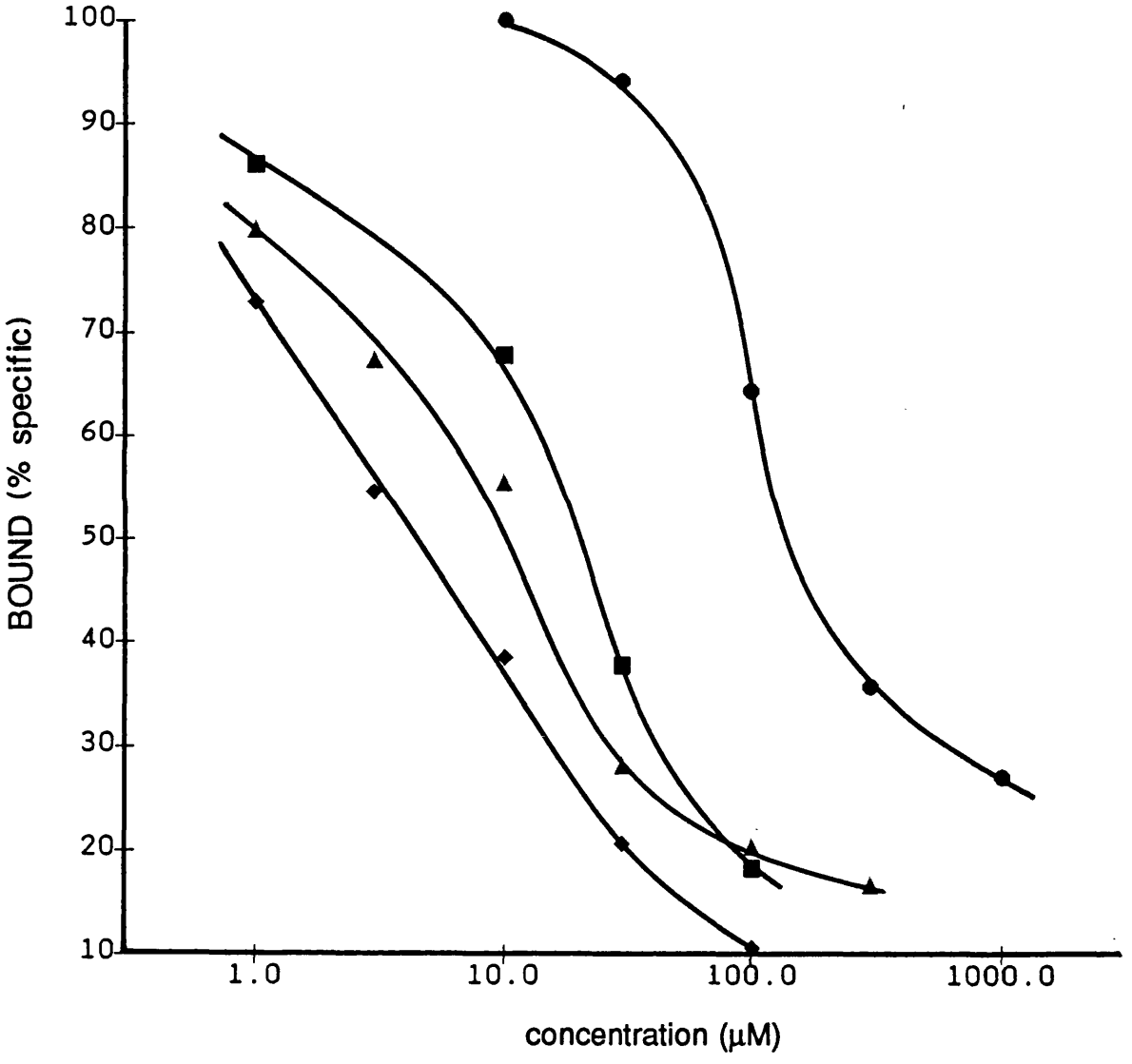

100
90
80
70
60
50
40
30
20
10
BOUND (% specific)
1.0
10.0
100.0
1000.0
concentration (μM)

Table 3.0 **Characterisation of $GABA_B$ receptor agonists and antagonists in rat brain tissue**

COMPOUND	n	IC_{50} (nM)	
		$GABA_B$	$GABA_A$
AGONISTS			
Isoguvacine	3	n.t.	73.0 ± 7.6
3-APA	5	3.39 ± 0.6	1055 ± 282
3-APA	*3*	*2.38 ± 0.4*	*2142 ± 363*
(-)-baclofen	*3*	*22.9 ± 3.6*	*n.t.*
(-)-baclofen	6	33.9 ± 4.9	n.t.
(±)-baclofen	3	195.7 ± 49.9	n.t.
(+)-baclofen	2	12,000 ± 500	n.t.
ANTAGONISTS		IC_{50} (μM)	
Phaclofen	3	240.7 ± 34.0	>1000
Saclofen	4	18.8 ± 0.8	>100
2-OH-Saclofen	3	3.5 ± 0.8	>100
CGP 35348	2	12.5 ± 1.0	>100

IC_{50} values represent the mean ± s.e.m. of triplicate determinations in each experiment. The concentration of [^{3}H]GABA used for synaptic membrane receptor binding was 5nM. In sections of rat brain (values in italics) the concentration of radioligandwas increased to 50nM. (n.t. = not tested).

Figure 3.4

Characterisation of (-)-[^{125}I]iodopindolol binding to beta-adrenoceptors in rat cerebral cortex. The abilities of isoprenaline (Δ), the β_1-adrenoceptor antagonist, CGP 20712A (●) and the β_2-adrenoceptor antagonist, ICI 118,551 (■) to displace (-)-[^{125}I]iodopindolol (150pM) from central cortical beta-adrenoceptors was assessed by direct counting of the bound radioligand using an 'LKB Clinigamma' counter. The amount of displacement of radiolabel at a given concentration was expressed as a percentage of the total specific binding (defined in the presence of 200μM (-)-isoprenaline). Fitting of the data to either one site or two site models was achieved with the Graph-Pad 'Inplot' computer package and the possible improvement of the fit to a two site model was examined using a partial 'F' test. Data points represent the mean of three separate experiments for which standard errors were less than 5% of the mean value (omitted for clarity). The IC_{50} values for isoprenaline, CGP 20712A and ICI 118,551 are summarised in Table 3.1.

ordinate: (-)-[^{125}I]iodopindolol bound (% specific)
abscissa: concentration (nM) of displacing ligand

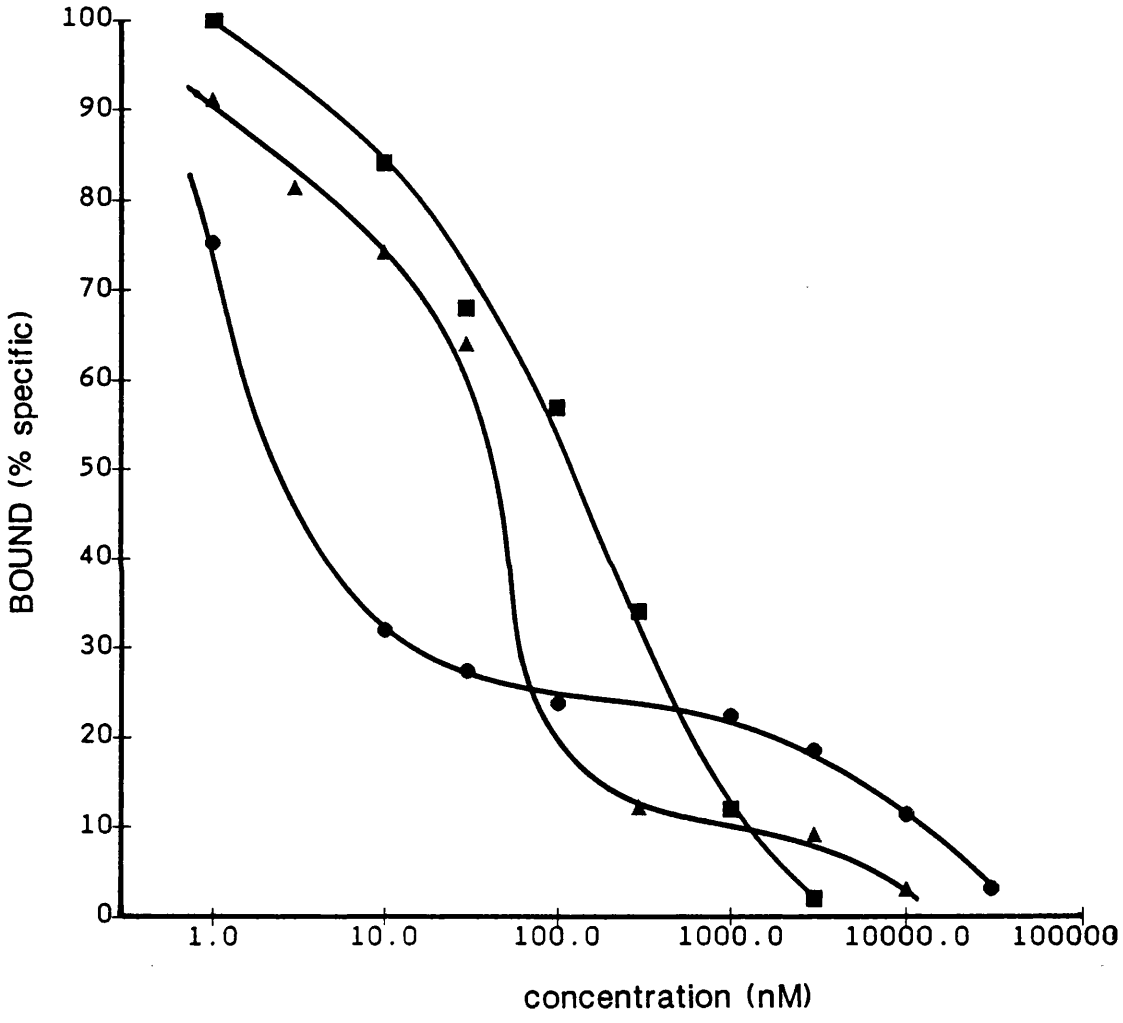

100
90
80
70
60
50
40
30
20
10
0
BOUND (% specific)
1.0
10.0
100.0
1000.0
10000.0
100000
concentration (nM)

Table 3.1 **Characterisation of central β_1- and β_2-adrenoceptors via the interaction of isoprenaline, CGP 20712A and ICI 118,551 with the binding of [^{125}I]iodopindolol to sections of rat cerebral cortex**

Compound	$IC_{50\ (1)}$	$IC_{50\ (2)}$	$B_{max\ (1)}$	$B_{max\ (2)}$
Isoprenaline	59.4 ± 1.10	---	100[a]	---
CGP 20712A	4.62 ± 0.32	11110 ± 730	82.3[b]	17.7[b]
ICI 118,551	6.89 ± 0.44	282 ± 13.7	30.1[c]	69.9[c]

IC_{50} concentrations are expressed in nM and represent the mean ± s.e.m. of three separate experiments. B_{max} values are calculated as the percentage of the total beta-adrenoceptor population, the data having been fitted to either a one site or two site model using the Graph-Pad 'In-Plot' computer package. The improvement of the fit to a two site model was examined via a partial 'F' test for which statistical values are denoted as:-

a $P<0.05$ 1 site > 2 sites
b $P<0.01$ 2 sites > 1 site
c $P<0.05$ 2 sites > 1 site

Figure 3.5 opposite: page 103

Autoradiograms of β-adrenoceptor binding in rat brain. Total (β_1 and β_2) (-)-[^{125}I]iodopindolol binding (150pM) is shown in images A and B whereas non-specific binding was defined by (-)-isoprenaline (200μM) (C,D). β_1-adrenoceptor binding (E,F) was defined in the presence of the β_2-adrenoceptor antagonist, ICI 118,551 (50nM) whilst β_2-adrenoceptor binding was resolved in the presence of the β_1-adrenoceptor antagonist, CGP 20712A (100μM). Note the absence of binding in the combined presence of ICI 118,551 and CGP 20712A (I,J). The para-sagittal and coronal sections are approximately represented by plates 81 and 30, respectively (Paxinos & Watson, 1986).

β_1-adrenoceptors predominated in the cerebral cortex (delineated into three regions denoted as laminae I, II-III and V-VI), hippocampus (CA1 region), caudate putamen (CPu), ventroposteromedial thalamic nucleus (VPM) and the gelatinosus thalamic nucleus (G). β_2-adrenceptors predominated in the molecular layer of the cerebellum (mol), the nucleus of the optic tract (OT), the optic tract (OPT), lamina IV of the parietal cortex (IV), olfactory tubercle (Tu), centromedial thalamic nucleus (CM) and the globus pallidus (GP).

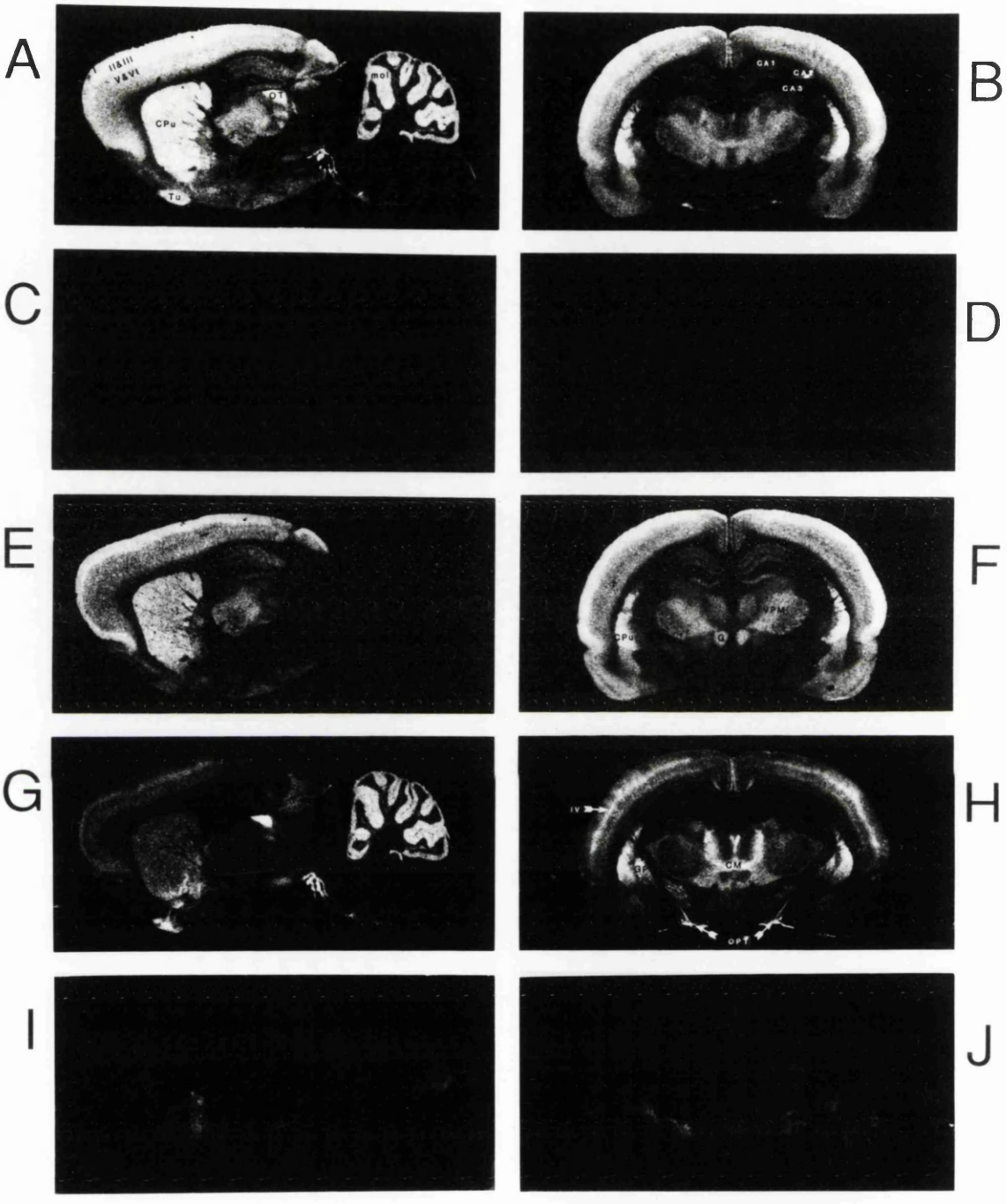
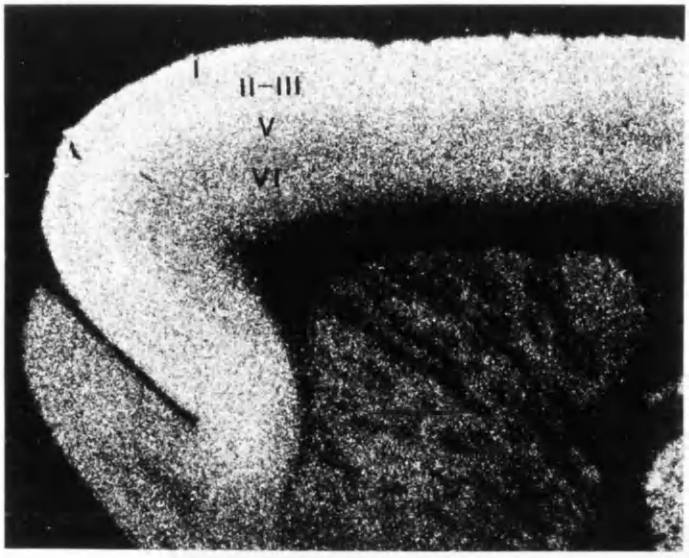

A
B
C
D
E
F
G
H
I
J
CPu
Tu
CA1
CA3
VPM
OM
OPT

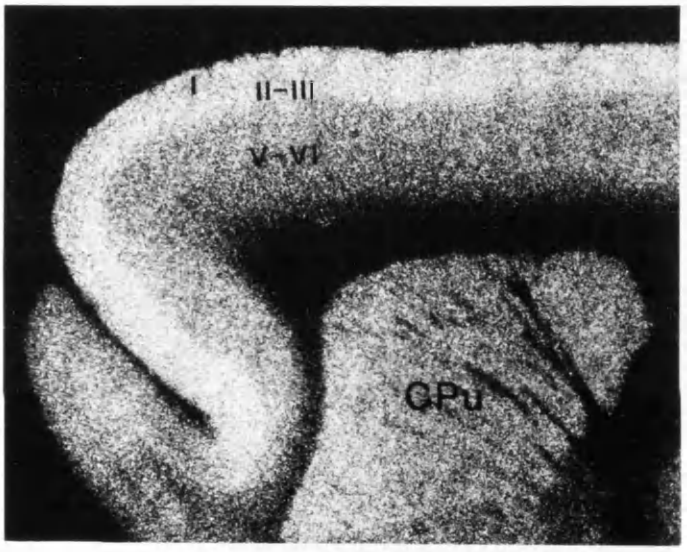
Figure 3.6 opposite: page 105

Autoradiograms showing $GABA_B$ and β-adrenoceptor binding sites in the frontal cortex of adjacent sections of rat brain. The selective binding of [^{3}H]GABA (50nM) to $GABA_B$ receptor binding sites was achieved in the presence of isoguvacine (40μM) and was clearly delineated in the frontal cortex into four distinct regions. These were designated as laminae I, II-III, V and VI.

The total β-adrenoceptor population was labelled by (-)-[^{125}I]iodopindolol (150pM) whilst the β_1-adrenoceptor subtype was resolved in the presence of the β_2-adrenoceptor antagonist, ICI 118,551 (50nM). In this instance, three laminar regions were detected and were denoted as laminae I, II-III and V-VI. A comparison of the binding densities of these binding sites is provided in Table 3.2.

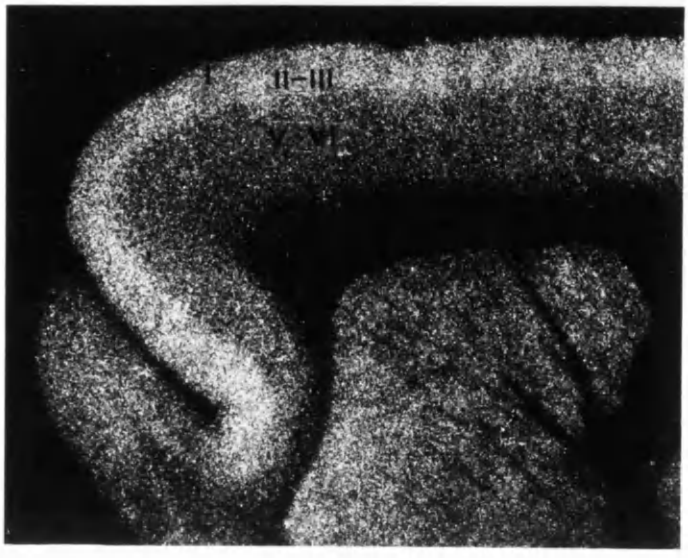


$GABA_B$



TOTAL

$\beta_1 + \beta_2$



β_1

Table 3.2 **Binding of (-)-[^{125}I]iodopindolol (150pM) to beta-adrenoceptors and [^{3}H]GABA (50nM) to $GABA_B$ receptor sites within sub-laminar regions of the rat frontal cortex**

Binding Site	(-)-[^{125}I]iodopindolol bound (fmol/mg tissue)		
	Lamina I	Laminae II-III	Laminae V-VI
Total (β_1 + β_2)	1.37 ± 0.09	1.62 ± 0.09	1.37 ± 0.06
$Beta_1$	0.99 ± 0.04	1.09 ± 0.04	0.79 ± 0.03
% $Beta_1$	72.6	67.4	57.4

Binding Site	[^{3}H]GABA bound (fmol/mg tissue)			
	Lamina I	Lam. II-III	Lamina V	Lamina VI
$GABA_B$	118.7 ± 6.7	145.0 ± 6.8	107.1 ± 6.5	68.6 ± 3.5

Values represent the mean ± s.e.m. of triplicate determinations (n=5) are were derived by densitometric analysis with the aid of a 'Quantimet 970' image analysis system.

Figure 3.7 opposite: page 108

Autoradiograms showing the binding of [^{3}H]BRL 43694 (10nM) to sequential coronal sections of the rat hindbrain performed in 5mM HEPES buffer (pH 7.4). The centre column represents total binding to sections cut in a caudal (bottom) to rostral (top) direction. The darkest region region, rich in 5-HT$_3$ receptor binding sites is the nucleus tractus solitarius (NTS). To the right of centre, cresyl fast violet staining to adjacent sections is shown. Note the position of the area postrema (AP) in relation to the nucleus tractus solitarius. Images to the left of centre represent non-specific binding defined in the presence of 100μM GR38032F. Autoradiograms were taken from tissue sections positioned approximately 13.3mm to 14.3mm posterior to bregma.

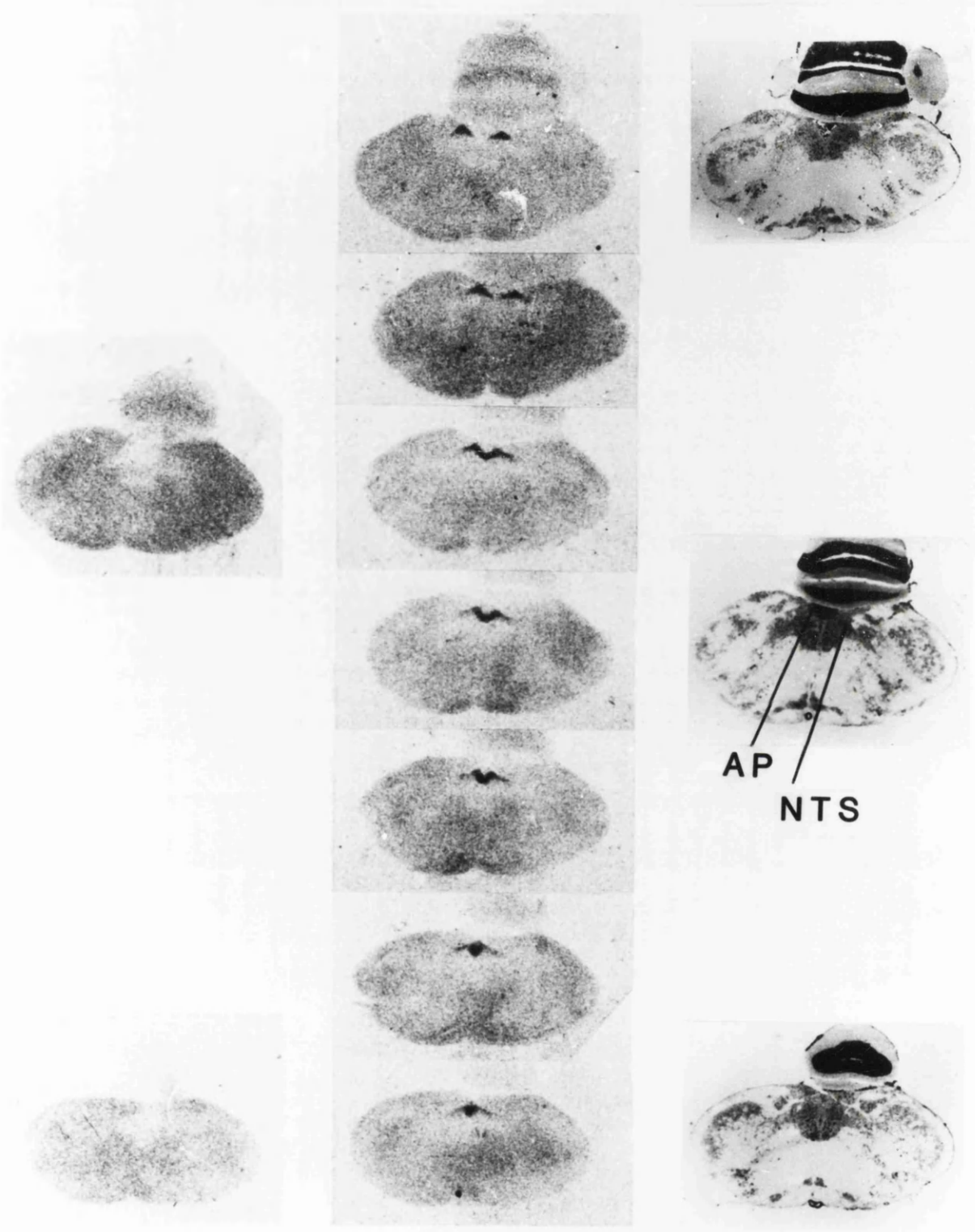

AP
NTS

Figure 3.8 opposite: page 110

Autoradiograms showing the binding of [^{3}H]BRL 43694 (10nM) to sequential horizontal sections of the rat hindbrain performed in 5mM HEPES buffer (pH 7.4). The centre profile represents total binding to sections cut in a dorsal (bottom) to ventral (top) direction. The darkest region is the nucleus tractus solitarius (NTS), which in more dorsal sections surrounds the area postrema (AP), lying at the caudal end of the fourth ventricle. Cresyl fast violet staining to adjacent sections is shown to the right of centre whilst images to the left represent non-specific binding, defined in the presence of GR38032F (100μM). Autoradiograms were taken from tissue sections positioned approximately 1.90mm above the interaural line.

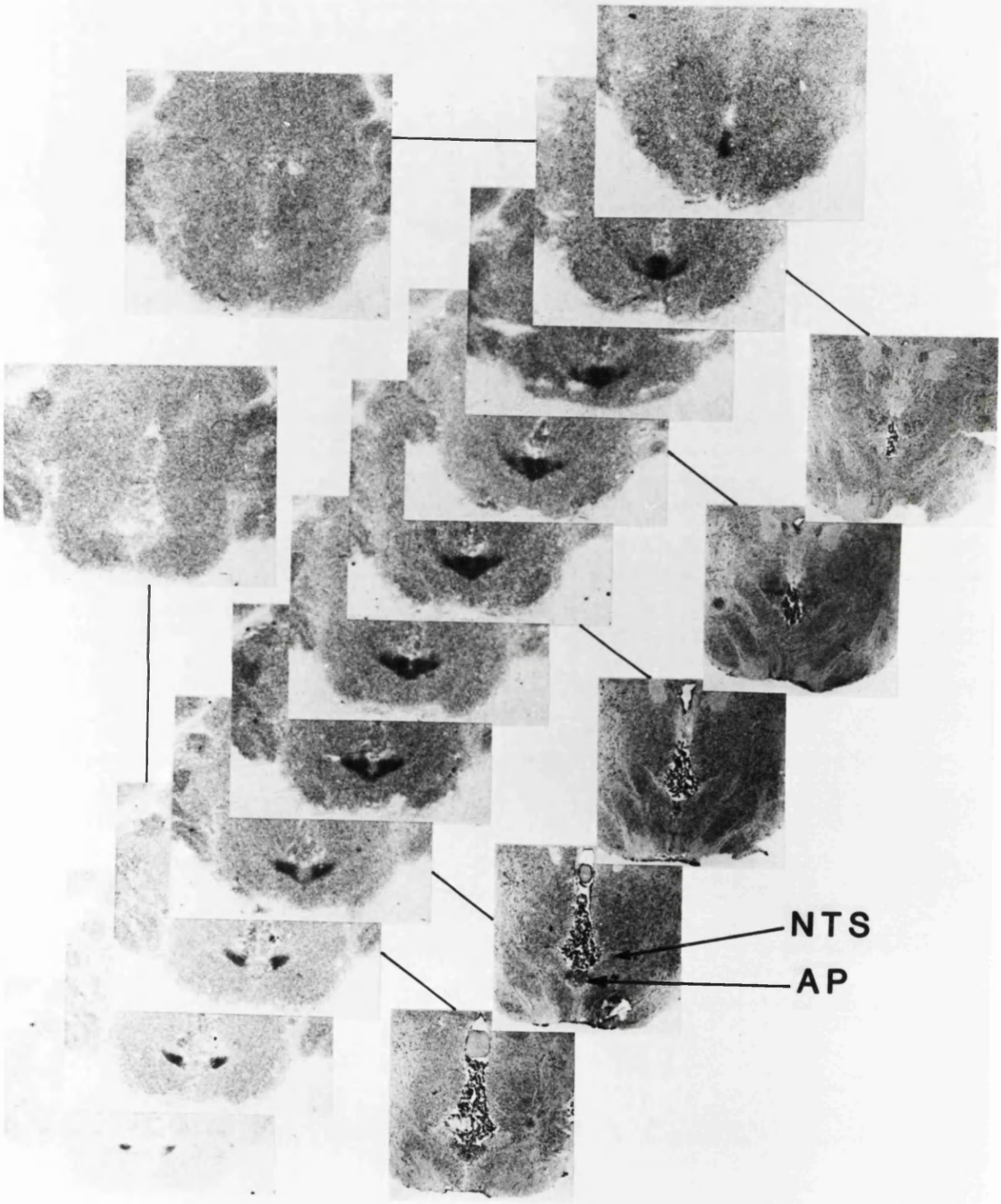
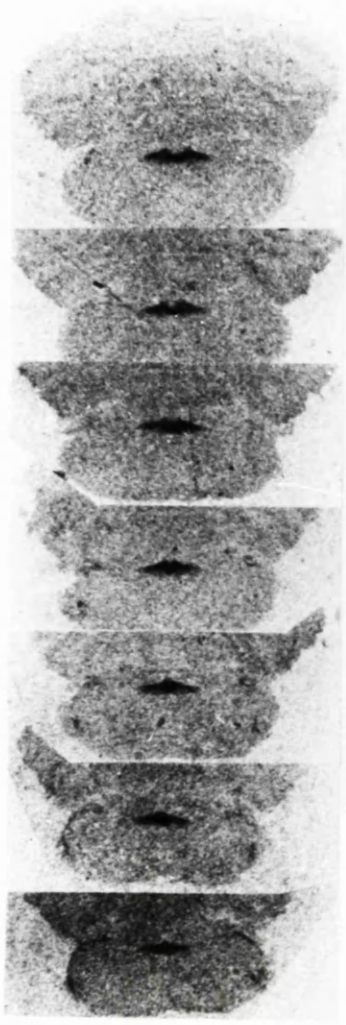

NTS
AP

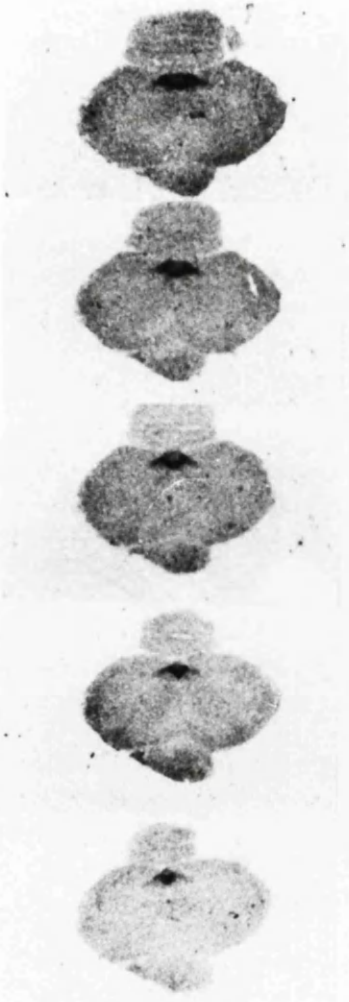
Figure 3.9 opposite: page 112

An autoradiographical comparison of the binding of the four 5-HT_3 receptor radioligands, [^{3}H]BRL 43694, [^{3}H]GR65630, [^{3}H]zacopride and [^{3}H]quipazine to sequential coronal sections of rat hindbrain. In each case, the concentration of radioligand employed was 10nM and each assay was performed in 5mM HEPES buffer (pH 7.4). Despite relatively high levels of background binding, it is immediately apparent that each radioligand bound to high densities of recognition sites within the nucleus tractus solitarius. The apparent lower lipophilicity of [^{3}H]zacopride also enabled the identification of 5-HT_3 recognition sites within the area postrema, the dorsal motor nucleus of the vagus nerve and the nucleus of the spinal tract of the trigeminal nerve. For a more detailed representation of this 5-HT_3 receptor site localisation, see Figure 3.10. Densitometric analysis of this 5-HT_3 radioligand binding is provided in Table 3.3.

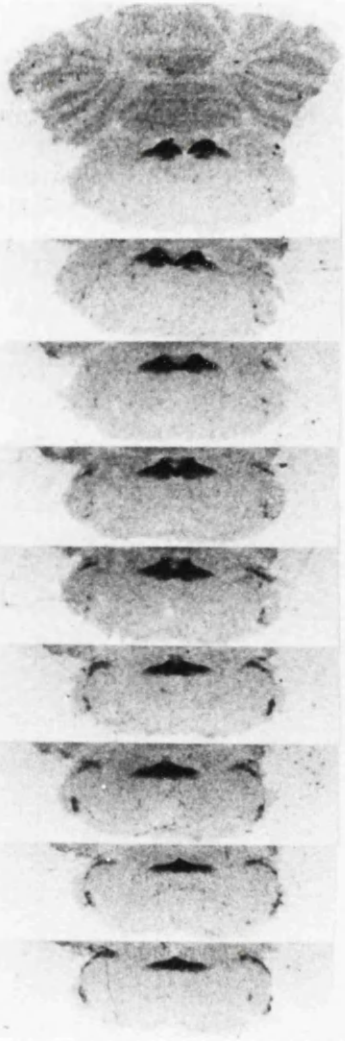
[^{3}H]-BRL 43694



[^{3}H]-GR 65630



[^{3}H]-ZACOPRIDE



[^{3}H]-QUIPAZINE

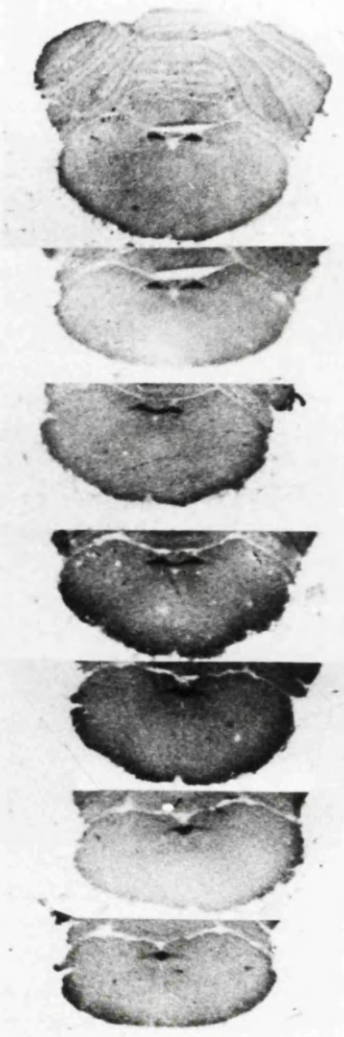
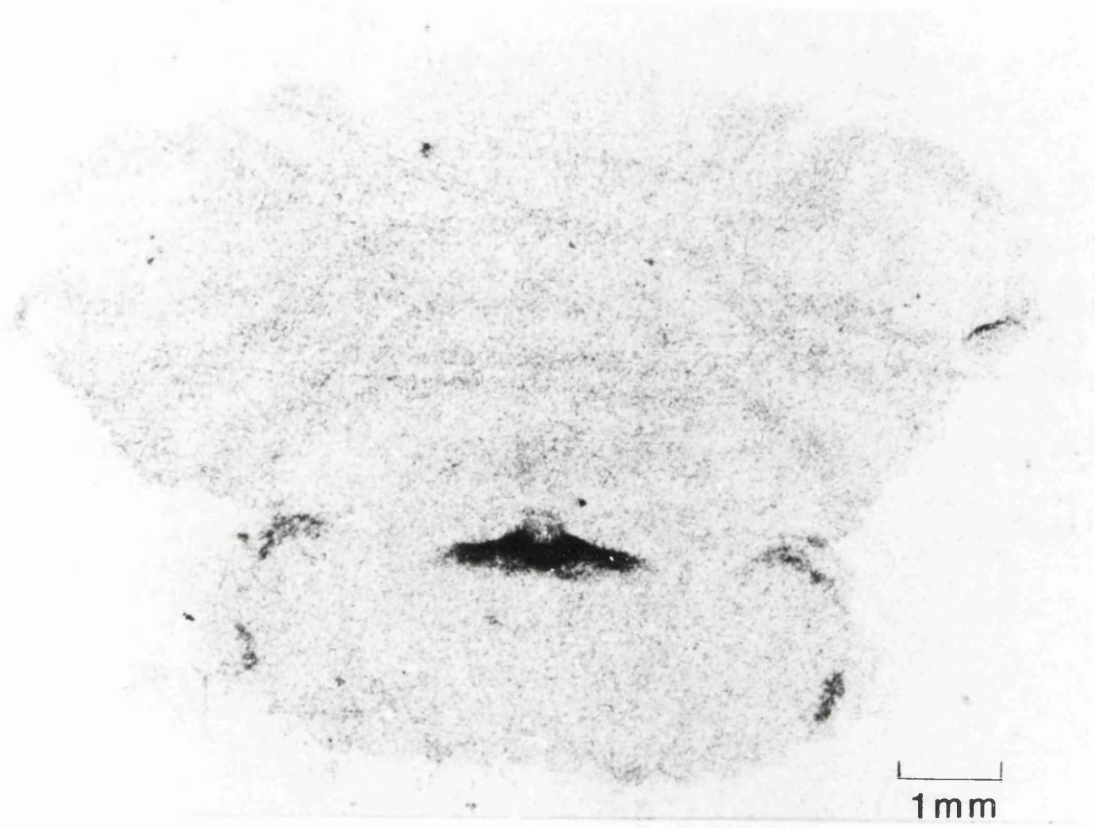


Figure 3.10 opposite: page 114

Autoradiogram of [^{3}H]zacopride (1nM) binding to a coronal section of the rat hindbrain together with a schematic morphological representation. The highest density of specific 5-HT_3 receptor binding was evident in the commissural nucleus tractus solitarius (cNTS) with lower levels in the area postrema (AP), the dorsal motor nucleus of the vagus nerve (DMX) and the nucleus of the spinal tract of the trigeminal nerve (nSpV). CB = cerebellum.


1mm

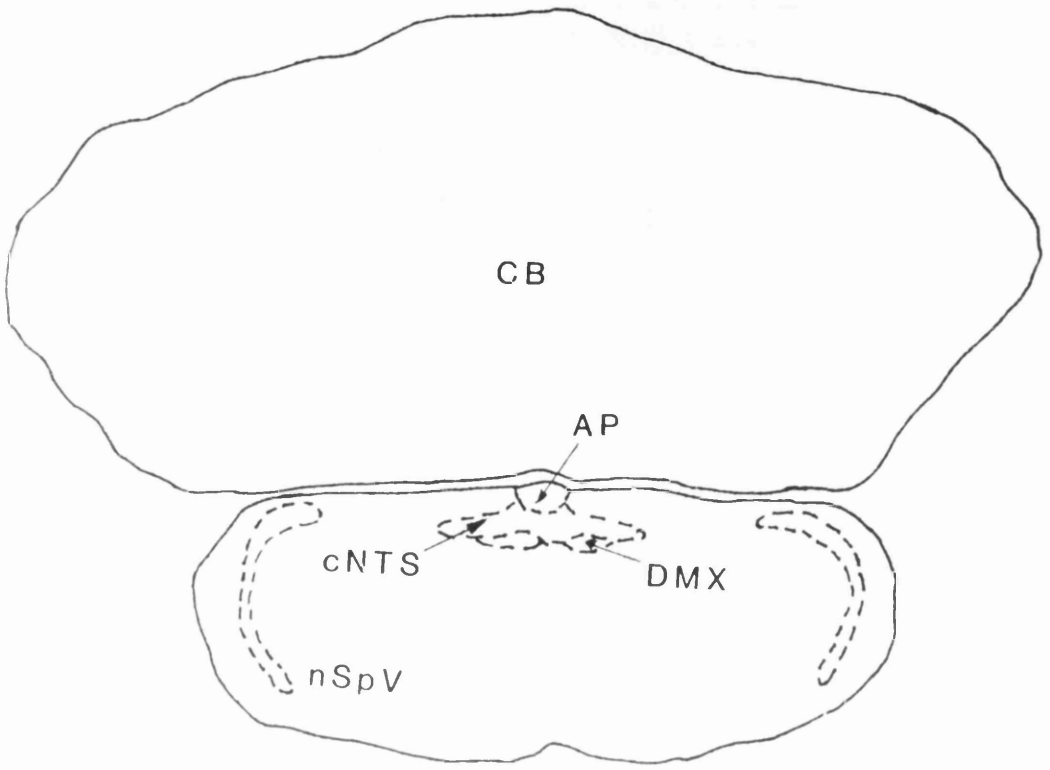

CB
AP
cNTS
DMX
nSpV

Figure 3.11 opposite: page 116

Autoradiography of [^{3}H]GR 65630 binding to sequential coronal sections of rat hindbrain. Two radioligand concentrations of 0.2nM and 10nM were compared using respective HEPES buffer concentrations of 50mM and 5mM. The qualitative nature of the specific 5-HT_3 receptor binding was similar in both cases in that the highest densities of recognition sites were associated with the nucleus tractus solitarius. However, the higher buffer concentration (50mM) conferred the advantage of dramatically reducing the amount of background binding, thus enabling a clearer visualisation of specific binding sites. Densitometric analysis of this binding is provided in Table 3.3.

[^{3}H]-GR 65630

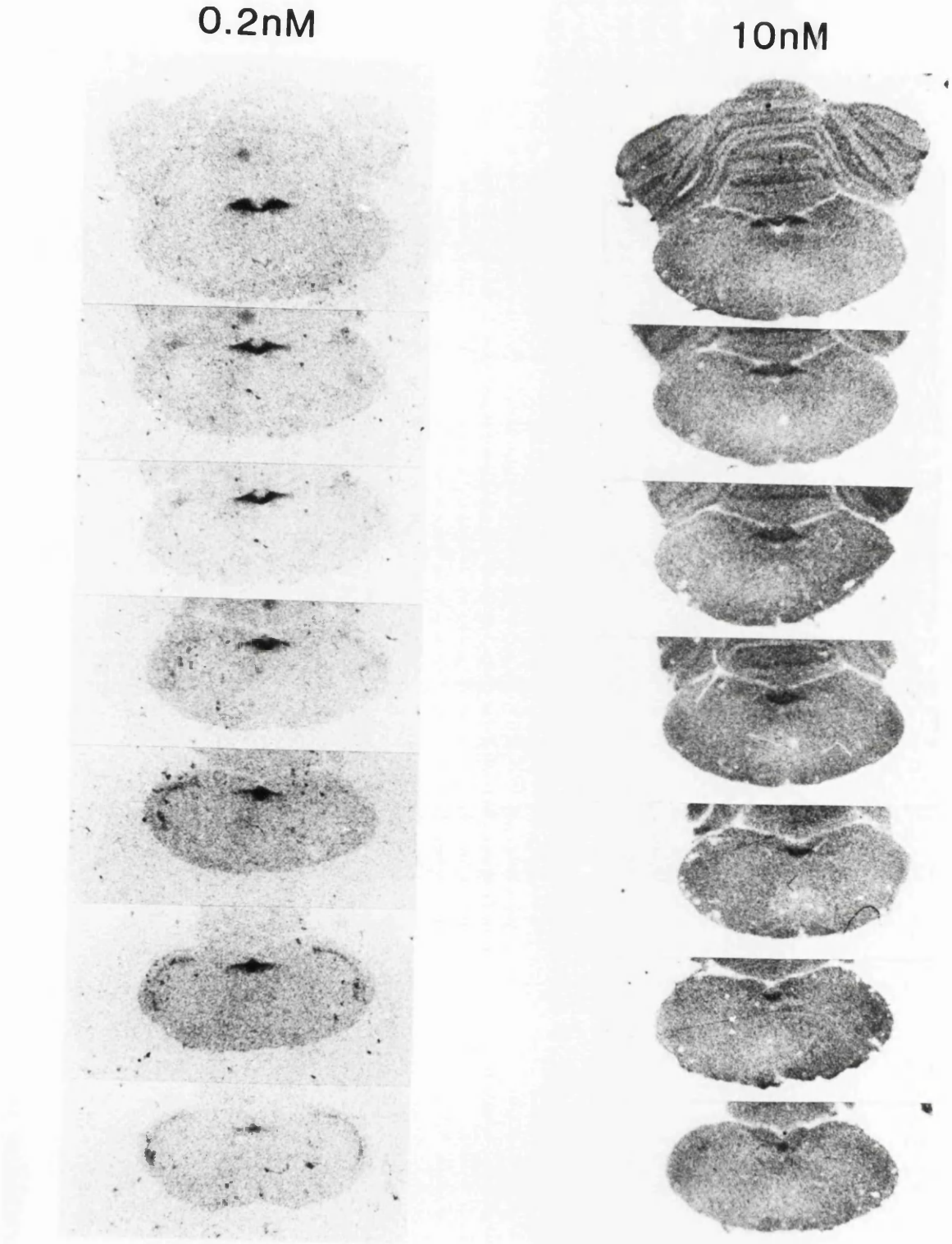


Table 3.3 **Densitometric analysis of 5-HT_3 receptor radioligand binding in rat hindbrain**

[^{3}H]Ligand	nM	n	NTS	AP	DMNX	nSpV
BRL 43694	10	5	630.8 ± 9.0	130.3 ± 32.3		
	1	5	545.0 ± 30.9	100.0 ± 12.8	detectable	detectable
GR65630	10	8	196.6 ± 10.5	90.2 ± 6.9		
	1	6	149.1 ± 7.0	43.3 ± 5.0		
	0.2	6	104.4 ± 3.3	18.0 ± 2.7	detectable	detectable
Zacopride	10	4	342.6 ± 16.6	82.7 ± 11.7		
	1	6	344.4 ± 9.6	77.9 ± 17.4	131.5 ± 8.8	83.1 ± 5.1
Quipazine	10	4	572.5 ± 41.9	114.9 ± 13.7		
	1	5	259.9 ± 4.0	37.6 ± 7.8	not detected	not detected

Values are expressed as fmol/mg tissue and represent the mean ± s.e.m. of at least 3 determinations from each rat. Assays were generally performed in 5mM HEPES buffer with the exception of [^{3}H]GR65630 (0.2nM) for which the HEPES concentration was increased to 50mM.

NTS (nucleus tractus solitarius), AP (area postrema), DMNX (dorsal motor nucleus of the vagus nerve), nSpV (nucleus of the spinal tract of the trigeminal nerve).

Figure 3.12

(A) Saturation analysis of [^{3}H]BRL 43694 binding in the nucleus tractus solitarius of the rat brain. A comparison of the binding in both 5mM (O) and 50mM (●) HEPES buffer (pH 7.4) was assessed autoradiographically over a radioligand concentration range of 0.375 - 3nM. Data points represent the mean ± s.e.m. of three separate experiments for which triplicate determinations were made. (B) Scatchard transformation of specific [^{3}H]BRL 43694 binding (defined in the presence of 100µM GR38032F) revealed no significant differences in B_{max} values (502.6 ± 69.5 and 530.0 ± 50.1 fmol/mg tissue, 50mM and 5mM HEPES buffer, respectively). However, reducing the concentration of buffer from 50mM to 5mM, significantly increased the affinity of the ligand, denoted by respective K_D values of 0.92 ± 0.1nM and 0.38 ± 0.04nM.

ordinate: Bound/Free (fmol/mg tissue/nM)
abscissa: Bound (fmol/mg tissue)

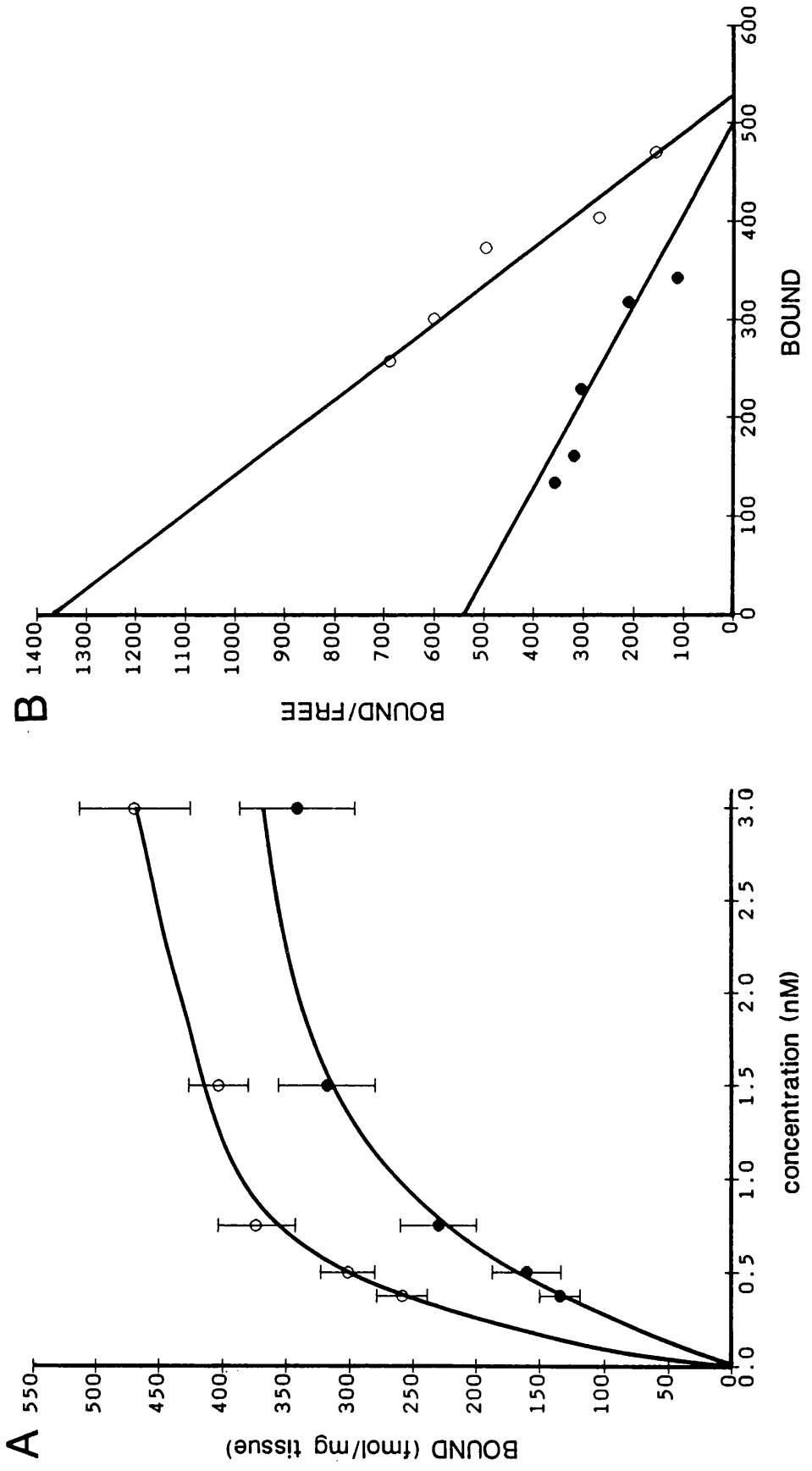

A
BOUND (fmol/mg tissue)
concentration (nM)
B
BOUND/FREE
BOUND

Figure 3.13

Pharmacological characterisation of [^{3}H]BRL 43694 binding in the nucleus tractus solitarius of rat hindbrain was assessed by autoradiographical competition experiments using a variety of 5-HT_3 receptor-selective or non-selective drugs. The amount of displacement of radiolabel at a given concentration was expressed as a percentage of the total specific binding (defined in the presence of 100µM GR38032F). Data points represent the mean of three separate experiments for which triplicate measurements were made (standard errors have been omitted for clarity). A summary of the IC_{50} values obtained is presented in Table 3.4.

ordinate: [^{3}H]BRL 43694 bound (% specific)
abscissa: concentration (nM) of displacing ligand

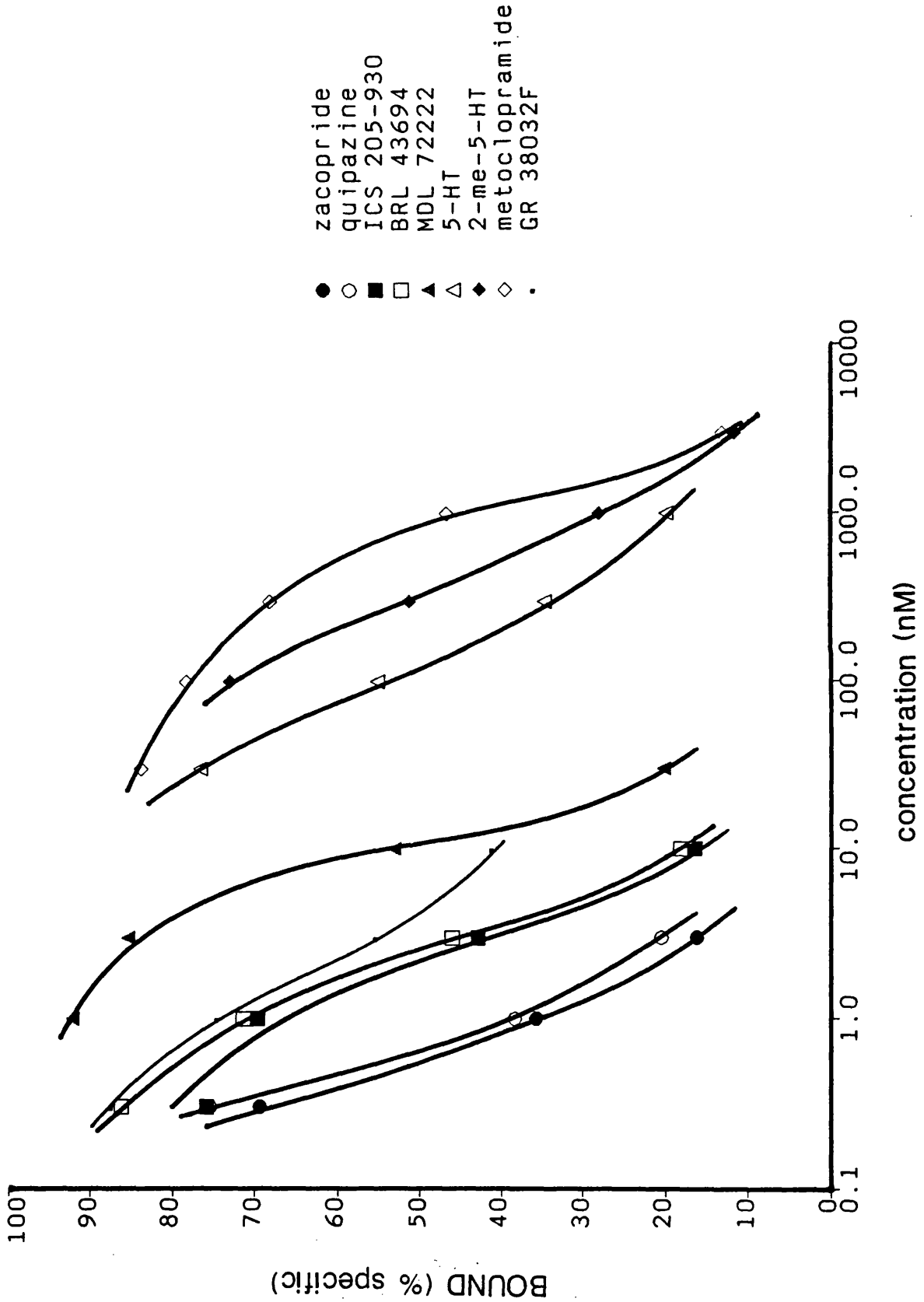

zacopride
quipazine
ICS 205-930
BRL 43694
MDL 72222
5-HT
2-me-5-HT
metoclopramide
GR 38032F
BOUND (% specific)
100
90
80
70
60
50
40
30
20
10
0
0.1
1.0
10.0
100.0
1000.0
10000
concentration (nM)

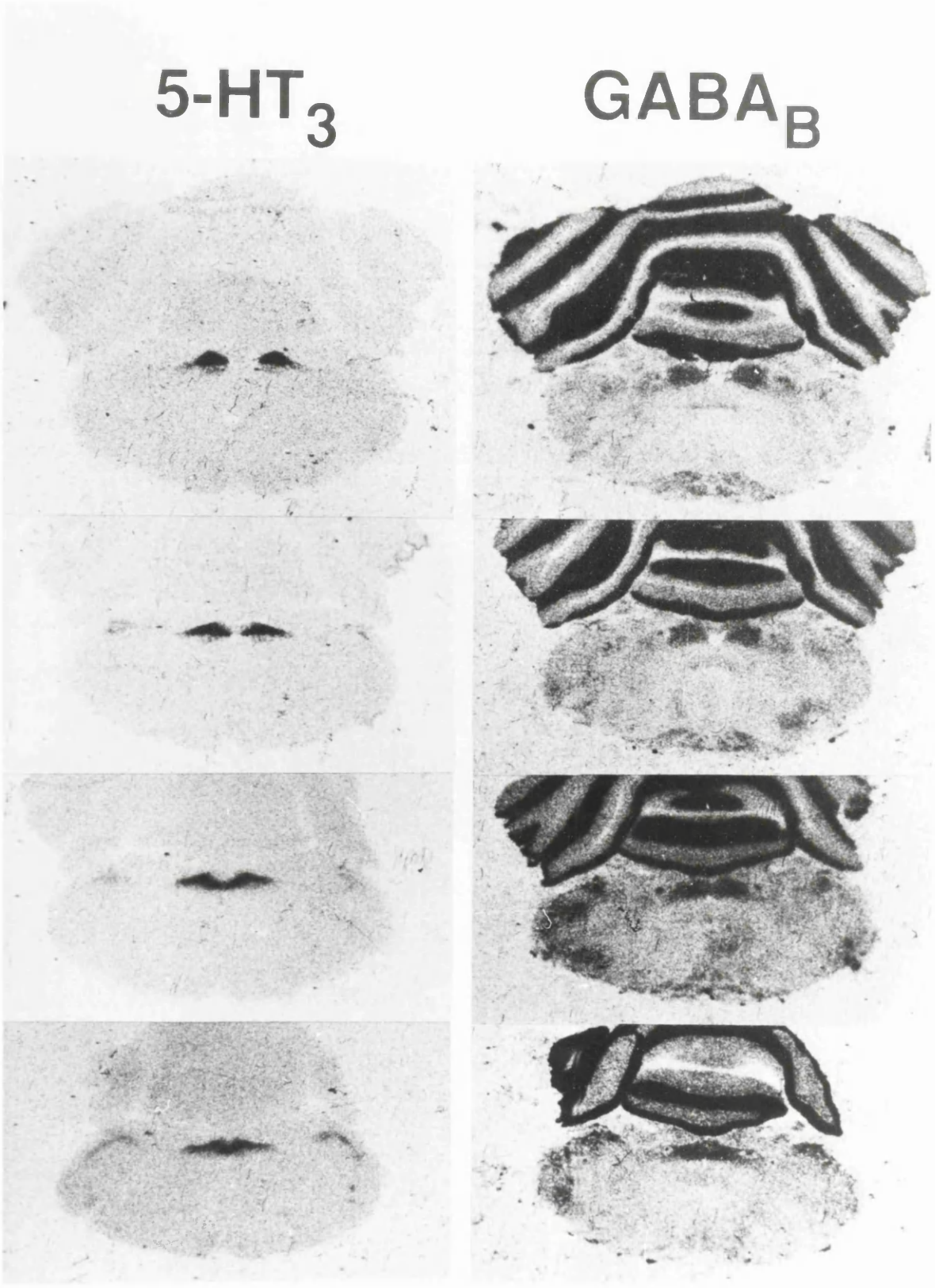
Table 3.4 **The pharmacological profile of [^{3}H]BRL 43694 (1nM) binding in the rat nucleus tractus solitarius assessed using receptor autoradiography**

COMPOUND	IC_{50} (nM)
Zacopride	0.60 ± 0.04
Quipazine	0.67 ± 0.09
ICS 205-930	2.27 ± 0.14
BRL 43694	2.42 ± 0.29
GR 38032F	4.72 ± 0.80
MDL 72222	12 ± 0.58
5-HT	134 ± 10.0
2-methyl-5-HT	325 ± 13.2
Metoclopramide	747 ± 78.8
8-OH-DPAT	>10,000
Methysergide	>10,000

IC_{50} values were derived from [^{3}H]BRL 43694 displacement curves and represent the mean (± s.e.m.) of three separate experiments, for which each point was performed in triplicate.

Figure 3.14 opposite: page 124

Colocalisation of 5-HT_3 and $GABA_B$ binding sites in the dorsal vagal complex of the rat hindbrain. 5-HT_3 sites were labelled by [^{3}H]BRL 43694 (1nM) and predominated in the nucleus tractus solitarius with lower levels in the dorsal motor vagal nucleus and the nucleus of the spinal tract of the trigeminal nerve. $GABA_B$ sites were labelled with [^{3}H]GABA (in the presence of 40μM isoguvacine) and were clearly evident in the dorsal vagal complex, however in contrast to 5-HT_3 sites, there was no precise demarcation between the nucleus tractus solitarius and the underlying dorsal motor vagal nucleus. $GABA_B$ binding sites were also observed in the nucleus of the spinal tract of the trigeminal nerve.


5-HT3
GABAB

DISCUSSION

An extensive mapping of the distribution of $GABA_B$ (and $GABA_A$) receptor binding sites throughout the rostro-caudal projection of the rat brain was initially provided by Bowery et al (1987). Although there was a fair degree of uniformity between the densities of both GABA receptor subtypes in the majority of regions examined, there were, nonetheless, a number of structures in which the concentration of $GABA_B$ receptor sites was significantly higher than those for $GABA_A$ sites. Most notably, these were the lateral posterior thalamic nucleus, the nuclei of the lateral amygdala and the medial habenula, the interpeduncular nucleus, superior colliculus, the molecular layer of the cerebellum, spinal trigeminal tract and the substantia gelatinosa of the spinal cord. From the autoradiograms presented, the current study has confirmed the majority of these distinctions providing also a closer examination of the differential localisation of $GABA_B$ binding sites within the frontal cortex relative to its laminar substructure.

In their report, Bowery et al (1987), described a uniform distribution of $GABA_B$ sites extending over the entire cerebral cortex which predominated in the four outermost layers and for which a single density quantification was made. A subsequent autoradiographical presentation of $GABA_B$ receptor sites subdivided the frontal cortex but only into laminae I and II, despite the complete resolution of the parietal cortex into its component six laminae (Chu et al, 1990). From the present study, it was now apparent that $GABA_B$ receptors are localised into four distinct regions which have been designated as laminae I, II-III, V and VI.

According to the meticulous topographical mapping of the rat cerebral cortex (Krieg 1946a), the frontal cortex comprises six 'Brodmann Areas' (10, 6, 4, 8, 8a and 11). Dorsomedially, it extends half-way back from the frontal pole although in the mediolateral direction, it rapidly shortens until at the lateral edge, it only extends to about one-quarter of the

posterior projection. Quantitative $GABA_B$ receptor density measurements in this study (see also Chapter 5) were restricted to area 10 (also described as frontal areas 1 and 2; Zilles & Wree, 1985) which covers the entire dorsal aspect from the frontal pole to approximately one-quarter of the rostro-caudal axis.

The cytoarchitecture and laminar structure of this region comprises a thin (~0.15mm) outer lamina I (molecular layer) whilst laminae II (outer granular) and III (outer pyramidal) collectively contain small crenated pyramidal cells (~0.3mm). A thicker (~0.6mm) pyramidal layer (lamina V) is densely packed with pyramidal cells which are periodically interspersed with larger granular cells whilst lamina VI (fusiform layer) which is similar in size to lamina V again consists of granule cells (Krieg, 1946b). It is, however, the lack of a prominent lamina IV, that provides a characteristically clear demarcation of the frontal cortex from its neighbouring parietal region (Zilles & Wree, 1985; Krieg, 1946b). This is not to say, however, that lamina IV does not 'exist' but rather its agranular nature poses difficulty in its demarcation from adjacent laminae (III and V). It is for this reason that $GABA_B$ sites in the frontal cortex were assigned to the four laminar regions previously mentioned.

Published reports of GABAergic neurones in the rat neocortex have concentrated largely on the occipital cortex, there being an apparent paucity of studies devoted purely to the frontal region. Morphological identification of neurones by immunohistochemistry with antibodies against GAD have located GAD-positive axosomatic 'baskets' (comprising axon terminals and varicosities) concentrated especially in laminae II-III of the occipital cortex, with lower densities in the molecular and deeper laminae (Wolff et al, 1984). Endogenously measured GABA in the same cortical region complemented this laminar distribution (Ishikawa et al, 1983). Moreover, the accumulation of [^{3}H]GABA by cerebral cortical neurones showed a predominance of radioligand in the cell bodies of stellate neurones within

laminae II-III. Uptake of the isotope into pyramidal cells, in contrast, was much lower (Hokfelt et al, 1972). Perhaps these studies provide a possible explanation for the predominance of $GABA_B$ receptor sites in laminae II and III.

Structure activity comparisons of $GABA_B$ receptor agonists and antagonists in brain tissue

Examination of the binding of [^{3}H]GABA to whole brain synaptic membranes demonstrated that the interaction of baclofen at $GABA_B$ sites was clearly stereoselective with the more potent displacing activity residing in the (-)-enantiomer and confirms previous reports by Bowery et al, (1983, 1985) and Bittiger et al, (1988). The greater selectivity of the $GABA_B$ receptor agonist, 3-APA over baclofen demonstrated in the present study was in close agreement with the initial report of this compound which was shown to be 20 times more potent than baclofen (Dingwall et al, 1987). Moreover, functional assays of $GABA_B$ receptor activation such as the baclofen-induced inhibition of either the cholinergic twitch responses of the guinea-pig ileum or the electrically-evoked contractions of the rat anococcygeus muscle have demonstrated the enhanced potency of this compound over the prototypic $GABA_B$ receptor agonist, baclofen (Hills et al, 1989).

The relative potencies of phaclofen, saclofen and 2-OH-saclofen to displace $GABA_B$ receptor site binding agreed closely with reports of the inhibition of [^{3}H]baclofen binding by Drew et al, (1990) and Al-Dahan et al, (1990) and also with their functional antagonistic actions *in vitro* models (Kerr et al, 1988; Kerr et al, 1989). Furthermore, the ability of CGP 35348 to inhibit [^{3}H]GABA binding closely paralleled the IC_{50} value of this compound (34μM) for the displacement of radiolabelled 3-APA reported by Olpe et al (1990).

Characterisation of central Beta-adrenoceptors

The high selectivity of CGP 20712A and ICI 118,551 for $beta_1$- and $beta_2$-adrenoceptors was clearly demonstrated by their biphasic inhibition of IPIN binding to sections of cerebral cortex. Whilst CGP 20712A was some 2400 times more selective for $beta_1$-adrenoceptors, ICI 118,551 possessed a blocking potency at $beta_2$-adrenoceptors some 40 times greater than at the $beta_1$-adrenoceptor subtype. The IC_{50} values obtained for these two antagonists were in close agreement with those reported by Beer et al (1988) in cortical membranes using the same radioligand as employed in the present study. The ability to selectively block one receptor subtype whilst leaving the other virtually unoccupied was clearly exemplified by autoradiographical analysis of these central beta-adrenergic sites. With particular regard to the concentration of ICI 118,551 used in this study (50nM) to visualise $beta_1$-adrenoceptors, this was in agreement with the membrane binding methodology of De Paermentier et al (1988) and Beer et al (1988) in addition to the autoradiographical studies of Rainbow et al (1984) and Ordway et al (1988) for which similar central beta-adrenoceptor distributions to those seen in the present study were observed. Although beta-adrenoceptor sites were identified in the frontoparietal cortex by Rainbow et al (1984), there was no indication of binding specifically to laminae II and III but rather a uniform density of sites was present over laminae I, IV and VI. The findings of the present study therefore extend existing reports and the localisation of beta-adrenoceptors closely parallels known markers of noradrenergic innervation to this region (Levitt & Moore, 1978).

$5\text{-}HT_3$ receptor distribution in mammalian hindbrain

Using the radioligand [^{3}H]ICS 205-930, initial reports of $5\text{-}HT_3$ receptor autoradiography identified areas of concentrated binding in regions of the mouse and human hindbrain that collectively have been termed the

dorsal vagal complex. These regions comprise the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus nerve (DMNX) and the area postrema (AP). Specific binding was also evident in the nucleus of the spinal tract of the trigeminal nerve (nSpV) (Waeber et al, 1988; Hoyer et al, 1989; Waeber et al, 1989). A concomitant report by Kilpatrick et al (1988b) of the binding of [^{3}H]GR65630 to rat hindbrain supported these findings in that dense 5-HT$_3$ recognition sites were found in the AP although regions surrounding this structure were devoid of any specific binding.

At this stage the possibility that these two radioligands were able to discriminate between discretely located 5-HT$_3$ receptor subtypes seemed a plausible explanation for these contrasting findings. In an attempt to explore this hypothesis further, the present study firstly provided a detailed autoradiographical analysis of the binding of [^{3}H]BRL 43694. Examination of the binding profiles of this radioligand from both the coronal and horizontal perspectives together with histological comparisons of adjacent sections using cresyl fast violet staining facilitated the identification of those regions displaying high levels of specific binding. Thus, at a concentration of 10nM, the NTS was found to contain the greatest densities of specific [^{3}H]BRL 43694 sites whilst those observed in the AP contributed to only one-fifth of the NTS value. Qualitatively, at least, the locations of these sites were similar to those in the mouse brain reported by Waeber et al (1988) and Hoyer et al (1989) labelled by [^{3}H]ICS 205-930. In contrast, however, the restricted labelling of the AP reported by Kilpatrick et al, (1988b) could not be achieved using [^{3}H]BRL 43694, which again, added weight to an argument in favour of 5-HT$_3$ receptor subtypes.

Although the reasons for the apparent discrepancies remained unclear, upon closer inspection of the assay conditions employed in the present study and that of Kilpatrick et al (1988b), subtle differences were evident. In view of the low specific activity (21Ci/mmol) of [^{3}H]BRL 43694 used initially, it was found that by reducing the HEPES buffer concentration

from 50mM (see Kilpatrick et al, 1988b) to 5mM, this had the effect of approximately doubling the amount of radioligand bound to the brain sections. The autoradiographical process would thus be facilitated by enhanced binding as would a higher radioligand concentration of 10nM. This value was considerably greater than that used by Kilpatrick et al (1988b) for [^{3}H]GR65630 (0.2nM) or by Waeber et al (1988) for [^{3}H]ICS 205-930 (1.5nM) and was in excess of the K_D of the ligand in cortical membranes (0.3nM) reported by Nelson & Thomas (1989). However, a lower concentration of 1nM produced a similar binding profile to that observed in the presence of 10nM [^{3}H]BRL 43694, with 5-HT_3 sites again predominating in the NTS. Furthermore, in the presence of either 50mM or 5mM HEPES, saturation analysis of [^{3}H]BRL 43694 binding to the NTS (up to a maximum radioligand concentration of 3nM), revealed no significant difference in the maximal number of binding sites between these two conditions. However, the affinity of this radioligand for 5-HT_3 sites within the NTS was significantly increased by the lower HEPES buffer concentration. To the author's knowledge, only one autoradiographical report of Scatchard analysis of [^{3}H]BRL 43694 binding exists (Reynolds et al, 1989b). Although in this instance, human brain tissue was employed, a K_D value of 1.21nM closely agreed with the present study (0.92nM). However, since quantitation of binding was measured against calibrated brain paste standards, in contrast to the plastic tritium standards (with tissue equivalent values) used in the current investigation, an accurate comparison of B_{max} values was difficult to extrapolate.

Thus it would appear that the concentration of either the buffer or radioligand employed did not influence the qualitative nature of [^{3}H]BRL 43694 binding in that the manipulation of these parameters failed to expose 5-HT_3 receptor subtypes in the dorsal vagal complex. This was consolidated further by comparing the binding of [^{3}H]GR65630 at concentrations of 0.2nM and 10nM to adjacent hindbrain sections in the presence of 50mM

and 5mM HEPES, respectively. Although non-specific binding was markedly lower using lower buffer concentration, the 5-HT_3 binding profile was similar under both conditions with sites predominating in the NTS.

Additionally, this study also examined the distribution of [^{3}H]zacopride and [^{3}H]quipazine binding sites in this region of the brain, which in keeping with the two 5-HT_3 receptor radioligands already mentioned, again identified a preponderance of sites within the NTS. These radioligands also bound to the AP, but which again, only represented approximately 20% of NTS levels. Moreover, the apparent low lipophilic nature of [^{3}H]zacopride enabled the clear autoradiographic visualisation of binding in the DMNX and the nSpV.

Since the delineation of the AP from the NTS in para-sagittal sections of rat brain is not so clearly defined as compared to the coronal and horizontal planes, this was the likely reason for the initial discrepancies in autoradiographic descriptions. A number of subsequent autoradiographical studies, however, in the brain of the rat (Pratt & Bowery, 1989), ferret (Reynolds et al, 1989a; Barnes et al, 1990b; Leslie et al, 1991), human (Reynolds et al, 1989b; Barnes et al, 1990a) and rodents (Perry, 1990) using a variety of 5-HT_3 receptor radioligands, has revealed that within the individual anatomical components of the dorsal vagal complex, the densities of 5-HT_3 sites are quite heterogeneous and that they appear to be associated with the vagal afferent fibres that terminate within this structure (Pratt & Bowery, 1989; Reynolds et al, 1989a; Leslie et al, 1991). Moreover, in all species it is the NTS that possesses the greatest concentration of 5-HT_3 sites and indeed this has been further characterised to represent the dorsomedial portion of this nucleus (Reynolds et al, 1989b; Barnes et al, 1990a; Leslie et al, 1991). This neuroanatomical region has been designated as the subnucleus gelatinosus, a term first described by Taber (1961) and which is synonymous with the area subpostrema proposed by Gwyn & Wolstencroft (1968). The possible functional relevance of these centrally-located 5-HT_3

receptor sites will be more fully discussed in the proceeding chapter.

Pharmacological characterisation of [^{3}H]BRL 43694 binding sites in the NTS was achieved with a number of 5-HT_3 receptor antagonists and agonists. The IC_{50} values obtained for these agents produced structure-activity relationships that were in close agreement with drug competition studies in homogenates of rat vagus nerve and AP (Kilpatrick et al, 1989) and in entorhinal cortex (Kilpatrick et al, 1990). Interestingly, the displacement of [^{3}H]BRL 43694 binding by GR38032F exhibited a dissimilar inhibition profile compared with, for example, ICS 205-930 and BRL 43694, in spite of the similar IC_{50} values of these compounds. Although a complete concentration range of GR38032F was not examined, the percentage displacement at 10μM amounted to around 60%, which contrasted markedly to an inhibition of binding in excess of 80% by the same concentration of both ICS 205-930 and BRL 43694. In their original paper identifying central 5-HT_3 receptor sites, Kilpatrick et al (1987) observed that the inhibition of [^{3}H]GR65630 binding by GR38032F (to a maximum of 90% of the total binding) could be resolved into two sites. This was also true of the structural analogues of GR38032F, notably, GR65630 and GR67330 (Kilpatrick et al, 1987, 1990). The inability of other 5-HT_3 receptor antagonists to interact with this second lower affinity site suggested that it is unrelated to the 5-HT_3 receptor. Indeed, since such non-specific 5-HT_3 sites are also present in the area postrema (Kilpatrick et al, 1989), these observations could contribute to the findings of the present study. However, a more detailed analysis of the interaction of GR38032F with 5-HT_3 receptor sites in the NTS over a much wider displacing concentration range, would provide more conclusive evidence in favour of such a hypothesis.

Colocalisation of 5-HT_3 binding sites with those for $GABA_B$ receptors in rat dorsal vagal complex

In view of the detailed mapping of central $GABA_B$ receptors in the rat

reported by Bowery et al (1987) and Chu et al (1990), it was perhaps surprising that such binding sites were not identified in the dorsal vagal complex. Since the magnitude of the rostral-caudal extent of the NTS is only in the region of 1mm (Paxinos & Watson, 1986), it would be relatively easy to omit sections containing this morphology. Nonetheless, in conjunction with 5-HT_3 receptor autoradiography, used as a marker of this hindbrain region, $GABA_B$ sites were positively identified. These autoradiographical findings corroborate immunocytochemical studies of glutamic acid decarboxylase immunoreactivity for which GABAergic cell bodies (perikarya) and axon terminals are richly distributed in the NTS, AP and DMNX (Blessing et al, 1984; Meeley et al, 1985; Mugnaini & Oertel, 1985).

CHAPTER 4

SIMILARITY OF THE DISTRIBUTION OF $GABA_B$ AND 5-HT_3 RECEPTORS IN THE RAT HINDBRAIN

INTRODUCTION

Autoradiographical analysis of the binding of the 5-HT_3 receptor ligands, [^{3}H]BRL 43694, [^{3}H]zacopride, [^{3}H]GR65630 and [^{3}H]quipazine to sections of the rat hindbrain, described in the preceding chapter has revealed that 5-HT_3 recognition sites predominate within the dorsal vagal complex of which the highest concentration was found in the nucleus tractus solitarius (NTS). Lower densities were also evident in the area postrema, the dorsal vagal motor nucleus and the nucleus of the spinal tract of the trigeminal nerve.

In the mammalian central nervous system, the NTS functions as the primary relay station for the integration of the majority of sensory afferent inputs from the periphery. Approximately 30,000 fibres terminate within the NTS constituting 80% of the Xth. (vagus) cranial nerve (Cottle, 1964). The cell bodies of general visceral afferent fibres are located in the nodose (inferior vagal) ganglion and provide sensory peripheral projections to the pharynx, larynx, trachea as well as thoracic and abdominal viscera. Their proximal central branches emanating from this ganglion enter the tractus solitarius and terminate within the nucleus of this tract (Craigie, 1963). The remaining vagal afferents (with cell bodies in the superior vagal ganglion) enter the medulla via the spinal trigeminal tract, terminating within its nucleus. Additionally, sensory afferent fibres arising from the Vth. (trigeminal) and VIIth. (facial) cranial nerves terminate in regions of the NTS, rostral to the obex whereas fibres from the IXth. (glossopharyngeal) and Xth. cranial nerves project more caudally to both medial and lateral regions (Beckstead & Norgren, 1979).

General visceral efferent outputs of vagal motor cells originate from the dorsal motor nucleus which lies subjacent to the NTS. This innervates thoracic and abdominal organs whilst special visceral efferent fibres (to the pharynx and larynx) project from the more ventrally situated nucleus

ambiguus (Craigie, 1963). The NTS together with the AP and the dorsal motor nucleus of the vagus collectively constitute the dorsal vagal complex from which much of the activity of the autonomic nervous system is influenced.

In addition to providing a location for sites that are identified by $5HT_3$ receptor radioligands, the dorsal vagal complex was also shown to possess both $GABA_A$ and $GABA_B$ binding sites. Although the neuroanatomical definition of these GABAergic loci was not as clearly defined as with 5-HT_3 receptor autoradiography, $GABA_B$ sites were nonetheless, evident in both the NTS and dorsal motor vagal nucleus, especially in sections located rostrally from the obex, whilst $GABA_A$ receptors appeared to be restricted to this latter subregion.

To determine the neuronal location of these sites in relation to pre- or postsynaptic terminals, autoradiography of [^{3}H]BRL 43694 and [^{3}H]GABA (under conditions to specifically identify $GABA_A$ and $GABA_B$ subsites) was performed following vagal deafferentation, achieved by unilateral nodose ganglionectomy. If recognition sites were located presynaptically on vagal terminals, an ipsilateral reduction in binding in the dorsal vagal complex would be predicted. Conversely, an increased binding density should arise if their location was postsynaptic to vagal terminals.

METHODS SURGICAL PROCEDURES

Nodose ganglionectomy

Rats were anaesthetised with chloral hydrate (400mgkg^{-1} i.p.). A mid-line incision of the skin of the ventral surface of the neck was made and the underlying layers of muscle retracted to reveal the right carotid artery. The vagus nerve was carefully separated from the artery and the closely

associated sympathetic nerve and traced back to the nodose ganglion. This was then extirpated by making one cut distally to the ganglion and one proximally through the vagus nerve. Following surgery, incisions were closed using wound clips and the rats allowed to recover from anaesthesia. Perfusion-fixation was performed after a period of 10 days had elapsed, allowing denervation to occur.

Hemisection of the nucleus tractus solitarius

Prior to nodose ganglionectomy, one further rat was anaesthetised as described above and positioned in a stereotaxic frame with the cranium at an angle of 45° below the horizontal. A mid-line incision was made through the skin, the neck muscles retracted, revealing the foramen magnum and the atlanto-occipital membrane, which was carefully incised to expose the brainstem. A microknife was positioned at the caudal end of the fourth ventricle, lowered 2mm into the brainstem and then moved 1mm in a rostral-caudal direction to hemisect the NTS.

RESULTS

Denervation studies with 5-HT_3 receptors

The effects of unilateral (right-sided) nodose ganglionectomy on the binding of [^{3}H]BRL 43694 (10nM) within the NTS are visualised autoradiographically in sequential coronally- (Figure 4.0) and horizontally- (Figure 4.1) cut sections. Specific binding of this radioligand in this region was significantly reduced ($P < 0.01$, Student's 't' test; n=6) from 366.7 $\pm$ 21.4 fmol/mg tissue (contralateral side) to 167.9 $\pm$ 21.9 fmol/mg tissue, ipsilaterally. Moreover, binding in the contralateral side of the NTS only afforded 58% of that seen in sham-operated controls (630.8 $\pm$ 9.0 fmol/mg tissue). This was also reflected in the area postrema where the density of

binding sites was again, significantly reduced by 49% ($P< 0.05$) as a consequence of denervation (Figure 4.2).

In one further animal, subjected to hemisection of the NTS prior to nodose ganglionectomy, 5-HT_3 sites were not evident in more caudal regions of the NTS (Figure 4.3). However, rostral to the hemisection cut, specific binding was reduced from 339.0 $\pm$ 8.6 fmol/mg tissue to 63.3 $\pm$ 23.8 fmol/mg tissue (triplicate determinations) (Figure 4.2).

Denervation studies with $GABA_A$ and $GABA_B$ receptors

Examination of the consequences of vagal denervation on $GABA_A$ and $GABA_B$ binding sites within the dorsal vagal complex was performed in conjuction with [^{3}H]BRL 43694 (1nM) autoradiography. The clear demarcation of 5-HT_3 sites previously demonstrated in this region was used to provide an anatomical marker for both GABA receptor subtypes.

Autoradiograms of 5-HT_3, $GABA_A$ and $GABA_B$ binding sites in the dorsal vagal complex in adjacent sections from sham-operated animals are shown in Figure 4.4. Again, 5-HT_3 sites predominated in the NTS (393.5 $\pm$ 40.4 fmol/mg tissue) and visually detectable binding was also apparent in the dorsal vagal motor nucleus. This latter region also possessed a high density of $GABA_A$ binding sites (154.5 $\pm$ 18.9 fmol/mg tissue) whilst in comparison, the NTS and dorsal motor vagal nucleus collectively contained a slightly lower $GABA_B$ site density (90.7 $\pm$ 15.9 fmol/mg tissue).

Following nodose ganglionectomy, 5-HT_3 sites in the ipsilateral NTS were significantly reduced by 55% from contralateral values ($P< 0.01$) which in turn, differed significantly ($P< 0.01$) from sham-treated animals (Table 4.0). In parallel with these findings, reductions in $GABA_B$ and $GABA_A$ sites amounting to 44% and 58%, also occurred in the ipsilateral portion of the NTS and dorsal vagal motor nucleus, respectively (Figure 4.5). Since no significant changes in the numbers of these GABAergic sites within the

molecular and granular layers of the cerebellum ensued, the effects of vagal lesioning appeared to be confined to those brainstem nuclei examined (Table 4.0).

Figure 4.0 opposite: page 141

The effect of right-sided unilateral nodose ganglionectomy on the binding of [^{3}H]BRL 43694 (10nM) to sequential coronal sections of rat hindbrain, performed 10 days following the lesion. Note the reduction in total binding on the ipsilateral (right-hand) side of the nucleus tractus solitarius compared to the contralateral side. Non-specific binding defined by GR38032F (100μM) is shown to the left of the total binding profile.

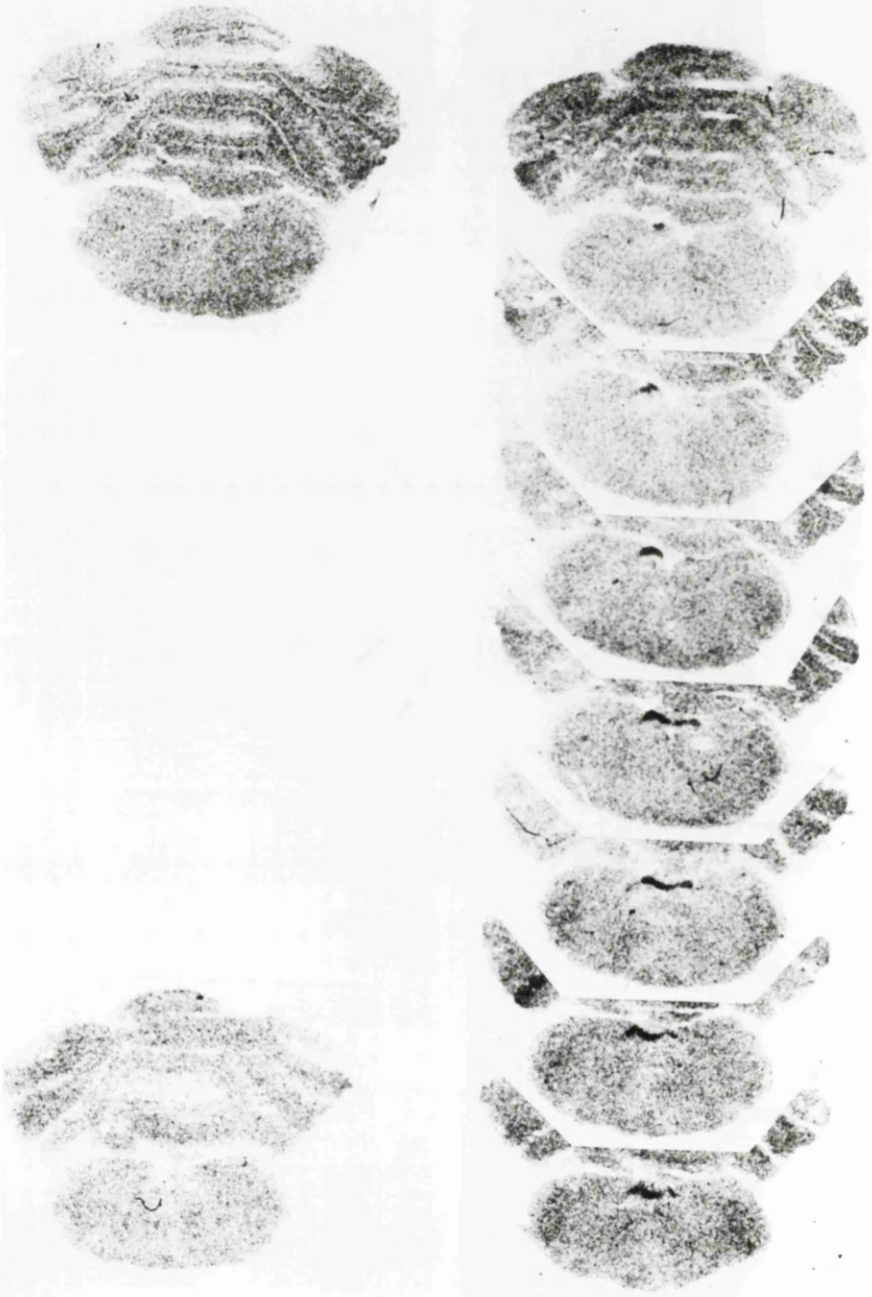


Figure 4.1 opposite: page 143

The effect of right-sided unilateral nodose ganglionectomy on the binding of [^{3}H]BRL 43694 (10nM) to sequential horizontal sections of rat hindbrain, performed 10 days following the lesion. Note the reduction in total binding on the ipsilateral (right-hand) side of the nucleus tractus solitarius compared to the contralateral side. Non-specific binding defined by GR38032F (100μM) is shown to the left of the total binding profile.

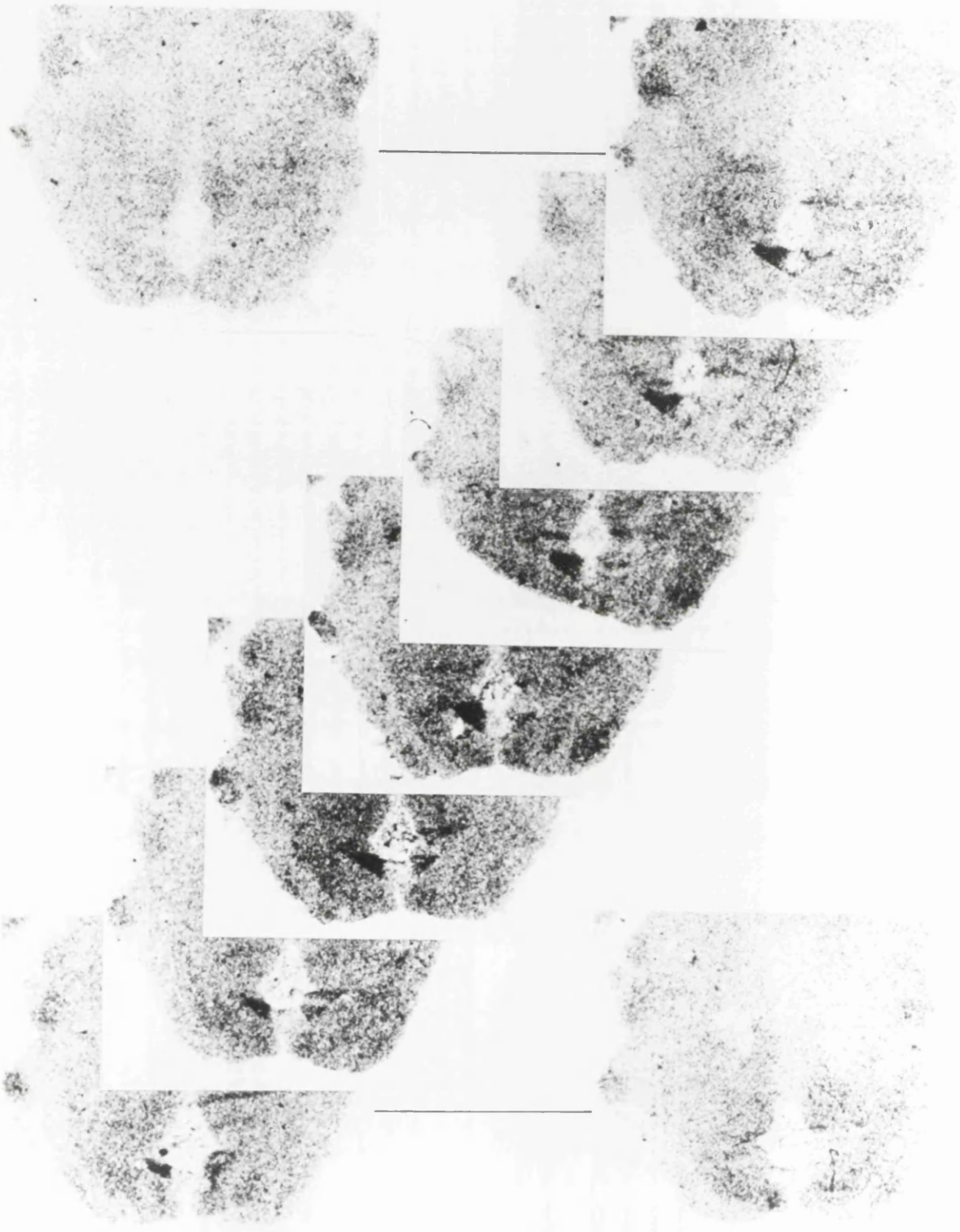


Figure 4.2

Summary of specific [^{3}H]BRL 43694 (10nM) binding densities (fmol/mg tissue) in the entorhinal cortex (Ent. Ctx.), nucleus tractus solitarius (NTS) and the area postrema (AP) of the brains of sham-operated and unilaterally nodose-ganglionectomised rats. Values represent mean $\pm$ s.e.m. of measurements from 5 rats/treatment group for which triplicate determinations were made. Also shown are values for one further rat, subjected to hemisection of the nucleus tractus solitarius prior to ganglionectomy.

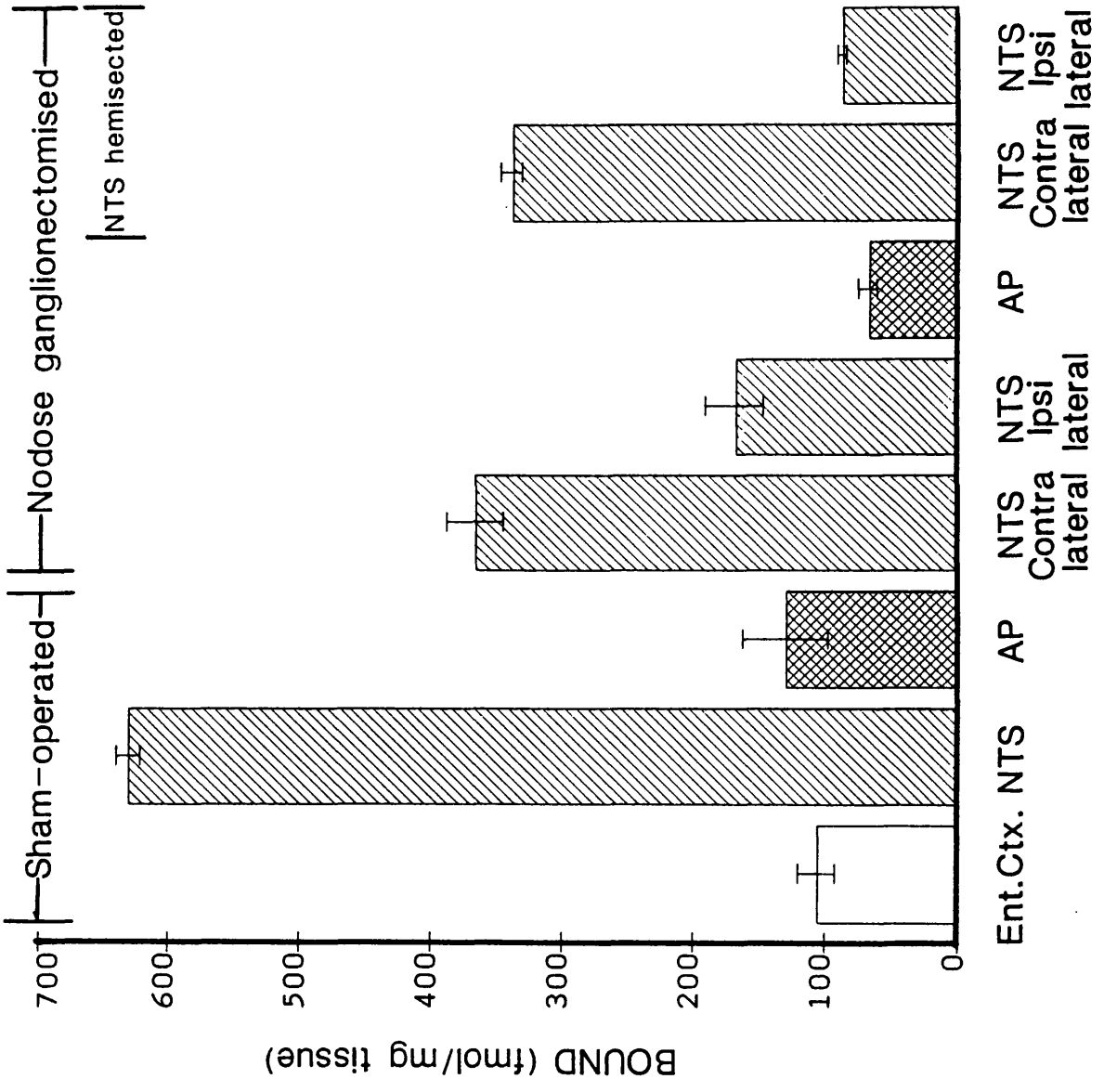

Sham-operated
Nodose ganglionectomised
NTS hemisected
BOUND (fmol/mg tissue)
700
600
500
400
300
200
100
0
Ent.Ctx.
NTS
AP
NTS Contra lateral
NTS Ipsi lateral
AP
NTS Contra lateral
NTS Ipsi lateral

Figure 4.3 opposite: page 147

The effect of hemisection of the nucleus tractus solitarius followed by unilateral nodose ganglionectomy, on the binding of [^{3}H]BRL 43694 (10nM) to sequential coronal sections of rat hindbrain. With the exception of the contralateral side of more rostral sections, note the absence of binding within the nucleus tractus solitarius. Non-specific binding defined by 100μM GR38032F is shown to the left of centre.

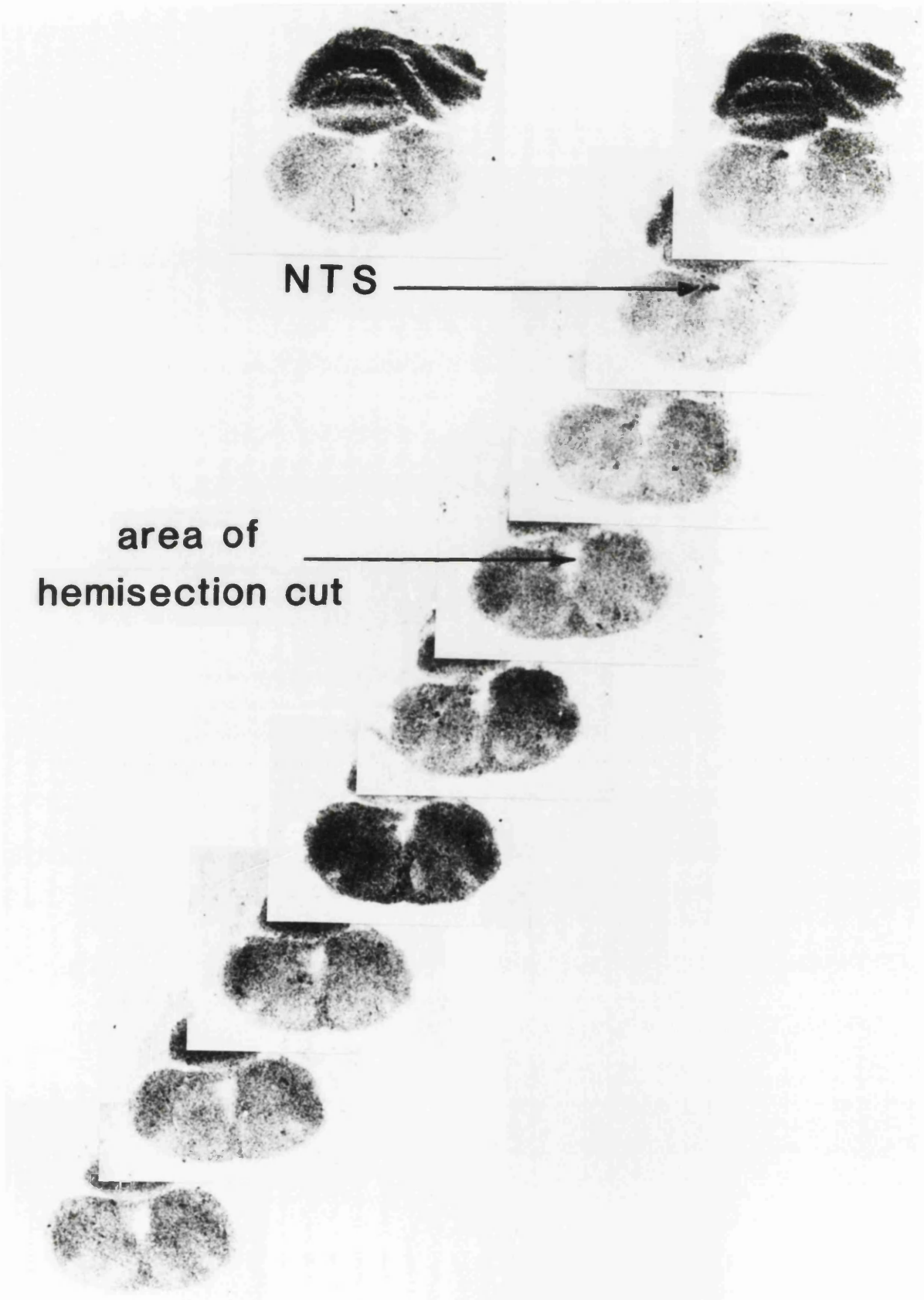
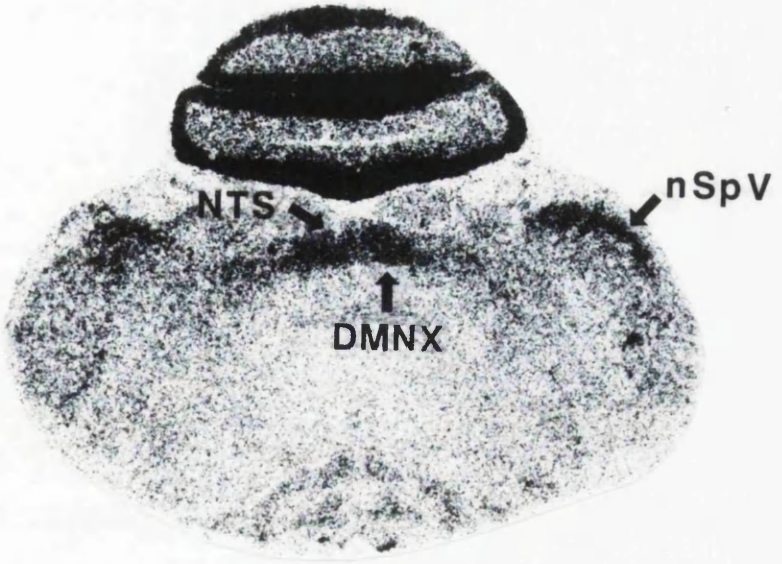

NTS
area of
hemisection cut

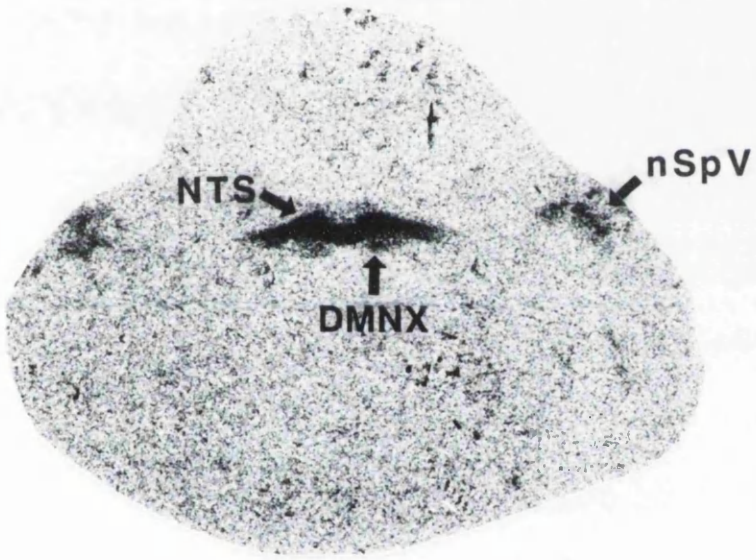
Figure 4.4 opposite: page 149

Colocalisation of $GABA_B$, $GABA_A$ and 5-HT_3 receptor binding sites in the dorsal vagal complex of the hindbrain of a control rat. 5-HT_3 sites were labelled by [^{3}H]BRL 43694 (1nM) and predominated in the nucleus tractus solitarius (NTS) with lower levels in the dorsal motor vagal nucleus (DMNX) and the nucleus of the spinal tract of the trigeminal nerve (nSpV). Whilst $GABA_B$ sites were clearly evident throughout the dorsal vagal complex, in contrast to 5-HT_3 receptor sites, there was no precise demarcation between the NTS and the underlying DMNX. Conversely, $GABA_A$ receptor sites appeared to be concentrated in the brainstem, solely within the DMNX, and unlike $GABA_B$ sites, were absent from the nSpV.

$GABA_B$



$5\text{-}HT_3$



$GABA_A$

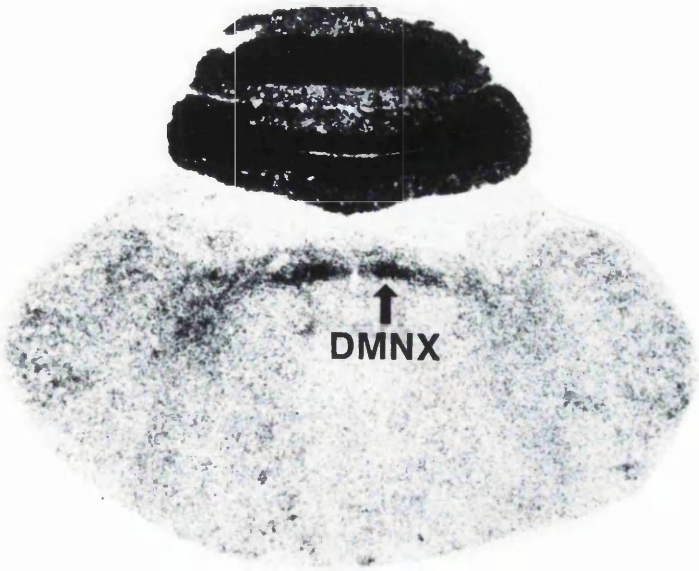
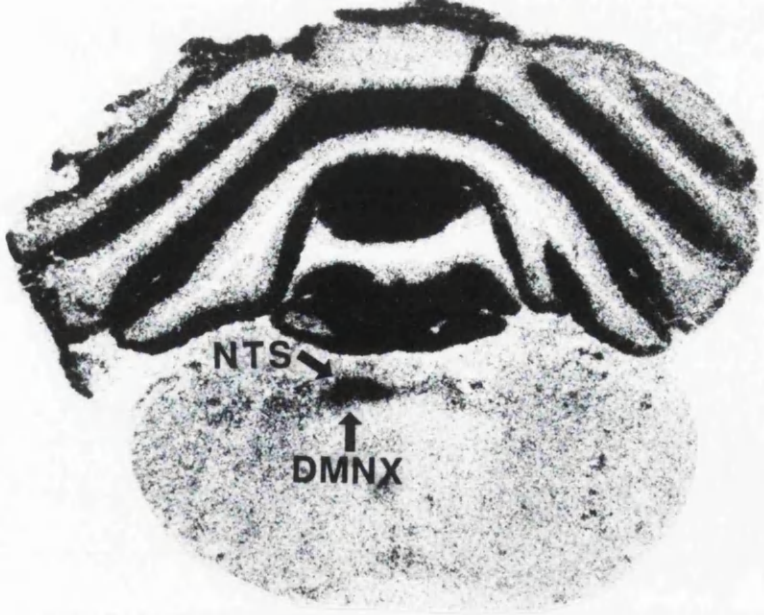


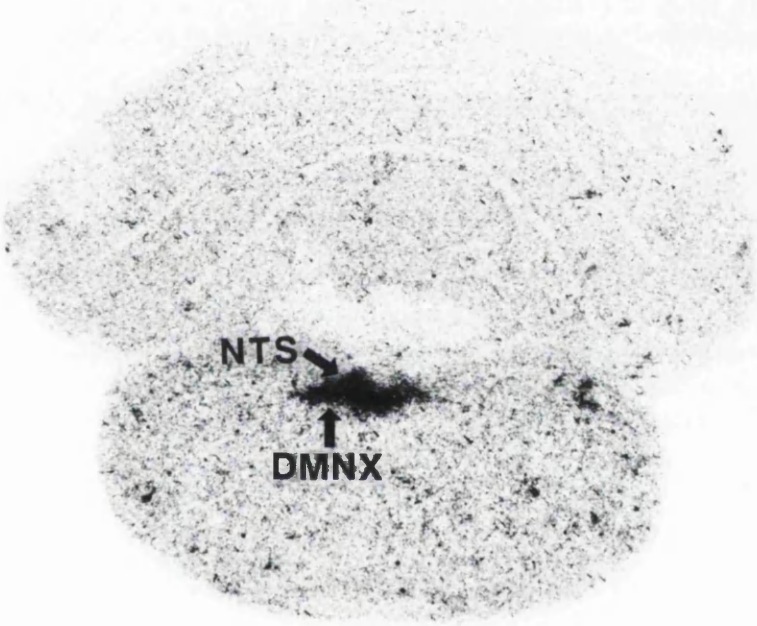
Figure 4.5 opposite: page 151

The effect of right-sided unilateral nodose ganglionectomy on the binding of [^{3}H]BRL 43694 to 5-HT$_3$ receptors and of [^{3}H]GABA under conditions to specifically label both GABA$_B$ and GABA$_A$ receptor sites, performed 10 days following the lesion. Note the reduction in binding on the ipsilateral (right-hand) side of the nucleus tractus solitarius (5-HT$_3$ receptors), the dorsal vagal complex (GABA$_B$ receptors) and the dorsal motor nucleus of the vagus nerve (GABA$_A$ receptors).

$GABA_B$



$5\text{-}HT_3$



$GABA_A$

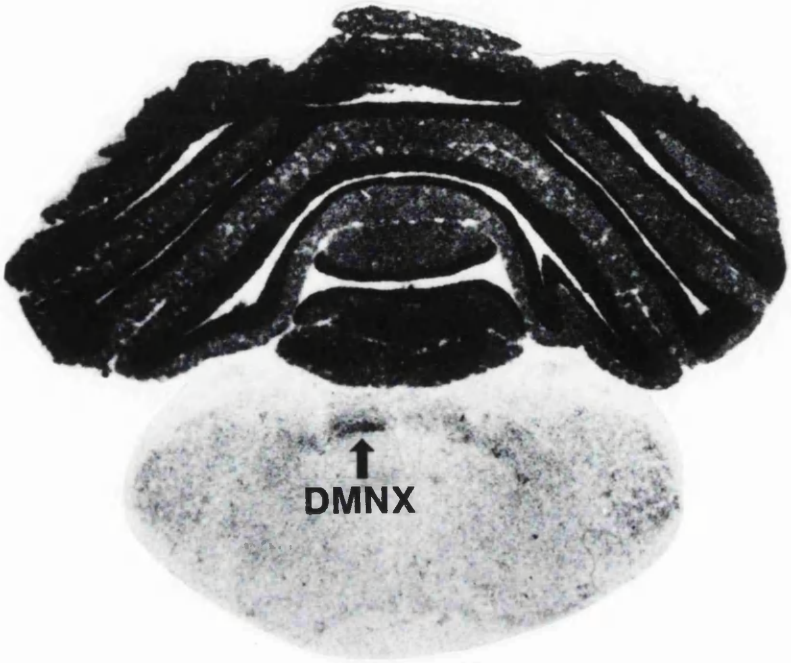


Table 4.0 **The effect of unilateral nodose ganglionectomy on the binding of [^{3}H]BRL 43694 (1nM) to 5-HT$_3$ sites and [^{3}H]GABA (50nM) to GABA$_B$ and GABA$_A$ sites in the dorsal vagal complex of the rat hindbrain.**

RECEPTOR SITE	Sham-operated			Nodose ganglionectomised			
	DVC	Cerebellum (layer)		DVC		Cerebellum (layer)	
		Molecular	Granular	Contralateral	Ipsilateral	Molecular	Granular
5-HT$_3$	393.5 ± 40.4	not detected	not detected	263.0 ± 4.1[a]	118.5 ± 2.4[b]	not detected	not detected
GABA$_B$	90.0 ± 16.2	244.5 ± 17.9	51.2 ± 6.8	59.1 ± 8.6	33.1 ± 4.4[c]	206.4 ± 17.4	50.2 ± 4.8
GABA$_A$	154.5 ± 18.9	202.1 ± 20.2	412.7 ± 17.7	87.6 ± 10.3[a]	36.5 ± 4.6[b]	168.0 ± 15.9	387.0 ± 13.0

Binding density values are expressed as fmol/mg tissue and represent the mean ± s.e.m. of at least 3 separate experiments.

The term dorsal vagal complex (DVC) refers to the following subregions:-

5-HT$_3$ sites; nucleus tractus solitarius
GABA$_B$ sites; nucleus tractus solitarius plus dorsal vagal motor nucleus
GABA$_A$ sites; dorsal vagal motor nucleus

a $P < 0.05$, contralateral DVC compared to sham-operated animals
b $P < 0.01$, ipsilateral DVC compared to contralateral DVC
c $P < 0.05$, ipsilateral DVC compared to contralateral DVC

DISCUSSION

5-HT_3 receptor association with vagal afferent terminals

Lesioning of the afferent vagal input to the medullary region of the rat brain by extirpation of the nodose ganglion, dramatically reduced the densities of 5-HT_3 recognition sites labelled by [^{3}H]BRL 43694 throughout the ipsilateral portion of the nucleus tractus solitarius (NTS) and also the area postrema (AP). Furthermore, specific binding in the contralateral side of the NTS was decreased by around 50% when compared with sham-operated controls. This reduced binding in the NTS supports the contention that, as within the area postrema, at least 50% of 5-HT_3 binding sites are associated with presynaptic vagal afferent terminals.

The virtual absence of 5-HT_3 sites after ganglionectomy, preceded by chronic hemisection of the NTS, substantiates the histological findings of Leslie et al (1982b) where vagal afferent fibres were shown to cross the mid-line bi-directionally (see below). Sectioning of these neuronal tracts thus acts to further denervate afferent terminals with a subsequent loss of presynaptic 5-HT_3 binding sites. After this treatment, the only residual binding was seen in the contralateral side of the NTS, lying subjacent to the floor of the fourth ventricle and rostral to the hemisection. Binding to the ipsilateral, side amounting only to 10% of the control, may reflect the presence of postsynaptic sites or alternatively, a lack of complete terminal degeneration after 10 days.

Although 5-HT_3 receptor antagonists, when injected into this medullary region of the ferret brain (Higgins et al, 1988), exhibit potent antiemetic activity against cytotoxic chemotherapy, it is unlikely, at least in the rat which lacks an emetic response, that such 5-HT_3 sites represent functional receptors for gastric emetic stimuli. This does not, however, preclude their involvement in the integration of other vagal sensory

information.

In contrast, the ferret, as already mentioned, readily responds to emetic stimuli but like the rat, possesses a high density of 5-HT_3 recognition sites again within the NTS (Leslie et al, 1991; Barnes et al, 1990b), which are reduced following unilateral nodose ganglionectomy and abolished as a consequence of subdiaphragmatic vagotomy (Leslie et al, 1991). Such sites within the dorsal vagal complex of this species could therefore be functionally linked to the coordination of emesis. Whole body X-irradiation produces two phases of emesis in the ferret; an initial 30 minute epoch, which is abolished both by abdominal vagotomy and by the 5-HT_3 receptor antagonist, BRL 24924. This is followed by a second, 60 minute vagally-independent phase but which is ameliorated by larger doses of BRL 24924 (Andrews & Hawthorn, 1987). These authors concluded that BRL 24924 acted at an 'extra-abdominal' site to prevent this second emetic epoch which may possibly be represented by the AP, although denervation studies that were subsequently described may also account for the possible involvement of presynaptic 5-HT_3 sites within the NTS (the present study; Leslie et al, 1991).

The sensation of nausea plus the ensuing emetic episodes that arise as a consequence of certain olfactory, gustatory or gastric sensory stimuli result from a complex sequence of both somatic and autonomic phenomena. Such stimuli are integrated in a region of the brainstem that has popularly become known as the vomiting centre, which is in essence, a rather simplistic description of a series of complex interactions between the NTS, the parvicellular reticular formation together with visceral and somatic motor nuclei (Brizzee & Mehler, 1986; Willems & Lefebvre, 1986). The somatic components of the reflex include licking and deglutition, plus contractions of the diaphragm and abdominal muscles which give rise to retching and to the actual expulsion of gastric contents. A large number of autonomic responses also accompany the act of vomiting; increased

salivation, gastric relaxation followed immediately by retro-peristaltic duodenal/pyloric spasm together with changes in respiration, heart rate and blood pressure.

Having discussed the essential characteristics of the emetic response, it would perhaps now seem pertinent to ascertain the mechanisms through which cytotoxic agents, such as cisplatin provoke emesis. Also, to gain further insight into understanding how 5-HT_3 receptor antagonists prevent emesis, a more detailed structural analysis of the dorsal vagal complex in relation to central neuroanatomical structures known to be involved in nausea and vomiting should be appreciated.

Serotonergic mechanisms in cisplatin-induced emesis

The original studies of 5-HT_3 receptor-mediated inhibition of cisplatin-induced emesis in ferrets (Miner & Sanger, 1986; Costall et al, 1987b; Higgins et al, 1988) and X-radiation (Andrews & Hawthorn, 1987) suggested that such events occur as a consequence of enhanced serotonergic function induced by certain chemotherapeutic regimes. Furthermore, inhibition of the synthesis or release of 5-HT by pretreatment with reserpine, para-chlorophenylalanine (pCPA) or fenfluramine has been shown to antagonise cisplatin-induced emesis in addition to markedly reducing 5-HT levels in the AP (Barnes et al, 1988b). These authors also reported that since cisplatin failed to increase concentrations of 5-HT or its metabolite 5-HIAA in this central region, it was more likely that the effects of cisplatin occurred at peripheral sites which function to activate tha vagal input to the AP. In support of this, 5-HT and 5-HIAA concentrations are altered in the ferret intestinal mucosa after exposure to cisplatin (Gunning et al, 1987) suggesting that cytotoxic therapy may indeed cause the release of 5-HT, presumably from mucosal enterochromaffin cells.

Thus, blockade of 5-HT_3 receptors within the gastrointestinal tract by 5-HT_3 receptor antagonists could quite feasibly mediate an antiemetic effect,

however, such sites are not in isolation since discrete injections of these antagonists into the AP itself, also inhibit cisplatin-induced emesis (Higgins et al, 1988). Surprisingly, these authors also found that the 5-HT_3 receptor agonist, 2-methyl-5-HT, at doses up to 30 micrograms, was ineffective at evoking a full emetic response (although some retching and abdominal symptoms were apparent). The hydrophilic nature of this compound may have prevented sufficiently high concentrations from fully penetrating the neurones of the AP although it was also suggested that such central 5-HT_3 sites might have a purely permissive function, allowing emesis to occur but upon activation, do not initiate the emetic reflex.

Although 5-HT_3 recognition sites are present in the AP, far higher densities are located within the NTS of the rat (the present study), ferret (Reynolds et al, 1989a; Barnes et al, 1990b; Leslie et al, 1991), human (Reynolds et al, 1989b; Barnes et al, 1990a) and rodents (Perry, 1990). It is therefore to this region that the discussion shall now be directed.

With such a plethora of vagally-mediated autonomic phenomena which are manifested as components of the emetic response, it would seem highly unlikely that such actions are integrated by a homogeneous neuronal pathway. Indeed, the NTS is known to be a heterogeneous structure for which in the cat, eight subnuclei have been described. The dorso- and ventrolateral regions (known also as the nucleus parasolitarius) receive vagal respiratory afferents whilst cardiac fibres terminate dorsolaterally (Kalia & Mesulam, 1980). Dorsomedial subdivisions of this region (the nucleus commissuralis) receive baroreceptor information from the carotid sinus and aortic depressor nerves (Davies & Kalia, 1981). With regard to this discussion, it is, however, the gastric afferent projections that warrant particular attention, which in the cat terminate in a region of the solitary nucleus described as the area subpostrema (Gwyn et al, 1979). This term was used originally by Gwyn & Wolstencroft (1968), to distinguish a dorsal

part of the NTS which had high cholinesterase activity but which was poorly demarcated by stains for Nissl granules and myelin such as cresyl fast violet and luxol fast blue. It is also synonymous with the subnucleus gelatinosus portion of the NTS described by Leslie et al (1982a).

The autoradiographical distribution of 5-HT_3 recognition sites in the rat hindbrain predominated throughout the caudal-rostral extent of the NTS, with lower binding site densities being found in the AP and the dorsal motor nucleus of the vagus. At this point, it would perhaps seem appropriate to provide a detailed description of studies in which the neuronal tracing of vagal afferents has been examined with particular reference to the cytoarchitecture of the dorsal vagal complex.

Fine microstructure of the dorsal vagal complex

The use of horseradish peroxidase (HRP), which is transported transganglionically and anterogradely to label axon terminals as well as retrogradely to locate neuronal cell bodies, has proved invaluable for the identification of the sites of neuronal origin and insertion. Following injection of HRP into the right cervical vagus nerve, heavy anterograde labelling was seen in the ipsilateral side of the NTS and the nucleus commissuralis with lighter labelling within the AP and the contralateral side of the NTS (indicating that fibres cross over at the midline) (Leslie et al, 1982b). In addition, the ipsilateral dorsal motor nucleus of the vagus received a dense retrograde labelling which was also evident after injection of HRP into the stomach wall. This latter site of administration produced a similar but finer distribution of HRP throughout the nucleus commissuralis of the NTS in levels at and caudal to the AP. However, in contrast to cervical vagus nerve injections, no labelling was seen in the lateral or more rostral regions, providing possible confirmation of the fact that these regions primarily receive respiratory and cardiac inputs.

Secondly, since the AP was free from HRP activity following labelling of the subdiaphragmatic vagus, this may provide at least one explanation as to why the rat does not elicit a vomiting reflex, at least from gastric stimuli. This observation has been subsequently confirmed by Shapiro & Miselis (1985) and Altschuler et al (1989).

Based on this evidence, those vagal afferents that *do* arise in the AP of the rat may be attributable solely to its thoracic fibres and indeed the range of monoaminergic, peptidergic and amino acid neurotransmitters that have been identified in this structure, implicates its involvement in a variety of physiological functions (Leslie, 1985 & 1986, for reviews).

A cholera toxin-HRP (CT-HRP) conjugate is highly resistant to diffusion onto structures other than those innervated from its peripheral injection site and has enabled the identification of specific subnuclei of the rat NTS labelled by oesophageal and gastric afferents. Thus, whilst injection of CT-HRP into the oesophagus resulted in heavy labelling of the subnucleus centralis, administration to the stomach wall concentrated this neuronal tracer to the subnucleus gelatinosus (although lighter, anterograde labelling was also apparent in the medial and commissural subnuclei) (Altschuler et al, 1989) and confirmed the anatomical studies of this region in the cat (Leslie et al, 1982a). These findings at the light microscopic level were substantiated by Rinaman et al (1989) who extended their studies to examine the ultrastructure of the synaptic relationship between vagal afferent terminals and their associated motoneurones. Following injection of CT-HRP into the cervical vagus nerve and the stomach wall, electron microscopical examination revealed the presence of many synaptic contacts between labelled afferent fibres and the dendrites of labelled motoneurones but which were restricted to the subnucleus gelatinosus. Such vagovagal contacts in many other subnuclei of the NTS were not obvious after labelling of the cervical nerve despite a widespread afferent labelling.

The functional significance of this gastric monosynaptic vagal circuit

in the rat remains to be elucidated. Although the gastrointestinal tract possesses its own inherent reflex circuitry, such a vagovagal pathway could function as a feedback mechanism controlling enteric reflexes influenced by central inputs to other vagal motoneurones (Grundy et al, 1986). Indeed, this pathway may provide a further explanation for the absence of a vomiting reflex in the rat.

Whether or not 5-HT_3 receptors are presynaptically located on the afferent terminals of this particular monosynaptic circuit is open to question. In support of the present lesion studies, a widespread neuronal degeneration of axodendritic synapses throughout the tractus solitarius has been demonstrated 4 days after nodose ganglionectomy or transection of the IXth. and Xth. cranial nerves (Palkovits & Zaborszky, 1977). Alternately, Blackshaw et al (1987) proposed that it is the mechanosensitive rather than chemosensitive vagal afferent input that predominates in the evocation gastrointestinal reflexes. In this situation, the monosynaptic reflex described would respond to mechanoreceptor activation whilst chemoreceptors would stimulate additional vagal gastric afferents (Rinaman et al, 1989) possibly through disynaptic interneurones within the NTS.

Since the rat does not vomit, the precise role for 5-HT_3 sites in the NTS of this species is a matter for speculation. On the one hand, these sites may simply be dormant binding sites with no functional relevance. Conversely, they may play a hitherto unidentified physiological role totally unrelated to gastric disturbance. Rats do possess a highly conditioned taste aversion response to many noxious stimuli that induce emesis in other species, and which is believed to be coordinated by the AP (Coil & Norgren, 1981). It may be that gustatory and olfactory stimuli produce a first level defence mechanism that is sufficient to prevent further ingestion. Secondary lines of defence, such as gastric chemoreceptors or a functional chemoreceptor trigger zone in the AP may have simply become redundant

so that although gastric afferents in the rat respond to copper sulphate, vomiting does not occur (Davis et al, 1986).

Nevertheless, 5-HT_3 receptors are present in the subnucleus gelatinosus of species that do respond to emetic stimuli by vomiting (Reynolds et al, 1991) and these findings will hopefully provide an additional insight into a clearer understanding of the physiology of this phenomenon.

The association of GABA receptors with central vagal terminals

Concomitant with the unilateral reduction in 5-HT_3 binding sites within the dorsal vagal complex observed following nodose ganglionectomy, the densities of both $GABA_A$ and $GABA_B$ sites were similarly reduced in this region as a consequence of vagal lesioning. Again, the implications of these findings is that these sites are also located presynaptically. Although $GABA_A$ sites predominate in the dorsal motor vagal nucleus, this does not necessarily preclude their presence in the NTS. Compared with $GABA_B$ receptor autoradiography, the demarcation of $GABA_A$ sites in this latter region was not sufficiently clear to permit accurate quantitation. However, since $GABA_A$ sites in the dorsal vagal motor nucleus are reduced after vagal lesioning, it would appear that neurones of the vagus nerve that terminate in this nucleus form direct synaptic contact with efferent projections, thereby by-passing the NTS. To this end, HRP studies have revealed the existence of such a monosynaptic pathway within the dorsal vagal motor nucleus of the rat (Neuhuber & Sandoz, 1986).

Whilst there is a relative paucity of published reports regarding possible functional roles for $GABA_A$ and $GABA_B$ receptors specifically within the NTS/dorsal vagal complex, there is an increasing amount of evidence that associates both GABA receptor subtypes with vagally-dependent autonomic responses in the gastrointestinal and cardiovascular

systems. $GABA_B$ receptors have also been implicated in medullary respiratory control and these collective findings shall now be briefly reviewed.

Gastrointestinal responses to central GABAergic receptor activation

Both the $GABA_A$ receptor agonist, muscimol, as well as the $GABA_B$ receptor agonist, baclofen, stimulate gastric acid secretion in the rat following intravenous administration (Goto et al, 1985; Andrews & Wood, 1986; Hara et al, 1990a, 1990b) Baclofen also enhances gastric motility through a centrally-mediated action (Andrews & Wood, 1986; Fargeas et al, 1988).

Pharmacological specificity with regard to the findings of Hara et al (1990a) is open to interpretation since neither the selective $GABA_A$ and $GABA_B$ receptor antagonists, bicuculline and phaclofen, inhibited the secretagogue actions of muscimol and baclofen. In contrast, a structural analogue of GABA, 3-amino-3-phenylpropionic acid (3A3PPA), non-selectively blocked the actions of these GABA-mimetics, possibly by a novel category of GABA receptor. Moreover, since this compound failed to influence gastric secretion evoked by electrical vagal stimulation (in contrast to the effect of atropine), these authors suggested that it was likely to act at central receptor sites (Hara et al, 1990b).

These findings corroborate those of Andrews & Wood (1986) in that $GABA_B$ receptor-induced gastric acid secretion was abolished by bilateral cervical or abdominal vagotomy. Moreover, denervation in addition to pretreatment with atropine, also suppressed baclofen-enhanced gastric motility. From the antagonistic action of atropine, it would appear that the vagus nerve functions to innervate intramural excitatory muscarinic receptors. The precise neuronal circuitry that couples the central gastrokinetic and secretory activity of baclofen with its peripheral effect is at present, unclear. Whilst electrical stimulation of the vagus enhances

gastric motility (Pagani et al, 1985), since this effect is absent in decerebrate animals (Andrews & Wood, 1986), it is likely that a more rostral central location, such as the hypothalamus, probably provides a more relevant site of action (Grijalva et al, 1980).

The precise role of $GABA_A$ and $GABA_B$ receptors within the dorsal motor vagal complex with respect to the gastrointestinal system still remains to be elucidated. Their presynaptic location could signify an involvement with integrative feedback control mechanisms between the periphery and central modulatory sites. Such sites may be represented by muscarinic receptors since i.c.v. injections of atropine antagonises baclofen-stimulated duodenal activity (Fargeas et al, 1988). These authors also found that $GABA_A$ receptor activation by muscimol has an opposite action to the $GABA_B$ receptor-mediated response, in that intestinal motility is inhibited. This may be a consequence of increased vagal outflow through the nucleus ambiguus (Williford et al, 1981) although $GABA_A$ sites within this region were not obvious from the present autoradiographical study. It is perhaps intriguing that the centrally-mediated effects of GABA receptors on the gastrointestinal system appear to oppose those influenced by these receptors on myenteric neurones. Activation of $GABA_A$ receptors causes a cholinergically-mediated contraction of the guinea-pig ileum whereas baclofen depresses electrically evoked twitches (Kaplita et al, 1981; Giotti et al, 1983; Ong & Kerr, 1983; also Chapter 1, for extended discussion). It is therefore possible that centrally and peripherally $GABA_A$ and $GABA_B$ receptor-mediated actions function reciprocally through their respective receptors in the dorsal vagal complex. This is, of course, speculative and such a proposal would require further experimentation. Direct experimental evidence does exist however, implicating $GABA_A$ and $GABA_B$ receptors located in the NTS in the regulation of cardiovascular responses.

Cardiovascular responses to central GABAergic receptor activation

It is now well-established that proximal primary afferent baroreceptor neurones project from their cell bodies within the nodose ganglion. These fibres enter the medulla oblongata through the nucleus and the tractus of the spinal trigeminal nerve, finally terminating in the NTS at a level caudal to the obex (Palkovits & Zaborszky, 1977; Kalia & Mesulam, 1980). Bilateral transection of the brainstem in a position just lateral to the NTS disinhibits central control resulting in a hypertensive response (De Jong & Palkovits, 1975).

With regard to the role of GABA in the homeostatic maintenance of arterial blood pressure, following bilateral microinjection of the GABA uptake inhibitor, nipecotic acid, into the NTS of the rat, blood pressure was elevated (Catelli et al, 1987). With a view to identifying the receptor mechanisms through which this effect was mediated, Catelli & Sved (1988) found that the pressor responses elicited by nipecotic acid and (-)-baclofen in spontaneously hypertensive rats (SHR) were significantly higher than in normotensive controls. Conversely, there was no difference between the two species with respect to the enhancement of arterial pressure elicited by muscimol. The greater pressor enhancing actions of these GABA-mimetics in the SHR may be due to the increased densities of [^{3}H]baclofen binding sites in the NTS (Singh & Ticku, 1985). However, the lower pressor response mediated by i.c.v. injection of baclofen into the SHR, suggests that this agonist may have diffused to a site other than the NTS (Catelli & Sved, 1987).

Although bicuculline was ineffective at blocking the pressor response to nipecotic acid (Sved & Sved, 1989), the antagonistic action of phaclofen on the effects of this GABA uptake inhibitor as well as the agonist action of (-)-baclofen, signified the involvement of $GABA_B$ receptors, thus consolidating the physiological significance of their findings.

A further role for $GABA_A$ receptors in maintaining baseline arterial

pressure has been inferred by McWilliam & Shepheard (1988). These authors observed that iontophoretically applied GABA reduced the responsiveness of neurones located within the NTS after electrical stimulation of the carotid sinus nerve. Since bicuculline increased evoked activity it would appear that these neurones were subject to a degree of tonic inhibition by endogenous GABA.

Functional roles for both $GABA_A$ and $GABA_B$ receptors in the NTS are therefore clearly evident, however, it is unlikely that activation of these receptors is *directly* responsible for the pressor and depressor effects described. Since the vasopressin antagonist, [1-β-β-cyclapentamethylene-propionic acid 2(O-methyl)-tyrosine] arginine vasopressin, blocked the pressor response to both muscimol and (-)-baclofen (Catelli et al, 1987; Sved & Sved, 1988), this would indicate that GABAergic receptors in the NTS may have an integrative function to effect the release of vasopressin from the pituitary gland via the supraoptic nucleus of the hypothalamus. To this end, monosynaptic pathways from the NTS to the hypothalamus (Ricardo & Koh, 1978) and afferent projections from the lateral and paraventricular hypothalamus back to the NTS (Ross et al, 1981) add neuroanatomical support to this hypothesis.

One point to consider further concerns the vasopressor effects induced by microinjection of muscimol and baclofen into the NTS. These cardiovascular actions are likely to be site specific effects since they are not mimicked intracerebroventricularly, in fact, hypotensive and bradycardic responses are the usual consequences of i.c.v. administration of muscimol, isoguvacine (Bousquet et al, 1984) and baclofen (Bousquet et al, 1981). In this latter report, the hypotensive action of baclofen was prevented by glutamic acid (as well as by its analogue, kainic acid), which also possesses a vasopressive influence of its own. Moreover, in the presence of bicuculline and kainic acid, the hypotensive actions of muscimol are more potently inhibited than by bicuculline alone. Conversely, the action of isoguvacine,

whilst unaffected by bicuculline, was blocked by the mixture, an effect attributable therefore, to kainic acid (Bousquet et al, 1984). These findings suggested that hypotension arises as a consequence of the stimulation of both $GABA_A$ and $GABA_B$ receptors, probably by the inhibition of glutamate release. But would such a mechanism account for the hypertension induced by GABA-mimetics in the NTS? In contrast to the vasopressor effects of glutamic acid injected into the lateral ventricle (Bousquet et al, 1981), its administration directly into the NTS, leads to hypotension and bradycardia. Thus it follows that baclofen may elicit the opposite effect to this, again by the inhibition of glutamate release.

Recent evidence from neurochemical and lesioning studies substantiates a functional relationship between GABAergic and glutamatergic neurones within the NTS. Micropunches of brainstem incorporating the NTS, contain high concentrations of GABA and glutamic acid. Potassium-stimulated release of these amino acids from superfused micropunches is Ca^{2+}-dependent and significantly reduced following nodose ganglionectomy (Meeley et al, 1989). It is, however, unlikely that the released GABA originates from the cell bodies of primary afferent fibres in the nodose ganglion, since compared with glutamate concentrations in this region, levels of GABA are negligible (Meeley et al, 1989). These authors proposed that GABAergic neurones in the NTS are innervated by glutamatergic afferents and that the deafferentation of this excitatory input leads to a decreased GABA release from degenerated GABAergic cells.

Central respiratory control by $GABA_B$ receptors

Afferent innervation of the dorsal and ventrolateral regions of the NTS by respiratory vagal fibres (Kalia & Mesulam, 1980) has previously been indicated in this discussion. Microelectrophoretically-administered baclofen to the ventrolateral subdivision of the NTS depresses the firing of medullary inspiratory neurones in artificially ventilated anaesthetised cats

(Lalley, 1986) whilst the synchronised neuronal firing in phase with the ventilator pump, was abolished by vagotomy or by switching off the pump. Centrally-mediated inspiratory events can be monitored by the measurement of phrenic nerve activity for which a functional role of $GABA_B$ receptors in central respiratory control has been demonstrated Schmid et al, 1989). Intracerebroventricular administration of baclofen decreases the duration of inspiration whilst increasing the duration of expiration. In contrast to the effects of this agonist, the $GABA_B$ receptor antagonist, phaclofen, increases phrenic nerve activity presumably via facilitatory disinhibition of the inherent tone. Respiratory depression, hypotension and bradycardia induced by comparatively high doses of baclofen, are also blocked by phaclofen. These findings were not directly attributed to actions within the NTS and are more likely to be mediated by postsynaptic receptors on respiratory neurones. Since $GABA_B$ receptors hyperpolarise serotonergic neurones in the dorsal raphe (Innis et al, 1988), these effects may be mediated from this region. Indeed, such a site may explain the fact that low doses of baclofen administered intravenously (in contrast to i.c.v. injection) increase both the firing of ventrolateral neurones in the NTS and also phrenic nerve activity (Lalley, 1986).

In summary, chronic unilateral lesioning of the vagus nerve has been shown to reduce the densities of 5-HT_3, $GABA_B$ and $GABA_A$ binding sites on vagal terminals that synapse within the dorsal vagal complex, indicative of their presynaptic location on these afferent fibres. However, the fact that around 25% of the three binding site categories remained unaffected ipsilaterally following denervation, should not be overlooked. This could be attributed to an incomplete terminal degeneration or may reflect the possible existence of postsynaptic sites. Nonetheless, these centrally-located sites may be functionally relevant in the integration of many of the responses to autonomic activation described.

CHAPTER 5

AUTORADIOGRAPHICAL ANALYSIS OF $GABA_B$ RECEPTORS IN RAT FRONTAL CORTEX FOLLOWING REPEATED ANTIDEPRESSANT ADMINISTRATION

INTRODUCTION

Adaptations to the populations of central cortical monoamine receptors of rodents following chronic antidepressant administration are now well-documented; down-regulation of the number of both beta-adrenoceptors (Banerjee et al, 1977) and 5-HT_2 receptors (Peroutka & Snyder, 1980) having been consistently found with a variety of antidepressant classes.

Recently, however, focus has turned to the GABAergic system following the observations that levels of GABA are reduced in the cerebrospinal fluid (Gold et al, 1980) and the plasma (Berrettini et al, 1982) of depressed patients. This evidence, together with the fact that the GABA agonists, progabide and fengabine have antidepressant actions in animal models as well as clinically (Lloyd et al, 1983; Musch & Garreau, 1986) has directed attention to GABA receptors and in particular to the possible alterations in the $GABA_B$ receptor subtype following chronic antidepressant administration. This stemmed from the observations of Lloyd et al (1985) who demonstrated increased $GABA_B$ receptor binding to rat cerebral cortical membranes following 18-day subcutaneous infusions of a variety of antidepressants. These effects have been substantiated using intraperitoneal administration (Szekely et al, 1987) and also in mouse cortical membranes (Suzdak & Gianutsos, 1986) who also reported that the potency of NA-stimulated cAMP production by the $GABA_B$ receptor agonist, baclofen, was enhanced by chronic imipramine suggesting an increased functionality of $GABA_B$ receptors.

Not all researchers, however, have been able to demonstrate $GABA_B$ receptor up-regulation after such dosing regimes. Cross & Horton (1988) failed to observe any significant changes in $GABA_B$ binding sites following twice daily oral administration of desipramine or zimeldine for 21 days despite the fact that 5-HT_2 binding site densities were significantly reduced

in the same membrane preparations. Furthermore, it is interesting that although Szekely et al (1987) reported a significant increase in [^{3}H]GABA binding to $GABA_B$ sites in rat frontal cortical membranes after prolonged treatment with desipramine and imipramine, the binding of [^{3}H]baclofen was unaffected by these drugs. A more recent report demonstrated that whilst the motor-suppressant effects of the beta-adrenoceptor agonist, salbutamol, were attenuated by a number of antidepressants (including desipramine), such treatment regimes failed to alter the motor-impairment induced by the GABA agonists, progabide and (±)-baclofen (McManus & Greenshaw, 1991). Although these findings indicate that the beta-adrenergic and GABAergic systems do not interact in such behavioural paradigms, this should not necessarily preclude their mutual involvement in the neurochemistry of depression.

A summary of the findings from published investigations into the effects of chronic antidepressant administration on $GABA_B$ receptors is provided in Table 5.0. With the exception of one study (Suzdak & Gianutsos, 1986), membranes of frontal cerebral cortex provided the tissue substrate in receptor binding assays. It was hypothesised that if indeed $GABA_B$ receptor densities are increased following repeated antidepressant administration, it could be that certain undetected changes may arise from a discrete localisation within certain cerebral cortical laminae which would be considerably diluted in a membrane preparation. The aim of the following investigations, therefore, was to employ receptor autoradiography in an attempt to resolve these discrepancies. Since this technique offers the advantage of retaining the morphology of the brain intact, such an approach should enable the detection of possible changes within individual laminae of the frontal cortex.

It is anticipated that by presenting data from four studies, separately, a logical progression of experimental hypothesising will be appreciated. In all studies, autoradiographical density measurements were restricted to the

frontal cortex of rat brain which was taken to approximately represent the anterior one-third region of the cerebral cortex (as described by plate 82, (Paxinos & Watson, 1986).

Table 5.0 **Summary of published studies examining the effects of protracted antidepressant treatments on $GABA_B$ receptors in brain membranes**

REFERENCE	DRUGS	DOSE mg kg^{-1}	DURATION	DRUG-FREE PERIOD	REGION/ SPECIES	RADIOLIGAND CONDITIONS	RESULTS re: $GABA_B$ Bmax	ADDITIONAL FINDINGS
Pilc & Lloyd (1984)	Amitriptyline Desipramine Citalopram Viloxazine Pargyline	10 s.c. 5 s.c. 10 s.c. 10 s.c. 20 s.c.	18 days mini-pumps	24 hrs	Frontal Cortex Wistar Rat	[^{3}H] GABA (8-160nM) saturation	+ 63% ** + 39% * + 52% ** + 66% ** + 57% *	Only viloxazine increased $GABA_B$ binding acutely (10nM). No chronic effects on $GABA_A$ binding, GABA levels or GAD activity.
Lloyd *et al.* (1985)	Nomifensine Desipramine Desipramine Zimeldine	5 s.c. 1.25 s.c. 5 s.c. 10 s.c.	18 days mini-pumps	72 hrs	Frontal Cortex Wistar Rat	[^{3}H] GABA (10nM) 1-750µM GABA displacement	+ 16% + 41% - 3% [a] + 34% [a]	In frontal cortex DMI, MAP, VILOX, CITAL, ZIMEL, NOMI, AMI, PARG, TRAZ, MIAN (s.c.), ECS; FLUOX, PROG, FENG, VAL (i.p.) produced increases in $GABA_B$ binding of between 27% and 88%. In hippocampus DMI, VILOX and CITAL significantly increased $GABA_B$ binding.
Suzdak & Gianutsos (1986)	Baclofen Imipramine	10 i.p. 32 i.p.	14 days	24 hrs	Whole Cortex Mouse (CD1)	[^{3}H] GABA (1-1000nM) saturation	-16% *[b] -17% *[c] +24% *[b] +15% *[c]	Chronic baclofen reduced the baclofen-induced potentiation of NA-stimulated adenylyl cyclase whereas imipramine augmented the potentiation.

Szekely *et al.* (1987)	Desipramine Imipramine Maprotiline	10 i.p. 7.5 i.p. 10 i.p.	21 days twice daily	48 hrs	Frontal Cortex Sprague-Dawley Rat	[^{3}H] GABA (5-400nM) [^{3}H] (-)bac (5-150nM) saturation	[^{3}H]GABA [^{3}H](-)bac +28% ** no +24% ** change -6%	Antidepressants failed to effect the potency of (-)baclofen to inhibit forskolin stimulated adenylyl cyclase in membrane studies.
Cross & Horton (1988)	Desipramine Zimeldine Desipramine Zimeldine	1.25 & 5 p.o. 1.25 & 5 p.o. 5 & 10 p.o. 5 & 10 p.o.	21 days twice daily	24 hrs	Whole Cortex Frontal Cortex Wistar Rat	[^{3}H] GABA (1nM) 5-150nM GABA displacement	no change " " " " " "	DMI (5 & 10 mgkg^{-1} p.o.) and ZIMEL (10 mgkg^{-1} p.o.) significantly reduced 5-HT$_2$ binding sites in frontal cortex but not hippocampus.
Cross *et al.* (1988)	Post-mortem depressed suicide victims	—	—	—	Frontal/Temporal Cortex Human	[^{3}H] GABA (1nM) 5-300nM GABA displacement	no change in KD or Bmax of drug-free or AD-treated suicide victims	Kinetic parameters were also unaffected in hippo-campal membranes
Motohashi *et al.* (1989)	Lithium chloride Carbamazepine	1.5 mEqkg^{-1} 50 i.p.	14 days	24 hrs	Hippocampus Wistar Rat	[^{3}H](-)bac (10-80nM) saturation	+52% * +38% *	No changes in GABA$_B$ binding in frontal cortex or [^{3}H]muscimol binding in either region

a changes refer to the high affinity components of a curvilinear Scatchard plot.
b high affinity binding site
c low affinity binding site
* P<0.05 ** P<0.01 Student's "t" test

Abbreviations

DMI (desipramine); MAP (maprotiline); VILOX (viloxazine); CITAL (citalopram); ZIMEL (zimeldine); NOMI (nomifensine); AMI (amitriptyline; PARG (pargyline), TRAZ (trazodon
MIAN (mianserin); ECS (electroconvulsive shock); FLUOX (fluoxetine); PROG (progabide); FENG (fengabine); VAL (sodium valproate); NA (noradrenaline); GAD (glutamic ac
decarboxylase); [^{3}H](-)bac ([^{3}H](-)baclofen); AD (antidepressant).

METHODS **Drug-treatment protocols and $GABA_B$ receptor binding conditions**

First investigation

Male Wistar rats (160-200g) were dosed via oral gavage, once daily for 18 days (between 9.00 am and 11.00 am) with either imipramine ($10mgkg^{-1}$), mianserin ($10mgkg^{-1}$) or paroxetine ($5mgkg^{-1}$). Control animals received an equivalent volume of vehicle (distilled water). Following a 24 hour drug-free period, animals were prepared for perfusion-fixation as described in Chapter 2. The effects of these treatments on $GABA_B$ receptors were determined at a single concentration of [^{3}H]GABA (50nM).

Second investigation

Male Wistar rats (160-200g) were anaesthetised with halothane, incisions made through the skin on the back into which Alzet 2002 minipumps (Alza Corporation, USA) were subcutaneously implanted and the incisions closed with Michel clips. The minipumps were filled with 200µl of a concentration of imipramine sufficient to deliver a proposed daily infused dose of $10mgkg^{-1}$ for 14 days. Following the removal of the minipumps under halothane anaesthesia, animals were separated into two groups to compare drug-free periods of 24 and 48 hours. A second group of rats was injected intraperitoneally for 14 days with imipramine ($5mgkg^{-1}$) in order to compare this route of administration with that of subcutaneous infusion. These animals were prepared for perfusion-fixation after a further 24 hours and were subjected to halothane anaesthesia to control for its possible influence on the action of imipramine.

For this study, the binding kinetic parameters B_{max} and K_D were assessed using non-linear regression analysis (as described in Chapter 2) using a range of GABA concentrations at a fixed concentration of [^{3}H]GABA (50nM).

Third investigation

Male CFY rats (140-180g; Interfauna) caged in groups of 5 animals were dosed via the drinking water for 21 days with the aim of achieving a daily intake of $30mgkg^{-1}$ (amitriptyline), $20mgkg^{-1}$ (desipramine) and $10mgkg^{-1}$ (paroxetine). Water intake and body weight gain were monitored every two days and the drug concentrations dissolved in the drinking water adjusted accordingly.

In this study, the effects of antidepressant treatment on $GABA_B$ receptor binding parameters were determined using saturation analysis by varying the concentration of [^{3}H]GABA (25-400nM; 5 concentrations). In addition, binding to beta-adrenoceptors, using the ligand (-)-[^{125}I]iodopindolol (37.5-600pM; 5 concentrations) and resolved $beta_1$-adrenoceptors (in the presence of the $beta_2$-adrenoceptor antagonist, ICI 118551) was performed on adjacent sections.

This investigation formed part of a collaborative venture with Dr. D.R. Nelson and Mr. A.M. Johnson of SmithKline Beecham Pharmaceuticals, Harlow, Essex.

Fourth investigation

Male CFY rats (210-250g; Interfauna) were dosed for 21 days with the antidepressants described in the previous investigation as well as with (±)-baclofen ($10mgkg^{-1}$ p.o.). In contrast, however, drugs were administered by oral gavage instead of via the drinking water. Additionally, desipramine ($10mgkg^{-1}$ i.p.) and the $GABA_B$ receptor antagonist, CGP 35348 were injected in parallel with a third drug, (both at a concentration of $100mgkg^{-1}$ i.p.), which, in respect of a confidentiality agreement, will be referred to as Compound X. This compound may be another $GABA_B$ receptor antagonist.

The effects of all of these compounds on both $GABA_B$ receptor binding and beta-adrenoceptor binding were examined in addition to the $GABA_B$ receptor-mediated modulation of forskolin and noradrenaline-

stimulated adenylyl cyclase activity by baclofen (these latter data will be discussed in Chapter 6).

In all of the investigations, the calculations of drug concentrations were expressed in terms of their salt:base ratios.

RESULTS

First investigation

The effects of the chronic administration for 14 days of imipramine, mianserin and paroxetine on the binding of [^{3}H]GABA to $GABA_B$ receptors within the laminal components of the frontal cortex are summarised in Table 5.1. Clearly, $GABA_B$ binding sites are not homogeneously distributed throughout the frontal cortex; the highest density is found in lamina II and III with a progressively lower densities being observed in laminae I, V and VI.

None of the antidepressants administered in this study, significantly affected the binding of [^{3}H]GABA (at a concentration of 50nM) in any of the four laminal regions. Although it should be feasible to detect a possible antidepressant-induced alteration in binding density using a single radioligand concentration, it would, however be impossible to ascertain whether or not such a change was mediated by the modulation of either receptor number, receptor affinity, or both. The terms, B_{max} and K_D, which respectively denote these receptor binding parameters, are derived experimentally by varying the concentrations of either the radioligand or the non-radioactive displacing ligand. Such an approach was therefore implemented in the proceding studies.

Second investigation

Table 5.2 summarises the effects of imipramine ($10mgkg^{-1}$)

administered for 14 days either intraperitoneally or by continuous infusion from sub-cutaneously implanted osmotic minipumps on [^{3}H]GABA binding to $GABA_B$ receptors in rat frontal cortex. The heterogeneous distribution of $GABA_B$ sites within the frontal cortex is again evident from B_{max} values; the highest density (264.2 $\pm$ 26.8 fmol/mg tissue) being associated with laminae II and III.

Following a 24 hour (but not 48 hour) drug-free period, subcutaneous infusion of imipramine produced an apparent increase in $GABA_B$ binding sites in lamina I amounting to 28% above control (Figure 5.0). However, statistical comparisons (Student's 't' test; unpaired, 2-tailed) were unable to demonstrate that this change was significant (P = 0.074). This trend of increased $GABA_B$ binding was also apparent following intraperitoneal injections of imipramine ($5mgkg^{-1}$) where a non-significant increase of 35% was observed. Such changes were reflected to a lesser extent in laminae II and III whereas receptor affinity (K_D) was unaffected. Upon closer inspection of this displacement (Figure 5.0A), the apparent increase in $GABA_B$ binding sites was evident only in the absence of GABA (total binding) and at the lower concentrations (20, 50 & 250nM). The amount of [^{3}H]GABA displaced by higher concentrations of unlabelled GABA (950 & 3000nM) was in close proximity to the non-specific binding (defined by 100µM GABA). A Hill plot (Figure 5.0B) incorporating the data points for the three lower concentrations in control tissue, gave a Hill coefficient (n_H) equal to 0.946, indicative of a single binding site with an apparent K_D of 33nM.

The determination of B_{max} and K_D values using non-linear regression analysis involving the transformation of competitive binding data is an indirect method of derivation which therefore can be expected to increase experimental variation and indeed, this was evident from the relatively large standard errors. For these reasons, it is generally considered that greater accuracy is achieved using saturation analysis over a range of

radioligand concentrations which directly yields the kinetic binding parameters. Such an approach has been employed in the proceding study, where in an attempt to achieve more consistent plasma drug levels than would be expected following once or twice daily injections, drugs were administered chronically via the drinking water. It was anticipated that such a dosing regime would allow the observation of similar $GABA_B$ receptor up-regulations to those reported by Lloyd et al (1985) following subcutaneous infusion.

Third investigation

In this study, drugs were administered via the drinking water and the effects of such treatments on water intake and bodyweight gain were monitored every second day. The mean volume of water consumed/day/rat was significantly reduced ($P<0.05$; Student's 't' test) by 19%, 31% and 55% in paroxetine-, amitriptyline- and desipramine-treated animals, respectively (Figure 5.1A). On the basis of the amount of water consumed by each treatment group/day, the actual mean ($\pm$ s.e.m) daily doses received were 26.2 $\pm$ 0.2 $mgkg^{-1}$ (amitriptyline), 17.1 $\pm$ 0.3 $mgkg^{-1}$ (desipramine) and 8.3 $\pm$ 0.1 $mgkg^{-1}$ (paroxetine). Despite a reduction in water intake, bodyweight increases of both amitriptyline- and paroxetine-treated animals were not significantly different from control values. By contrast, however, the weight of rats receiving desipramine was significantly lower than controls from day 5 although the rate of weight gain was similar after week 1 (Figure 5.1B).

a) $GABA_B$ receptor-modulation by antidepressants

The effects of amitriptyline, desipramine and paroxetine on the binding of [^{3}H]GABA to $GABA_B$ sites in the frontal cortex are visualised autoradiographically in Figure 5.2 whilst the kinetic parameters of this binding, derived from Scatchard analysis of saturable binding to the various

frontal cortical laminae are summarised in Table 5.3. By comparing the B_{max} values of $GABA_B$ binding in lamina I of control and antidepressant-treated rats (Figure 5.3), it was apparent that these sites were up-regulated by each of the three treatments, however, only the desipramine-induced increase of 32% above control, reached significance ($P<0.05$). The affinity of the ligand for its receptor was unaffected by these treatments with the exception of desipramine, which in lamina I, produced a significant increase in K_D of 47% ($P<0.05$; Student's 't' test).

In laminae II and III (possessing the highest density of $GABA_B$ sites, desipramine and paroxetine produced smaller but non-significant increases in B_{max} values whereas amitriptyline was without effect.

b) Beta-adrenoceptor modulation by antidepressants

The effects of amitriptyline, desipramine and paroxetine on the binding of (-)-[^{125}I]iodopindolol to both total ($beta_1$ and $beta_2$) and resolved $beta_1$-adrenoceptors are demonstrated in the autoradiograms of Figure 5.4. No apparent reduction in binding was produced by either amitriptyline or paroxetine in the frontal cortex, however, chronic treatment with desipramine produced a clear down-regulation of both total and $beta_1$-adrenoceptor binding in all laminae of this region. This is further exemplified by the representative Scatchard plots of beta-adrenoceptor binding to lamina I (Figure 5.5). The magnitude of the desipramine-induced down-regulation, determined by the comparison of B_{max} values, amounted to 25% below control in analyses of both total and $beta_1$ sites. These changes were highly significant ($P<0.01$) and occurred to the same extent in laminae II-III and V-VI. Although $beta_1$-adrenoceptor binding appeared to be reduced in all laminae, this decrease only reached significance in laminae V-VI.

Neither amitriptyline nor paroxetine reduced the density of beta-adrenoceptors, indeed with the exception of amitriptyline in laminae V-VI,

they produced small but non-significant increases in total and $beta_1$-adrenoceptor binding. No change in the affinity of the ligand for either of the sites was evident (Table 5.4).

Fourth investigation

The aim of this study was firstly to confirm the findings described in the preceding section, however, instead of administering the antidepressants dissolved in the drinking water, animals were dosed once daily via oral gavage.

a) Drug-induced modulation of $GABA_B$ receptor binding

Since this study involved drug administration by both oral and intraperitoneal routes, control values for [^{3}H]GABA binding to $GABA_B$ sites were derived from the mean of six animals; three dosed orally and three dosed intraperitoneally, there being no significant difference between these two groups.

The kinetic binding parameters B_{max} and K_D were derived by Scatchard analysis of the binding of four concentrations of [^{3}H]GABA (300, 150, 75 and 37.5nM) and these data are summarised in Table 5.5. It is first worth noting the close reproducibility of the control B_{max} and K_D values throughout the frontal cortical laminae in this study as compared to those of the previous investigation.

Chronic treatment with desipramine for 21 days significantly increased the B_{max} of $GABA_B$ binding sites in lamina I by 34%. By contrast, amitriptyline and paroxetine (as well as the $GABA_B$ receptor agonist, (±)baclofen ($10mgkg^{-1}$)), following a similar dosing regime, appeared to increase the B_{max} of $GABA_B$ binding (although to a lesser extent to that observed with desipramine), the values were not significantly different from control, confirming the findings of the previous study.

In parallel with oral administration, intraperitoneal injection of

desipramine (10mgkg^{-1}) also up-regulated $GABA_B$ binding sites in lamina I by 49% above control whilst the $GABA_B$ receptor antagonist, CGP 35348, (100mgkg^{-1}), was ineffective at increasing $GABA_B$ receptor numbers under the conditions employed in this experiment, prolonged administration of Compound X, at an equivalent dose, mediated a significant enhancement (+55%) of $GABA_B$ binding in lamina I, comparable to that produced by desipramine.

It is interesting that in both this, and the preceding study, significant changes in the number of $GABA_B$ receptors within the frontal cortex, induced by either desipramine or Compound X, were restricted to the outer lamina I. Whilst smaller increases in B_{max} values were observed in laminae II and III, these changes failed to reach significance whereas in the deeper laminae (V and VI) of the cortex, such changes, if observed at all, were only marginal.

With the exception of Compound X, none of these treatments significantly altered the affinity of [^{3}H]GABA for $GABA_B$ sites in any of the frontal cortical laminae studied. It is perhaps of interest, that as observed in the previous study, chronic oral administration of desipramine increased (albeit non-significantly) the K_D in lamina I by 52%. A greater significant reduction in receptor affinity was produced by Compound X, denoted by an increased K_D of 89% above control. Thus, it would appear that the ability of this compound to markedly up-regulate $GABA_B$ receptors possibly occurs at the expense of a reduction in the affinity of the endogenous ligand for its receptor. The effects of these chronic treatments are shown in the colour-coded images of Figure 5.6 as well as graphically by Scatchard plots (Figure 5.7).

b) Drug-induced modulation of beta-adrenoceptor binding

Figure 5.8 shows the effects of the repeated administration of antidepressants, CGP 35348 and the putative $GABA_B$ receptor antagonist,

Compound X, on the beta-adrenoceptor population of the frontal cortex assessed autoradiographically. The binding of (-)-[^{125}I]iodopindolol was examined over 5 radioligand concentrations (300, 150, 75, 37.5, 18.75pM) and the amount bound at each concentration was transformed into Scatchard plots of total and beta$_1$-adrenoceptor binding (Figures 5.9 and 5.10) from which the kinetic binding parameters B_{max} and K_D were derived (summarised in Table 5.6).

The desipramine-induced beta-adrenoceptor down-regulation observed in the former study was further substantiated in this study where binding was reduced from around 25-31% in all laminae of the frontal cortex. As observed previously, paroxetine failed to influence the beta-adrenoceptor population. Conversely, like desipramine, amitriptyline also significantly down-regulated beta-adrenoceptors throughout the frontal cortex by between 18% and 27%, which is in marked contrast to the lack of effect of this drug seen earlier. Similarly, oral administration of the GABA$_B$ receptor agonist, baclofen, also produced a beta-adrenoceptor down-regulation of a similar magnitude to that produced by amitriptyline.

Resolution into the beta$_1$-adrenoceptor subtype, revealed that with the exception of paroxetine, the observed drug-induced modifications described, were attributable to this subsite in all frontal cortical laminae. Moreover, with the exception of desipramine (i.p.) in laminae II and III, no significant changes in receptor affinity were observed.

Concomitant with the effect of oral administration, when injected intraperitoneally, desipramine produced large general significant reductions of around 50% in both total and resolved beta$_1$-adrenoceptors. Both the GABA$_B$ receptor antagonist, CGP 35348, and Compound X, mediated a down-regulation of the total beta-adrenoceptor population to a similar degree as observed with amitriptyline. However, the resolved beta$_1$-adrenoceptor subtype was not significantly affected by either treatments which may implicate the preferential involvement of beta$_2$-adrenoceptors in

this response.

Furthermore, both $GABA_B$ receptor antagonists and desipramine significantly increased the affinity of the total beta-adrenoceptor population (with the exception of CGP 35348 in laminae II and III) for the ligand, (-)-[^{125}I]iodopindolol, as exemplified by reduced K_D values of between 14% and 40%. In contrast, the affinities of resolved $beta_1$-adrenoceptors were unaffected by such treatments (with the exception of desipramine in laminae II and III). By ranking the individual B_{max} values for $GABA_B$ receptors in desipramine- and Compound X-treated rats with the corresponding B_{max} values for beta-adrenoceptors, no positive correlation was apparent (Spearman's rank correlation coefficient, $r_S = 0.13$; $P>>0.05$).

Table 5.1 **The effects of imipramine, mianserin and paroxetine administered orally for 14 days on [^{3}H]GABA binding (50nM) to $GABA_B$ receptors in rat frontal cortex (First Investigation)**

TREATMENT	DOSE $mgkg^{-1}$	n	Bound (fmol/mg tissue)			
			Lamina I	Laminae II-III	Lamina V	Lamina VI
Control		5	29.2 ± 3.0	39.1 ± 3.2	19.1 ± 2.0	8.3 ± 1.4
Imipramine	10 p.o.	4	27.7 ± 3.8	34.0 ± 4.1	19.9 ± 2.9	8.8 ± 1.1
Mianserin	10 p.o.	4	21.5 ± 4.2	31.2 ± 4.0	14.5 ± 2.4	6.6 ± 1.1
Paroxetine	5 p.o.	4	22.9 ± 4.1	33.2 ± 4.5	16.5 ± 3.6	10.8 ± 3.0

Figure 5.0

Second Investigation. (A) Histogram showing the effects of imipramine on $GABA_B$ receptor binding in the rat frontal cortex (lamina I). Imipramine was administered for 14 days either intraperitoneally ($5mgkg^{-1}$; cross-hatched bars) or by continuous infusion from sub-cutaneously implanted 'Alzet 2002' osmotic minipumps, calibrated to deliver a daily dose of $10mgkg^{-1}$. For this method of administration two drug free periods of 24 hours (filled bars) and 48 hours (horizontal bars) were compared. Open bars represent control animals. The kinetic binding parameters, K_D and B_{max}, were assessed using non-linear regression analysis as described in Chapter 2 using a range of GABA concentrations (20, 50, 250, 950 and 3000nM) at a fixed concentration of [^{3}H]GABA (50nM). Non-specific binding was defined in the presence of 100μM GABA).

(B) A Hill plot of the binding of [^{3}H]GABA (50nM) to lamina I of the rat frontal cortex, based on the data points for the three lower non-radioactive GABA concentrations (●) gave a Hill coefficient of 0.946. This was indicative of a single binding site with an apparent K_D of 33nM. Since the data points for the two higher GABA cencentrations (950 and 3000nM; O) were in close proximity to non-specific binding, they were not incorporated into the plot.

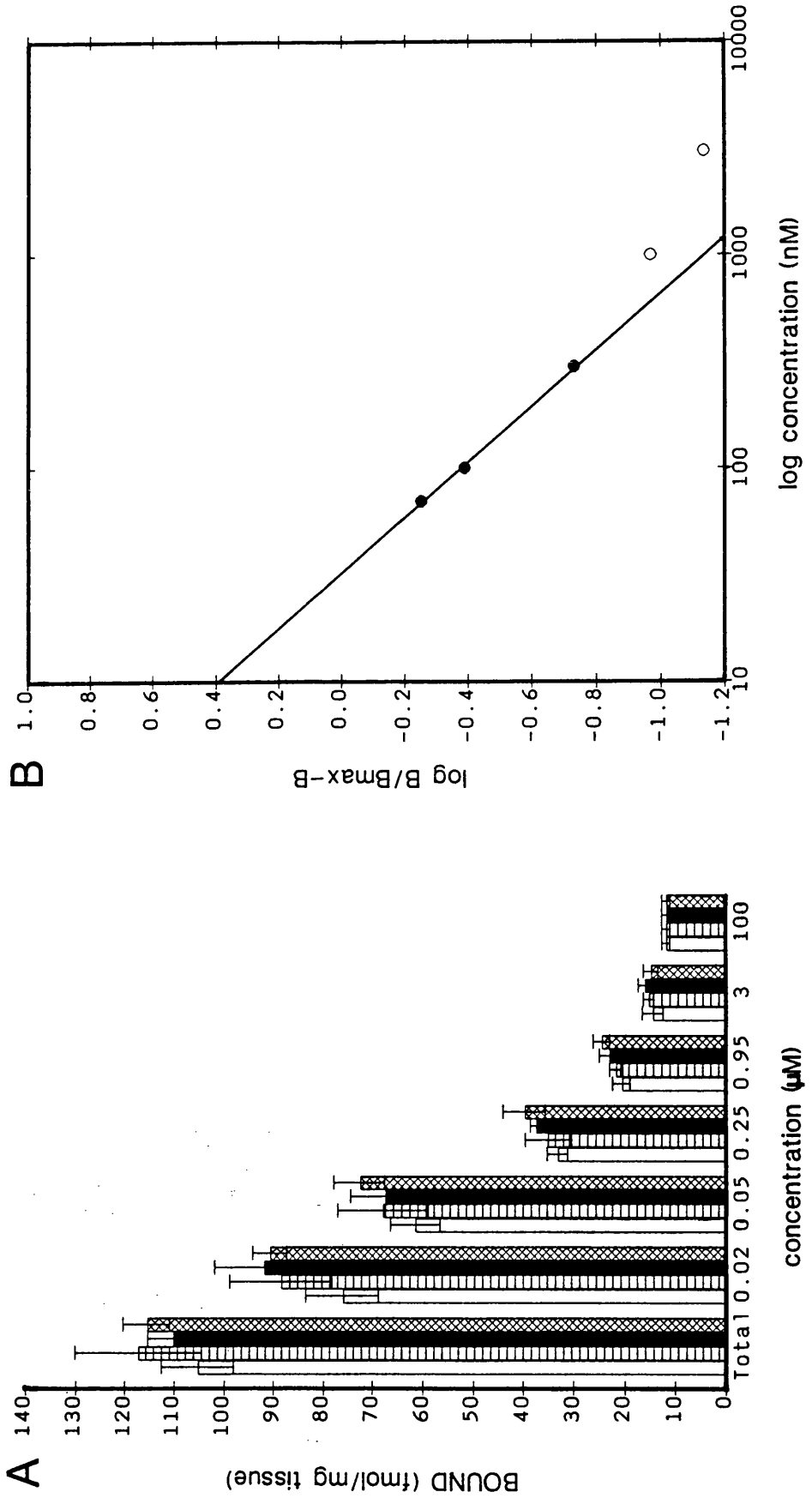

A
BOUND (fmol/mg tissue)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Total 0.02 0.05 0.25 0.95 3 100
concentration (μM)
B
log B/Bmax-B
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
10 100 1000 10000
log concentration (nM)

Table 5.2 **The effect of imipramine, administered intraperitoneally and via subcutaneously implanted minipumps for 14 days, on [^{3}H]GABA binding to $GABA_B$ sites in rat frontal cortex (Second Investigation)**

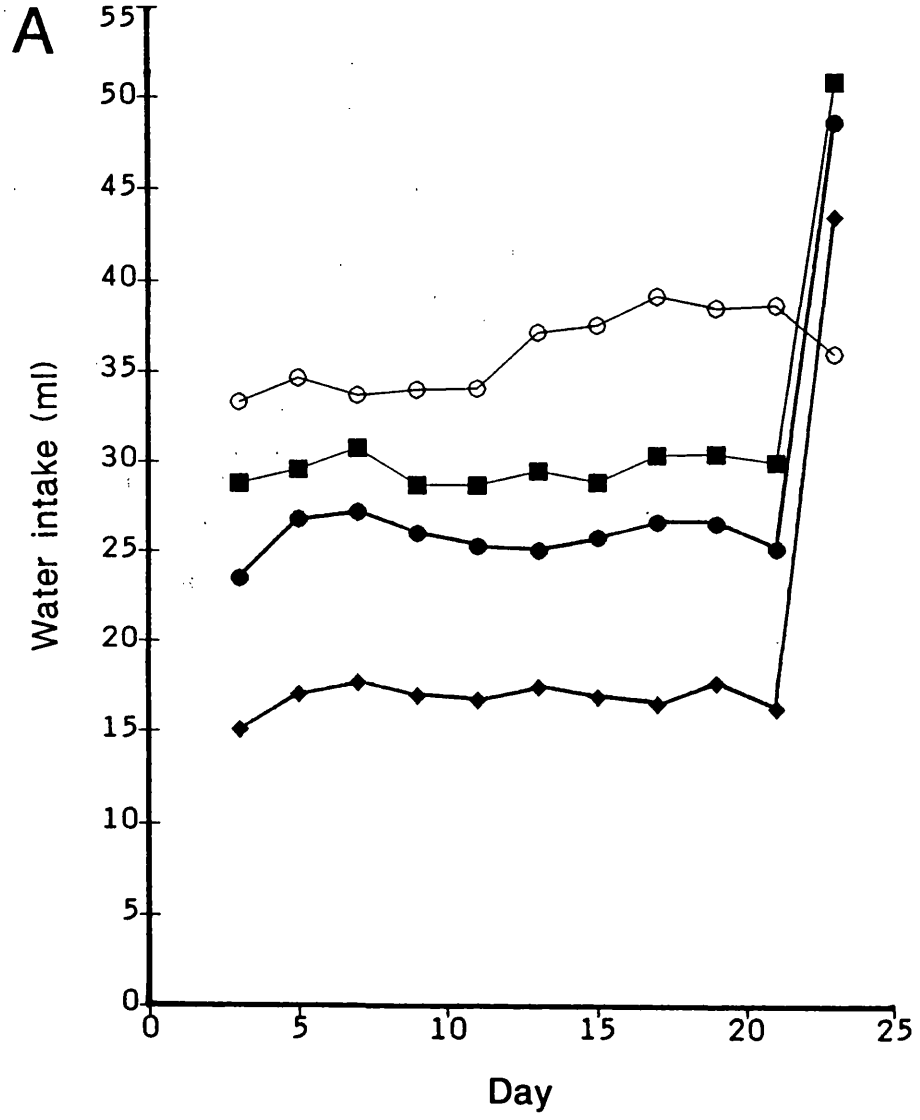
TREATMENT	n	Lamina I	Laminae II-III	Lamina V	Lamina VI
		B_{max} (fmol/mg tissue)			
Control	5	211.9 ± 23.0	264.2 ± 26.8	197.4 ± 19.2	139.9 ± 18.6
Imipramine (1)	5	270.8 ± 29.8	305.4 ± 22.4	217.3 ± 23.5	134.4 ± 17.3
Imipramine (2)	5	231.4 ± 48.7	292.2 ± 55.0	215.9 ± 49.0	n/c
Imipramine (3)	4	286.9 ± 42.0	319.2 ± 69.3	209.8 ± 69.3	151.3 ± 12.2
		K_D (nM)			
Control	5	77.5 ± 10.3	86.5 ± 15.9	90.6 ± 20.2	117.3 ± 24.1
Imipramine (1)	5	90.0 ± 19.1	95.2 ± 14.0	91.8 ± 15.6	110.2 ± 20.6
Imipramine (2)	5	81.8 ± 26.5	82.0 ± 36.6	118.0 ± 42.2	n/c
Imipramine (3)	4	86.5 ± 15.9	95.8 ± 25.2	90.7 ± 46.6	114.8 ± 14.2

(1) 10mgkg^{-1} s.c., 24 hour drug-free period
(2) 10mgkg^{-1} s.c., 48 hour drug-free period
(3) 5mgkg^{-1} i.p., 24 hour drug-free period

n/c not calculated

Figure 5.1

Third Investigation. The effect of the administration of amitriptyline (26.2 ± 0.2 $mgkg^{-1}$; ●), desipramine (17.1 ± 0.3 $mgkg^{-1}$; ♦) and paroxetine (8.3 ± 0.1 $mgkg^{-1}$; ■), via the drinking water for 21 days on (A) water intake and (B) bodyweight increase. A significant reduction in the daily water intake per rat was observed when compared to control animals (○) after treatment with both amitriptyline and desipramine from day 1 to day 21 and with paroxetine treatment after day 7 ($P < 0.05$; Student's 't' test; n = 5 rats/treatment). However, only the bodyweight of rats receiving desipramine was significantly lower after day 5 ($P < 0.05$) although the rate of weight gain was similar after week 1.


A
Water intake (ml)
55
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Day

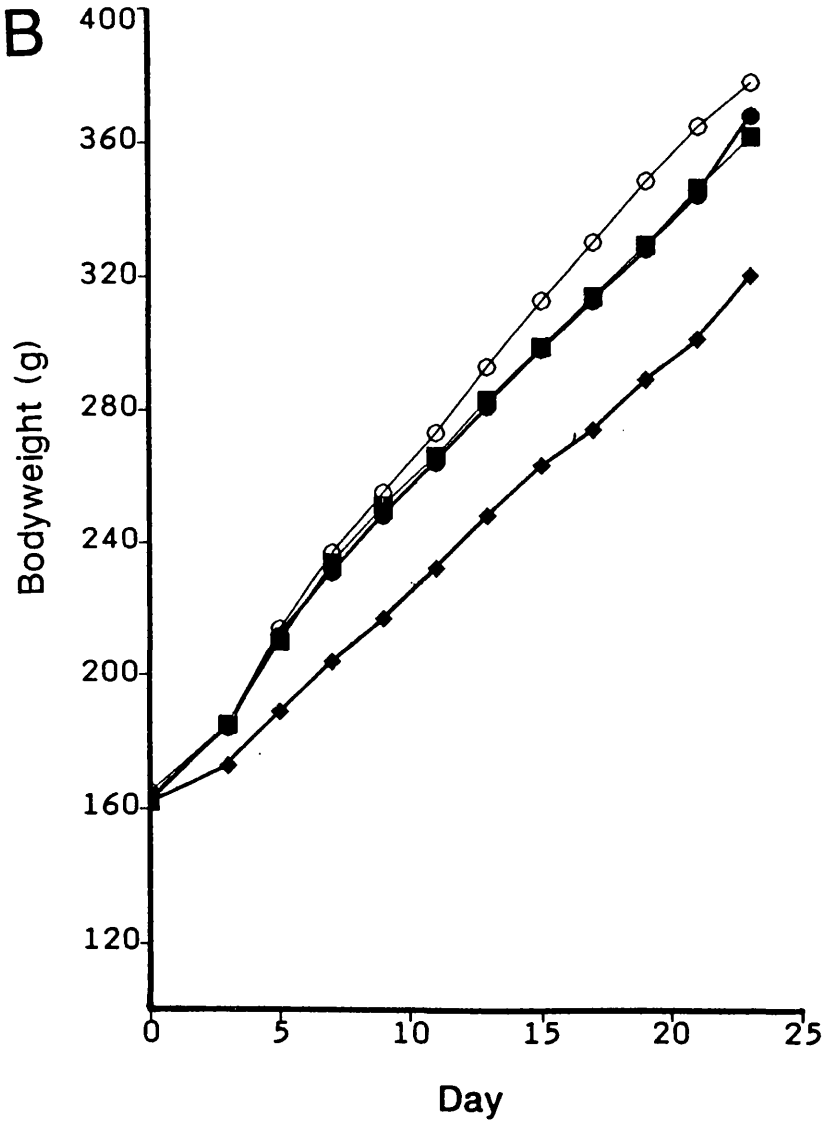
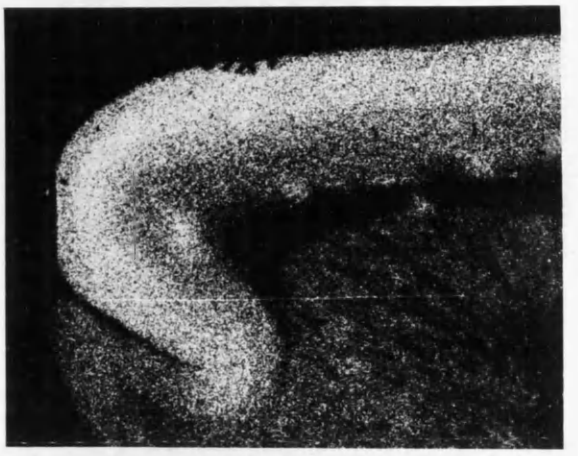

B
Bodyweight (g)
400
360
320
280
240
200
160
120
0
5
10
15
20
25
Day

Figure 5.2 opposite: page 190

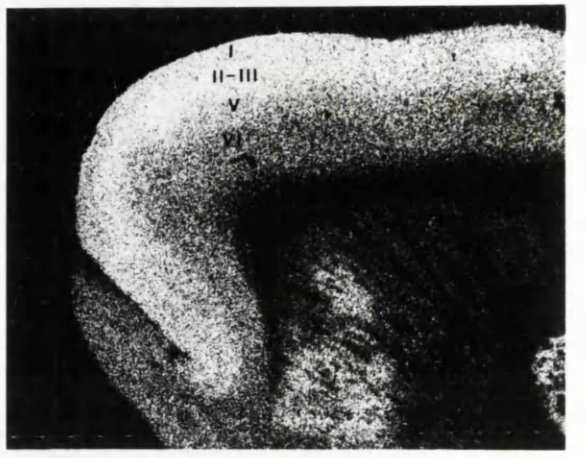
Third Investigation. Autoradiograms showing the binding of [^{3}H]GABA (50nM) to $GABA_B$ receptor sites in the frontal cortex of control and antidepressant-treated rats. Amitriptyline (26.2 $\pm$ 0.2 $mgkg^{-1}$), desipramine (17.1 $\pm$ 0.3 $mgkg^{-1}$) and paroxetine (8.3 $\pm$ 0.1 $mgkg^{-1}$), were administered for 21 days via the drinking water. Following a 24 hour drug-free period, animals were prepared for perfusion-fixation (as described in Chapter 2) prior to $GABA_B$ receptor autoradiography. Sections were incubated for 20 minutes with [^{3}H]GABA (25-400nM) and the selective labelling of $GABA_B$ receptors was achieved in the presence of 40µM isoguvacine whilst non-specific binding was defined by 100µM (-)-baclofen. Following incubation, sections were washed for two, three second periods in ice-cold 50mM Tris-HCl buffer (pH 7.4) containing 2.5mM $CaCl_2$ and dried rapidly in air before apposition to tritium-sensitive film.

Of particular interest is the discrete increased $GABA_B$ receptor density in the outer lamina I of the frontal cortex following protracted treatment with desipramine. Neither amitriptyline nor paroxetine produced any significant up-regulatory signal for $GABA_B$ receptor sites in this region. A summary of the kinetic parameters of this binding is provided in Table 5.3.

CONTROL



DESIPRAMINE



AMITRIPTYLINE

PAROXETINE

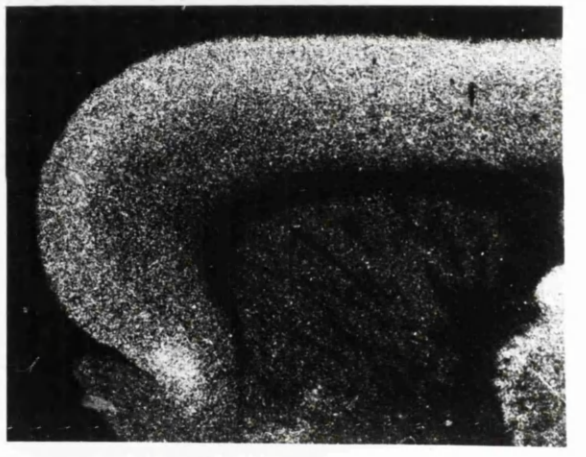


Figure 5.3

Third Investigation. Scatchard plots of $GABA_B$ receptor binding in the frontal cortex (lamina I) of control (O; r=0.91) and amitriptyline- (26.2 $\pm$ 0.2 $mgkg^{-1}$ ●; r=0.90), desipramine- (17.1 $\pm$ 0.3 $mgkg^{-1}$ ♦; r=0.89) or paroxetine- (8.3 $\pm$ 0.1 $mgkg^{-1}$ ■; r=0.90) treated rats. The kinetic parameters, K_D and B_{max} of the binding of [^{3}H]GABA (at concentrations of 25, 50, 100, 200 and 400nM) were derived by linear regression analysis and data points represent the mean of 5 animals for which triplicate determinations were made. A summary of these kinetic parameters in all frontal cortical laminae is provided in Table 5.3.

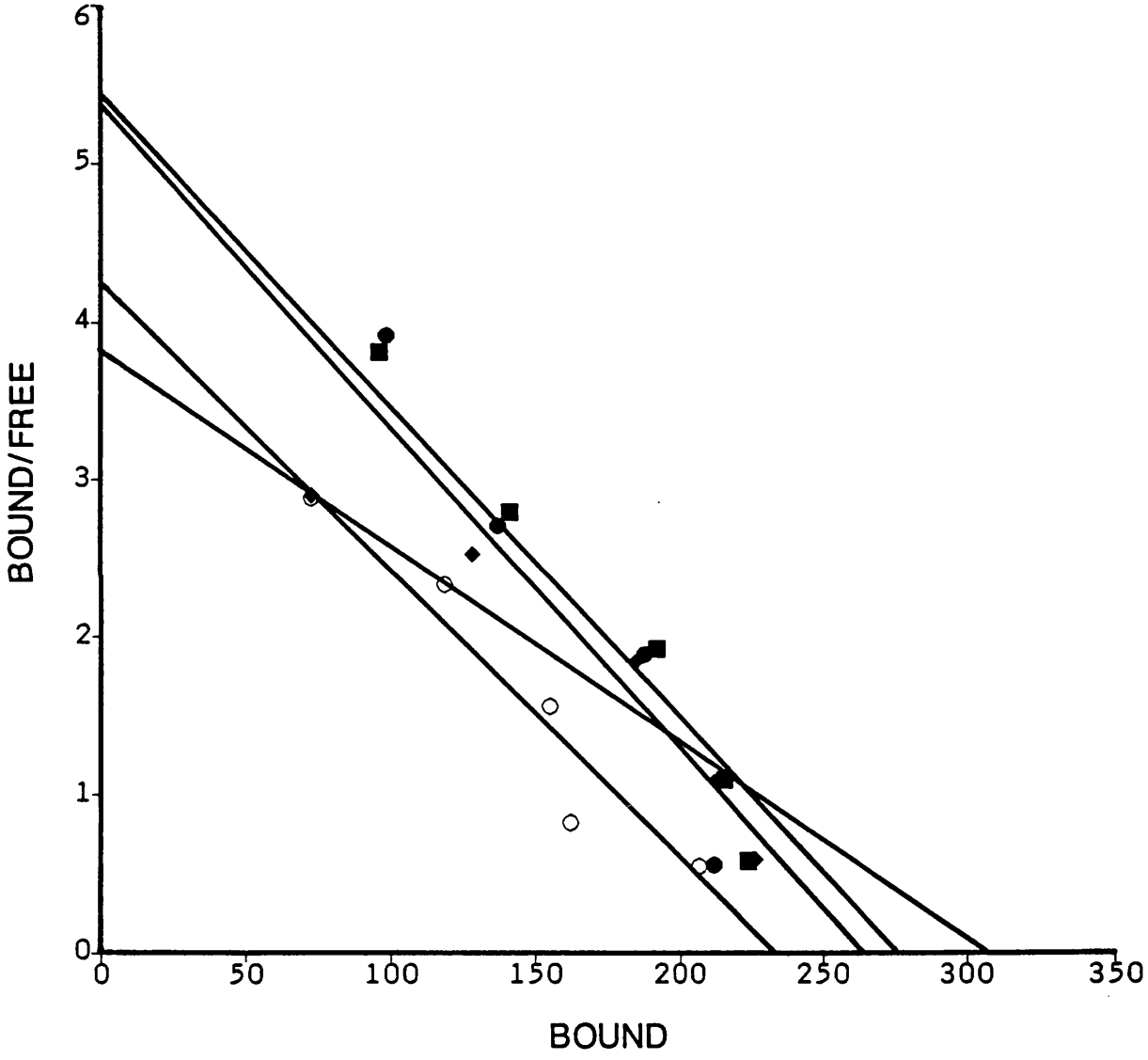

BOUND/FREE
BOUND
0
1
2
3
4
5
6
0
50
100
150
200
250
300
350

Table 5.3 **The effects of antidepressants, administered via the drinking water for 21 days, on [^{3}H]GABA binding to $GABA_B$ sites in rat frontal cortex (Third Investigation)**

TREATMENT	DOSE $mgkg^{-1}$	Lamina I	Lamina II-III	Lamina V	Lamina VI
		B_{max} (fmol/mg tissue)			
Control		232.8 ± 16.9	280.4 ± 11.7	213.9 ± 16.5	194.1 ± 15.7
Amitriptyline	26.2 p.o.	264.0 ± 19.1	284.3 ± 15.3	214.7 ± 9.5	189.1 ± 9.0
Desipramine	17.1 p.o.	307.2 ± 26.6*	312.7 ± 15.7	245.8 ± 11.2	198.3 ± 14.0
Paroxetine	8.3 p.o.	275.5 ± 23.2	304.3 ± 19.9	241.2 ± 17.8	205.3 ± 15.1
		K_D (nM)			
Control		54.5 ± 2.7	54.6 ± 3.7	62.3 ± 10.1	98.6 ± 13.4
Amitriptyline	26.2 p.o.	49.0 ± 12.6	45.9 ± 9.3	43.3 ± 6.8	87.6 ± 13.2
Desipramine	17.1 p.o.	79.9 ± 12.6*	68.2 ± 8.4	79.3 ± 10.3	105.6 ± 20.0
Paroxetine	8.3 p.o.	50.5 ± 8.8	58.4 ± 12.2	62.4 ± 8.1	98.3 ± 13.7

* $P<0.05$ Student's 't' test (unpaired; 2-tailed)
Values represent mean ± s.e.m. (n=5)

Figure 5.4 opposite: page 195

Third Investigation. Autoradiograms of total (β_1 and β_2) and resolved β_1-adrenoceptor binding in para-sagittal sections of the brains of control (A,B) and antidepressant-treated rats. Amitriptyline (26.2 $\pm$ 0.2 $mgkg^{-1}$; E,F), desipramine (17.1 $\pm$ 0.3 $mgkg^{-1}$; C,D) and paroxetine (8.3 $\pm$ 0.1 $mgkg^{-1}$; G,H), were administered for 21 days via the drinking water. Following a 24 hour drug-free period, animals were prepared for perfusion-fixation (as described in Chapter 2) prior to beta-adrenoceptor autoradiography. Sections were incubated for 60 minutes with (-)-[^{125}I]iodopindolol (37.5 - 600pM). Non-specific binding was defined in the presence of 200µM (-)-isoprenaline whilst the selective labelling of β_1-adrenoceptors was achieved in the presence of the β_2-adrenoceptor antagonist, ICI 118,551 (50nM). Following incubation, sections were washed for two, five minute periods in ice-cold Tris-saline buffer and then dried rapidly in air prior to apposition to 'Hyperfilm' (Amersham).

Of particular interest is the desipramine-induced down-regulation of both total- and β_1-adrenoceptor binding in all laminae of the frontal cortex. Amitriptyline and paroxetine were without significant modulatory effect. A summary of the kinetic parameters of this binding is provided in Table 5.4.

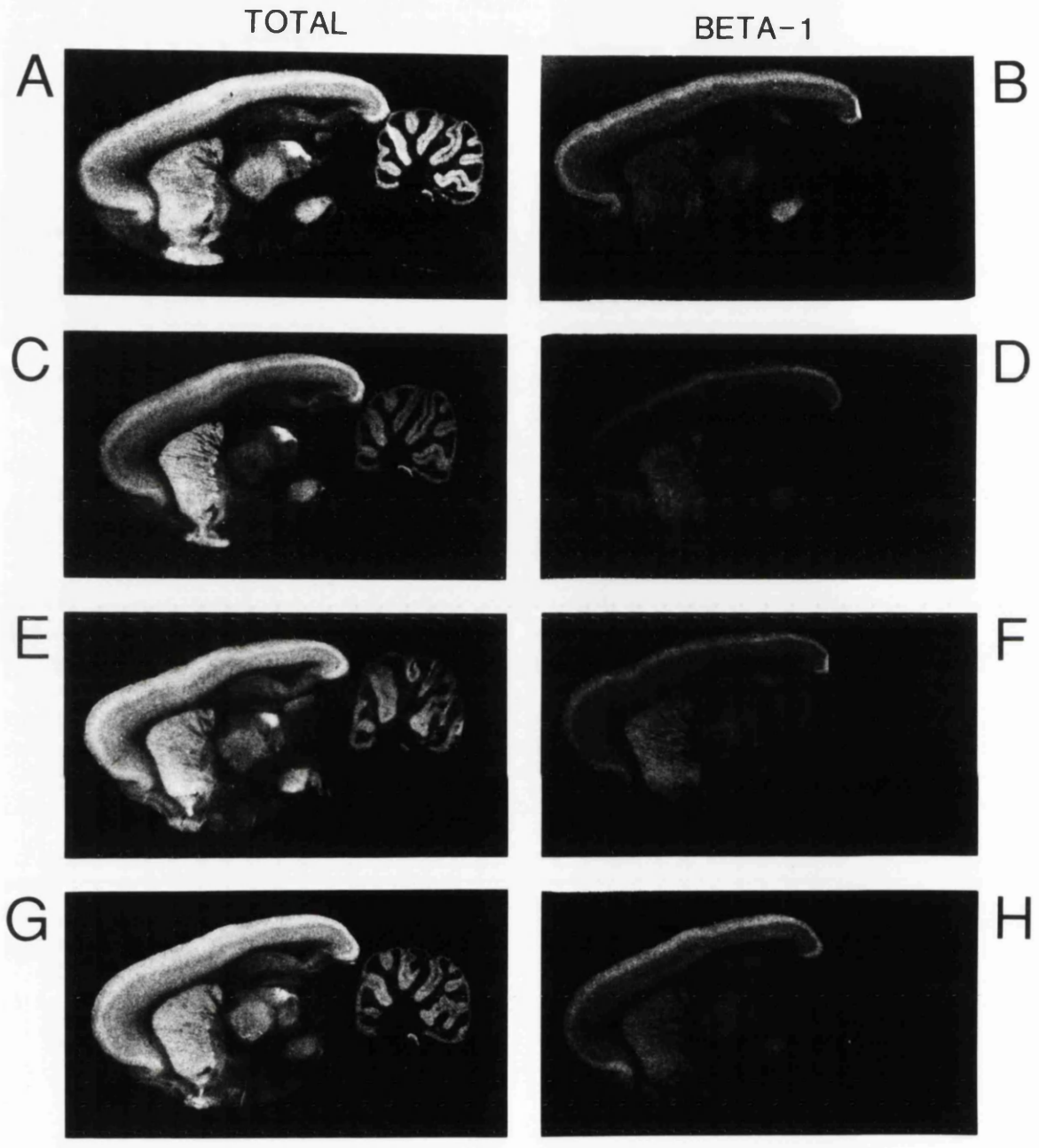

TOTAL
BETA-1
A
B
C
D
E
F
G
H

Figure 5.5

Third Investigation. Scatchard plots of (A) total (β_1 and β_2) and (B) resolved β_1-adrenoceptor binding (in the presence of 50nM ICI 118,551) in the frontal cortex (lamina I) of control (O; r=0.96 & 0.87, total & β_1, respectively) and amitriptyline- (26.2 $\pm$ 0.2 $mgkg^{-1}$ ●; r=0.95 & 0.93), desipramine- (17.1 $\pm$ 0.3 $mgkg^{-1}$ ♦; r=0.89 & 0.88) or paroxetine- (8.3 $\pm$ 0.1 $mgkg^{-1}$ ■; r=0.89 & 0.90) treated rats. The kinetic parameters, K_D and B_{max} of the binding of (-)-[^{125}I]iodopindolol (at concentrations of 37.5, 75, 150, 300 & 600pM) were derived by linear regression analysis and data points represent the mean of 5 animals for which triplicate determinations were made. A summary of these kinetic parameters in all frontal cortical laminae is provided in Table 5.4.

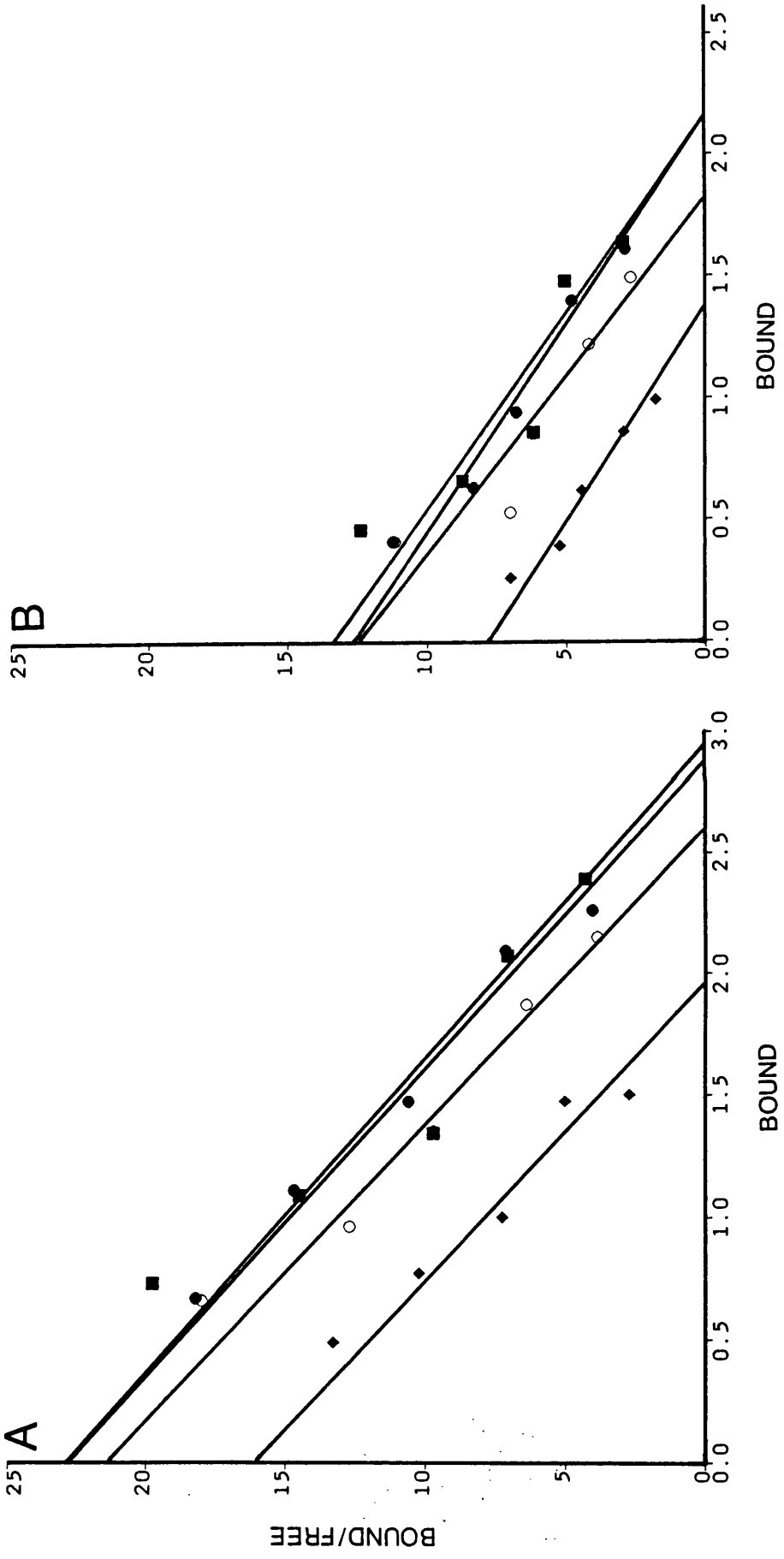

A
B
BOUND/FREE
BOUND
BOUND
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
5
10
15
20
25

Table 5.4 **The effects of antidepressants, administered via the drinking water for 21 days, on (-)-[^{125}I]iodopindolol binding to beta-adrenoceptors in rat frontal cortex (Third Investigation)**

TREATMENT	DOSE	Total			$Beta_1$		
		Lamina I	Laminae II-III	Laminae V-VI	Lamina I	Laminae II-III	Lamina V-VI
	$mgkg^{-1}$	B_{max} (fmol/mg tissue)					
Control		2.61 ± 0.12	3.34 ± 0.15	2.69 ± 0.11	1.83 ± 0.26	2.16 ± 0.26	1.68 ± 0.18
Amitriptyline	26.2 p.o.	2.90 ± 0.19	3.40 ± 0.16	2.56 ± 0.09	2.17 ± 0.12	2.50 ± 0.14	1.79 ± 0.09
Desipramine	17.1 p.o.	1.97 ± 0.12**	2.50 ± 0.09**	1.91 ± 0.04**	1.38 ± 0.09	1.82 ± 0.10	1.29 ± 0.09*
Paroxetine	8.3 p.o.	2.96 ± 0.19	3.49 ± 0.15	2.56 ± 0.10	2.16 ± 0.14	2.46 ± 0.11	1.70 ± 0.10
		K_D (pM)					
Control		122 ± 13	134 ± 17	128 ± 17	146 ± 15	134 ± 16	151 ± 15
Amitriptyline	26.2 p.o.	127 ± 16	126 ± 10	121 ± 11	172 ± 15	163 ± 18	181 ± 19
Desipramine	17.1 p.o.	122 ± 14	119 ± 9	117 ± 14	178 ± 11	163 ± 13	182 ± 9
Paroxetine	8.3 p.o.	130 ± 15	130 ± 12	109 ± 8	161 ± 14	148 ± 8	149 ± 11

* P<0.05 **P<0.01 Students's 't' test (unpaired; 2-tailed)
Values represent mean ± s.e.m. (n=5)

Figure 5.6

Fourth Investigation. Colour-digitised autoradiograms showing the binding of [^{3}H]GABA (50nM) to $GABA_B$ receptor sites in the frontal cortex of rats treated with antidepressants, CGP35348 and a putative $GABA_B$ receptor antagonist, Compound X. Rats were treated orally with amitriptyline ($30mgkg^{-1}$), desipramine ($20mgkg^{-1}$), paroxetine ($10mgkg^{-1}$) or baclofen ($10mgkg^{-1}$) whilst intraperitoneal injections of desipramine ($10mgkg^{-1}$), CGP 35348 ($100mgkg^{-1}$) or Compound X ($100mgkg^{-1}$) were also administered for a period of 21 days. Following a 24 hour drug-free period, animals were prepared for perfusion-fixation (as described in Chapter 2) prior to $GABA_B$ receptor autoradiography. Sections were incubated for 20 minutes with [^{3}H]GABA (37.5 - 300nM) and the selective labelling of $GABA_B$ receptors was achieved in the presence of 40µM isoguvacine whilst non-specific binding was defined by 100µM (-)-baclofen. Following incubation, sections were washed for two, three second periods in ice-cold 50mM Tris-HCl buffer (pH 7.4) containing 2.5mM $CaCl_2$ and dried rapidly in air before apposition to tritium-sensitive film. Colour-coded images of the resulting autoradiograms were generated using a Quantimet 970 image analysis system. Of particular interest is the discrete increased $GABA_B$ receptor density in the outer lamina I of the frontal cortex following protracted treatment with desipramine (p.o. and i.p.) and also with Compound X.

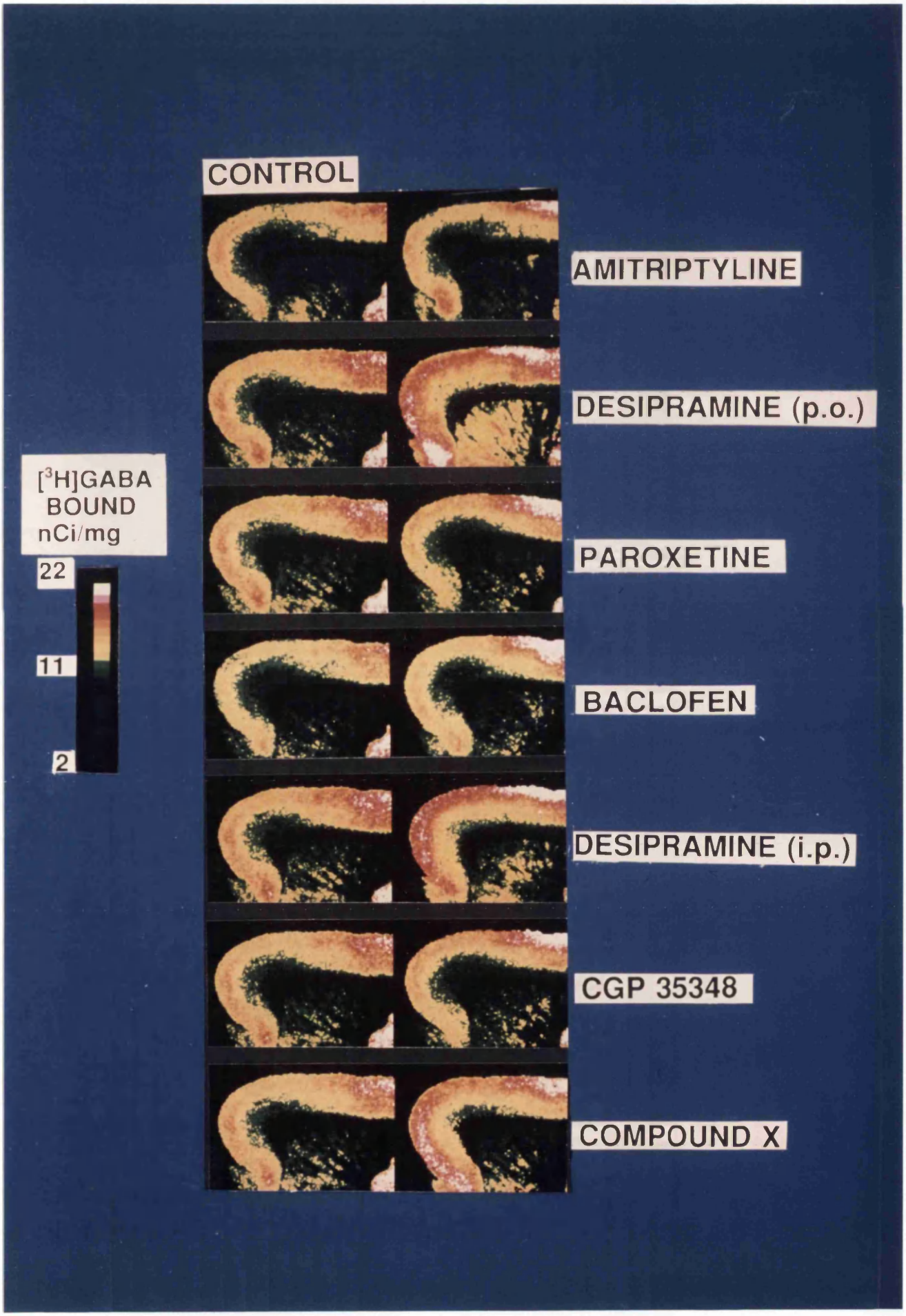

CONTROL
AMITRIPTYLINE
DESIPRAMINE (p.o.)
PAROXETINE
BACLOFEN
DESIPRAMINE (i.p.)
CGP 35348
COMPOUND X
[3H]GABA
BOUND
nCi/mg
22
11
2

Figure 5.7

Fourth Investigation. Scatchard plots of $GABA_B$ receptor binding in the frontal cortex (lamina I) of control (O; r=0.97) and (A) amitriptyline- ($30mgkg^{-1}$ ●; r=0.91), desipramine- ($20mgkg^{-1}$ ♦; r=0.84), paroxetine- ($10mgkg^{-1}$ ■; r=0.84) or baclofen- ($10mgkg^{-1}$ ▲; r=0.94) orally-treated rats. (B) Desipramine- ($10mgkg^{-1}$ ♦; r=0.96), CGP 35348- ($100mgkg^{-1}$ ▼; r=0.89) or Compound X- ($100mgkg^{-1}$ ●; r=0.97) intraperitoneally-treated rats. The kinetic parameters, K_D and B_{max} of the binding of [^{3}H]GABA (at concentrations of 37.5, 75, 150 and 300nM) were derived by linear regression analysis and data points represent the mean of 5 animals for which triplicate determinations were made. A summary of these kinetic parameters in all frontal cortical laminae is provided in Table 5.5.

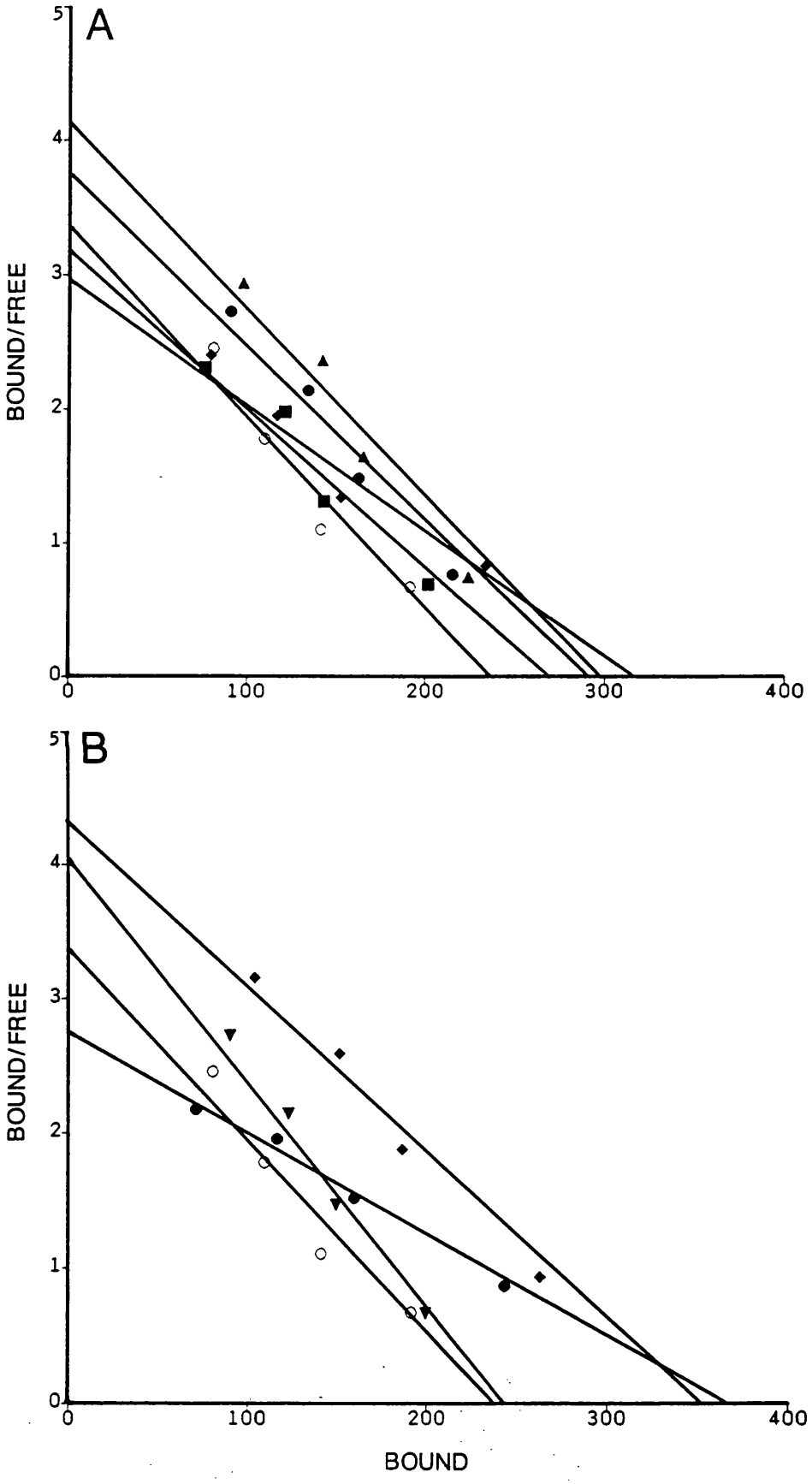

A
B
BOUND/FREE
BOUND/FREE
BOUND
0
1
2
3
4
5
0
100
200
300
400

Table 5.5 **The effects of repeatedly administered (21 days) antidepressants on the binding of [^{3}H]GABA to $GABA_B$ sites in rat frontal cortex (Fourth Investigation)**

TREATMENT	DOSE $mgkg^{-1}$	Lamina I	Laminae II-III	Lamina V	Lamina VI
		B_{max} (fmol/mg tissue)			
Control		236.6 ± 25.0	254.6 ± 42.3	228.8 ± 38.5	209.4 ± 22.2
Desipramine	20 p.o.	316.0 ± 26.0*	299.2 ± 26.2	252.0 ± 23.9	237.7 ± 27.3
Amitriptyline	30 p.o.	290.5 ± 40.9	283.5 ± 34.4	239.8 ± 22.2	232.6 ± 27.8
Paroxetine	10 p.o.	268.5 ± 38.7	299.1 ± 44.3	n/c	177.5 ± 29.2
Baclofen	10 p.o.	297.4 ± 24.9	266.4 ± 11.8	194.1 ± 10.1	250.4 ± 18.7
Desipramine	10 i.p.	352.0 ± 50.7*	275.1 ± 11.9	224.2 ± 4.8	190.8 ± 23.4
CGP 35348	100 i.p.	241.4 ± 7.4	255.2 ± 16.9	190.4 ± 6.9	161.1 ± 7.3
Compound X	100 i.p.	367.1 ± 36.1**	307.4 ± 17.3	259.4 ± 11.6	221.1 ± 9.8
		K_D (nM)			
Control		70.1 ± 16.1	59.2 ± 19.3	74.6 ± 22.3	85.8 ± 17.4
Desipramine	20 p.o.	106.5 ± 26.8	72.2 ± 14.9	76.7 ± 13.5	85.6 ± 17.5
Amitriptyline	30 p.o.	76.9 ± 13.6	55.6 ± 10.2	68.9 ± 7.6	103.8 ± 18.1
Paroxetine	10 p.o.	84.1 ± 24.0	76.4 ± 21.2	n/c	70.3 ± 31.2
Baclofen	10 p.o.	71.6 ± 1.5	48.5 ± 4.0	40.5 ± 6.6	111.3 ± 20.7
Desipramine	10 i.p.	81.0 ± 12.3	44.9 ± 11.2	55.9 ± 7.2	61.0 ± 24.4
CGP 35348	100 i.p.	59.5 ± 6.5	56.0 ± 8.2	41.7 ± 3.3	46.8 ± 6.4*
Compound X	100 i.p.	132.5 ± 20.3*	82.9 ± 10.1	90.8 ± 10.1	103.5 ± 10.9

* $P<0.05$ ** $P<0.01$ Student's 't' test (unpaired; 2-tailed; n = between 3 and 5 rats/group) n/c - not calculated

Figure 5.8 opposite: page 205

Fourth Investigation. Autoradiograms of total (β_1 and β_2) and resolved β_1-adrenoceptor binding in para-sagittal sections of the brains of rats treated with antidepressants, CGP35348 and a putative $GABA_B$ receptor antagonist, Compound X. Rats were treated orally with amitriptyline ($30mgkg^{-1}$), desipramine ($20mgkg^{-1}$), paroxetine ($10mgkg^{-1}$) or baclofen ($10mgkg^{-1}$) whilst intraperitoneal injections of desipramine ($10mgkg^{-1}$), CGP 35348 ($100mgkg^{-1}$) or Compound X ($100mgkg^{-1}$) were also administered for a period of 21 days. Following a 24 hour drug-free period, animals were prepared for perfusion-fixation (as described in Chapter 2) prior to beta-adrenoceptor autoradiography. Sections were incubated for 60 minutes with (-)-[^{125}I]iodopindolol (18.75 - 300pM). Non-specific binding was defined in the presence of 200µM (-)-isoprenaline whilst the selective labelling of β_1-adrenoceptors was achieved in the presence of the β_2-adrenoceptor antagonist, ICI 118,551 (50nM). Following incubation, sections were washed for two, five minute periods in ice-cold Tris-saline buffer and then dried rapidly in air prior to apposition to 'Hyperfilm' (Amersham). A summary of the kinetic parameters of this binding is provided in Table 5.6.

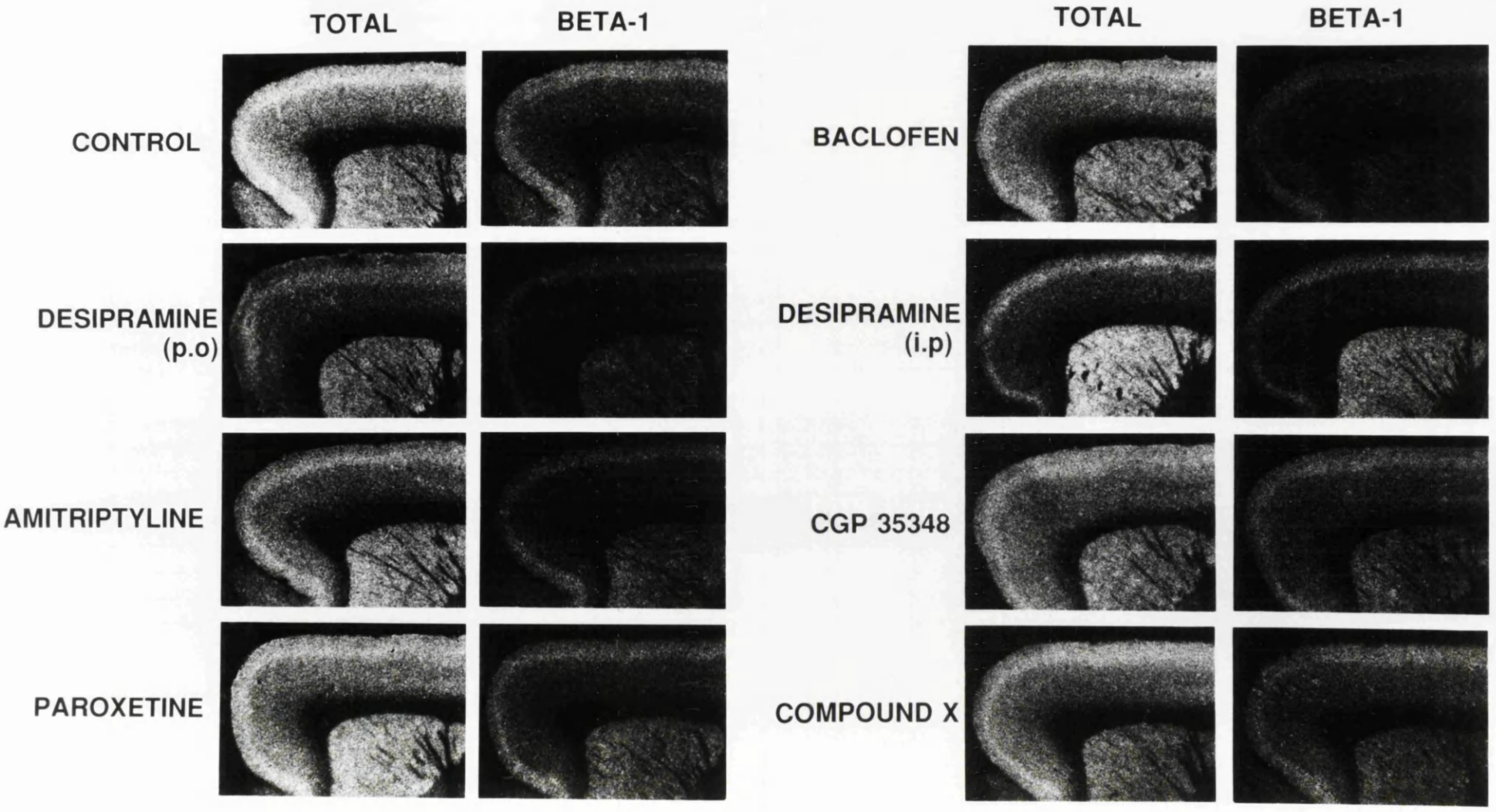

TOTAL
BETA-1
CONTROL
DESIPRAMINE (p.o)
AMITRIPTYLINE
PAROXETINE
TOTAL
BETA-1
BACLOFEN
DESIPRAMINE (i.p)
CGP 35348
COMPOUND X

Figure 5.9

Fourth Investigation. Scatchard plots of (A) total (β_1 and β_2) and (B) resolved β_1-adrenoceptor binding (in the presence of 50nM ICI 118,551) in the frontal cortex (lamina I) of control (○; r=0.86 & 0.89) and amitriptyline- (30mgkg^{-1} ●; r=0.93 & 0.97), desipramine- (20mgkg^{-1} ♦; r=0.87 & 0.89), paroxetine- (10mgkg^{-1} ■; r=0.95 & 0.99) or baclofen- (10mgkg^{-1} Δ; r=0.95 & 0.96) orally-treated rats. The kinetic parameters, K_D and B_{max} of the binding of (-)-[^{125}I]iodopindolol (at concentrations of 18.75, 37.5, 75, 150 and 300pM) were derived by linear regression analysis and data points represent the mean of 5 animals for which triplicate determinations were made. A summary of these kinetic parameters in all frontal cortical laminae is provided in Table 5.6.

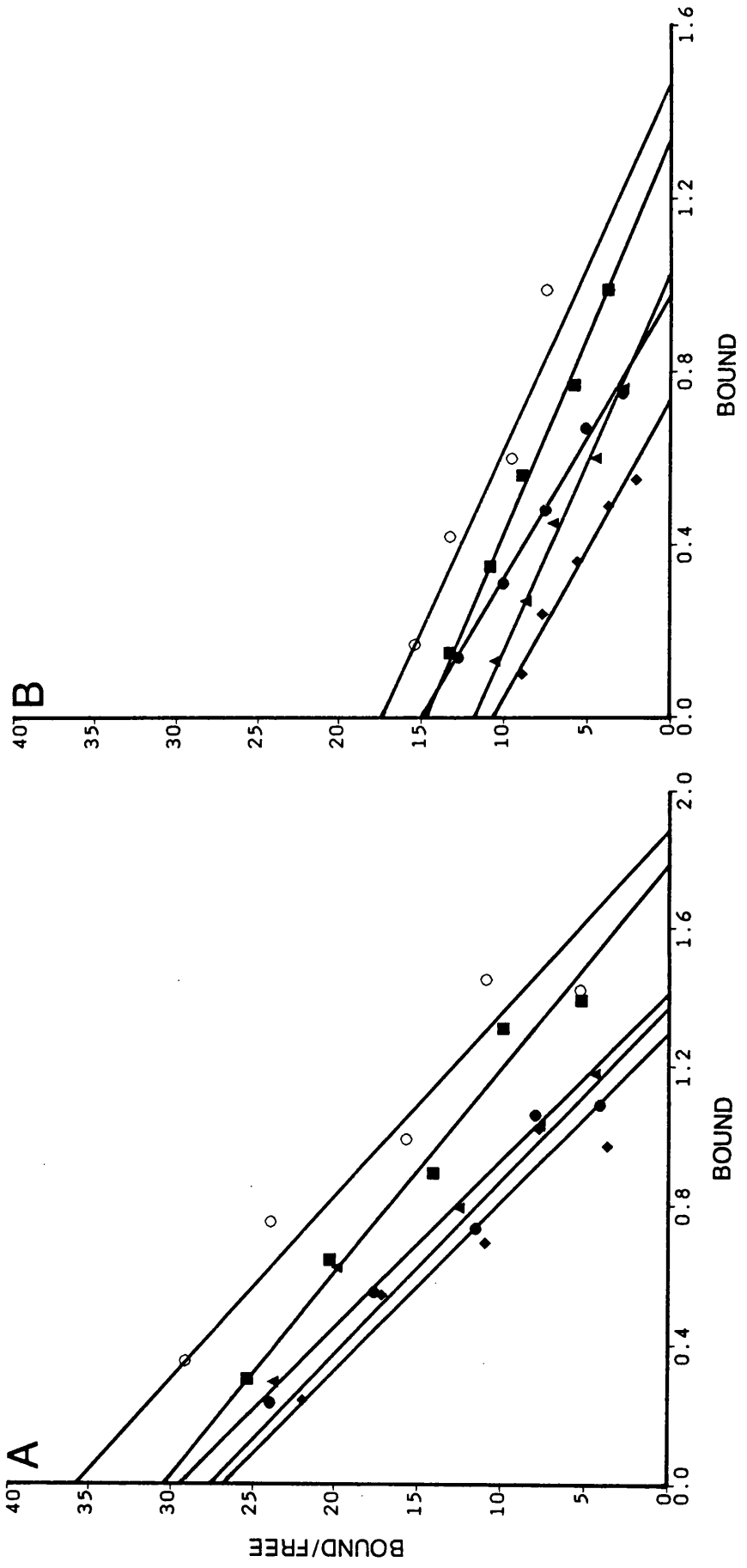

A
B
BOUND/FREE
BOUND
BOUND
0.0
0.4
0.8
1.2
1.6
2.0
0
5
10
15
20
25
30
35
40

Figure 5.10

Fourth Investigation. Scatchard plots of (A) total (β_1 and β_2) and (B) resolved β_1-adrenoceptor binding (in the presence of 50nM ICI 118,551) to the frontal cortex (lamina I) of control (○; r=0.86 & 0.89) and desipramine- ($10mgkg^{-1}$ ♦; r=0.95 & 0.81), CGP 35348- ($100mgkg^{-1}$ ▽; r=0.93 & 0.84) or Compound X- ($100mgkg^{-1}$ ●; r=0.90 & 0.83) intraperitoneally-treated rats. The kinetic parameters, K_D and B_{max} of the binding of (-)-[^{125}I]iodopindolol (at concentrations of 18.75, 37.5, 75, 150 and 300pM) were derived by linear regression analysis and data points represent the mean of 5 animals for which triplicate determinations were made. A summary of these kinetic parameters in all frontal cortical laminae is provided in Table 5.6.

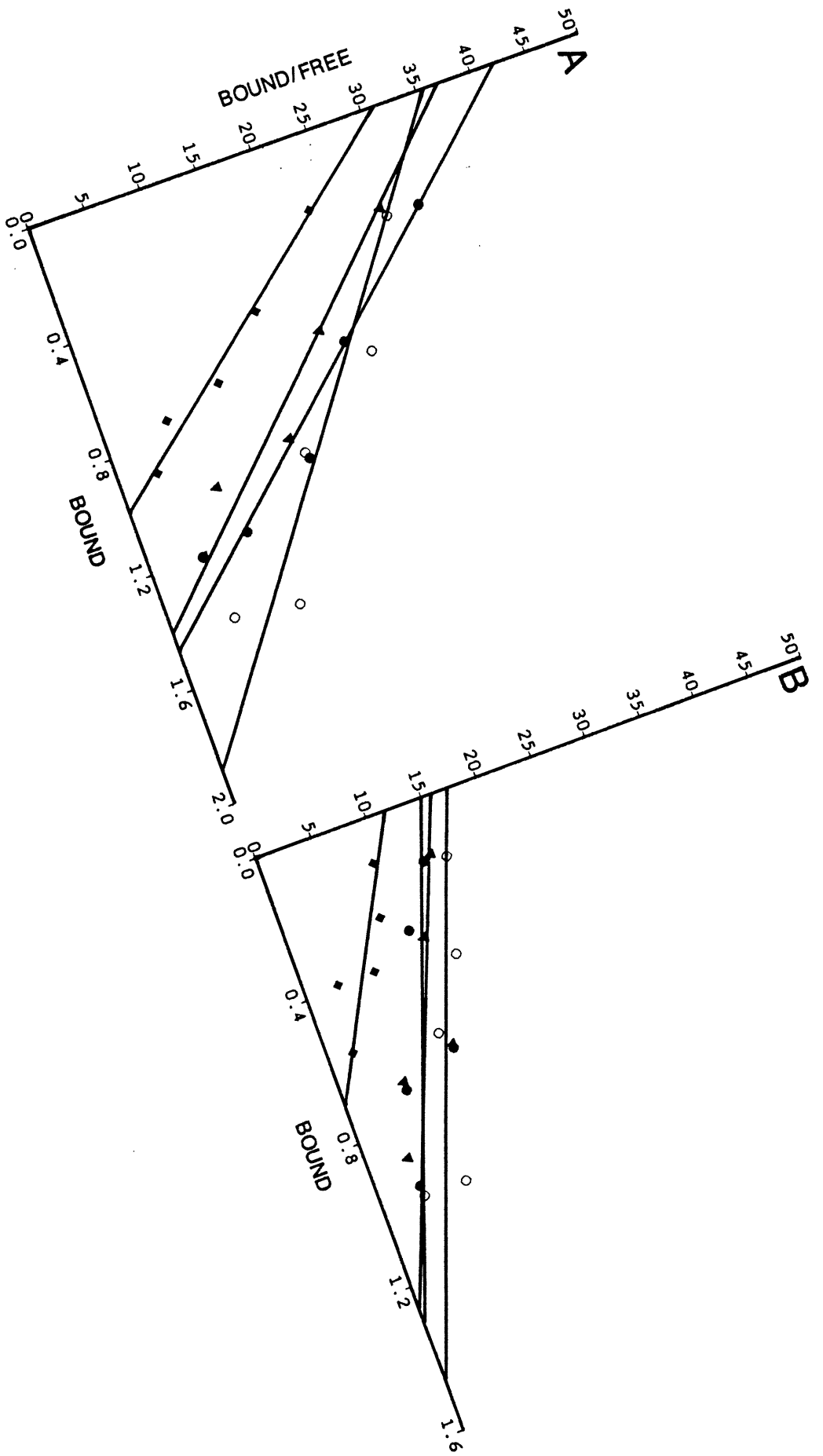

A
BOUND/FREE
0.0
5
10
15
20
25
30
35
40
45
50
0.4
0.8
1.2
1.6
2.0
BOUND
B
0.0
5
10
15
20
25
30
35
40
45
50
0.4
0.8
1.2
1.6
BOUND

Table 5.6 **The effects of repeatedly administered (21 days) antidepressants on (-)-[^{125}I]iodopindolol binding to beta-adrenoceptors in rat frontal cortex (Fourth Investigation)**

TREATMENT	DOSE mgkg^{-1}	Total			$Beta_1$		
		Lamina I	Laminae II-III	Laminae V-VI	Lamina I	Laminae II-III	Laminae V-VI
		B_{max} (fmol/mg tissue)					
Control		1.88 ± 0.06	1.92 ± 0.05	1.51 ± 0.02	1.46 ± 0.06	1.55 ± 0.05	1.15 ± 0.04
Desipramine	20 p.o.	1.29 ± 0.08**	1.44 ± 0.04**	1.09 ± 0.04**	0.73 ± 0.06**	0.92 ± 0.06**	0.62 ± 0.05**
Amitriptyline	30 p.o.	1.37 ± 0.06**	1.57 ± 0.07**	1.22 ± 0.07**	0.97 ± 0.10**	1.27 ± 0.11*	0.92 ± 0.07*
Paroxetine	10 p.o.	1.78 ± 0.01	1.80 ± 0.01	1.36 ± 0.04	1.33 ± 0.03	1.38 ± 0.05	0.94 ± 0.04
Baclofen	10 p.o.	1.41 ± 0.08**	1.51 ± 0.01**	1.16 ± 0.02**	1.02 ± 0.09	1.14 ± 0.08**	0.79 ± 0.09
Desipramine	10 i.p.	0.98 ± 0.09**	1.21 ± 0.05**	0.92 ± 0.03**	0.69 ± 0.03**	0.85 ± 0.03**	0.57 ± 0.04**
CGP 35348	100 i.p.	1.40 ± 0.04**	1.56 ± 0.05**	1.20 ± 0.04**	1.26 ± 0.12	1.37 ± 0.14	1.03 ± 0.09
Compound X	100 i.p.	1.46 ± 0.05**	1.59 ± 0.07**	1.18 ± 0.06**	1.30 ± 0.08	1.40 ± 0.08	0.98 ± 0.07
		K_D (pM)					
Control		52.5 ± 5.3	46.5 ± 4.0	41.4 ± 2.4	83.7 ± 9.6	73.4 ± 8.7	81.6 ± 6.8
Desipramine	20 p.o.	48.2 ± 5.9	46.9 ± 1.3	40.3 ± 1.3	68.4 ± 9.5	62.7 ± 3.9	70.2 ± 7.1
Amitriptyline	30 p.o.	49.7 ± 1.7	49.6 ± 2.8	47.0 ± 4.3	64.7 ± 3.1	79.3 ± 4.5	84.5 ± 7.6
Paroxetine	10 p.o.	58.5 ± 2.5	53.4 ± 2.8	42.1 ± 2.1	90.2 ± 8.1	76.9 ± 5.6	68.9 ± 4.8
Baclofen	10 p.o.	48.0 ± 10.7	39.1 ± 2.4	36.0 ± 2.9	86.2 ± 11.7	73.4 ± 8.1	64.0 ± 3.7
Desipramine	10 i.p.	31.2 ± 5.2*	31.0 ± 2.8*	30.0 ± 3.2*	58.9 ± 11.1	48.2 ± 5.0*	55.8 ± 14.5
CGP 35348	100 i.p.	37.8 ± 2.2*	38.8 ± 2.7	35.4 ± 1.2*	79.3 ± 12.8	67.4 ± 10.8	68.4 ± 6.1
Compound X	100 i.p.	34.6 ± 1.5*	32.6 ± 3.4*	27.7 ± 3.2**	86.7 ± 7.1	73.9 ± 12.0	64.4 ± 8.0

* P<0.05 ** P<0.01 Student's 't' test (unpaired; 2-tailed; n = between 3 and 5 rats/group)

DISCUSSION

The controversy surrounding the modulation of $GABA_B$ receptor populations by chronic treatment with antidepressants has remained largely unresolved since those research groups involved in such investigations have failed to produce any common definitive evidence in favour of such a hypothesis. From a summary of the main findings, predominantly from four laboratories (Table 5.0), it is immediately apparent that many methodological differences abound which may account for the existing discrepancies.

The use of subcutaneously implanted osmotic minipumps to administer antidepressants (Pilc & Lloyd, 1984; Lloyd et al, 1985) confers the advantage of effecting a slow drug infusion over a required period which would thereby serve to maintain more consistent plasma drug levels than would perhaps be expected following twice daily intraperitoneal injections (Szekely et al, 1987) or oral administration (Cross & Horton, 1988). Indeed, such an approach has facilitated the up-regulation of $GABA_B$ sites in rat frontal cortex by representatives of antidepressants from the majority of categories of biochemical mechanisms of action (Pilc & Lloyd, 1984; Lloyd et al, 1985). However, these effects cannot be solely attributed to this method of administration, since intraperitoneal injections of fluoxetine, sodium valproate, progabide and fengabine also increased $GABA_B$ binding sites (Lloyd et al, 1985), although such an observation does not provide an explanation as to why desipramine and zimeldine were without effect on $GABA_B$ receptors, despite mediating a significant reduction in $5\text{-}HT_2$ receptors (Cross & Horton, 1988).

Lloyd et al (1985), using Scatchard analysis of the displacement of [^{3}H]GABA (10nM) by a range of GABA concentrations, found that nomifensine and desipramine ($1.25 mg kg^{-1} day^{-1}$) increased the B_{max} of the high affinity $GABA_B$ binding component (although it was not indicated

whether or not this was a significant change). Similar findings were obtained for amitriptyline, citalopram, viloxazine and pargyline. However, inconsistencies arise since upon closer inspection, is not clear whether the GABA concentration range was, in fact, 1-750µM, (Methods section); or 1-750nM, (Results section) (Lloyd et al, 1985) and the data for the latter four antidepressants were reported initially in their first study (Pilc & Lloyd, 1984), for which the kinetic binding parameters were derived by saturation analysis of varied [^{3}H]GABA concentrations (8-160nM). Moreover, respective drug-free periods of 24 and 72 hours were used in these two studies.

Higher doses of desipramine (5mgkg^{-1}day^{-1}) and zimeldine (10mg kg^{-1}day^{-1}) unmasked a second, lower affinity $GABA_B$ site, not evident in controls, which was attributed to the possible induction of *de novo* protein synthesis by these treatments. This report of a biphasic $GABA_B$ Scatchard plot is not in isolation since Suzdak & Gianutsos (1986) also demonstrated that both high and low affinity $GABA_B$ receptors (K_D = 30nM and 500nM, respectively) in mouse cortical membranes were increased following treatment with imipramine. Conversely, [^{3}H]GABA concentrations of up to 400nM produced a single $GABA_B$ binding component for which the B_{max} was increased by the chronic administration of desipramine and imipramine (but not maprotiline) (Szekely et al, 1987). Surprisingly, these authors also found that $GABA_B$ sites labelled by [^{3}H]baclofen amounted to only 44% of those labelled by [^{3}H]GABA in the presence of bicuculline and which were unaffected by these treatments. Furthermore, the ability of (-)-baclofen to inhibit forskolin-stimulated adenylyl cyclase activity was equally, unchanged and from this, it was proposed that antidepressants may affect a low affinity $GABA_B$ receptor sub-population that is not necessarily coupled to G_i proteins.

In an attempt to arbitrate these conflicting reports, the four

investigations presented in this chapter have examined the effects of a number of chronically administered antidepressants on the $GABA_B$ receptor population in the frontal cortex of rat brain using receptor autoradiography. This approach has enabled a more detailed analysis of $GABA_B$ sites located within distinct laminal areas of this brain region and has therefore advanced the findings of existing studies in which only synaptic membranes of the frontal cortex had been employed.

Using a single [^{3}H]GABA concentration (50nM), the first investigation may perhaps be described as a pilot study in which $GABA_B$ receptor populations were examined following repeated (14 day) oral administration of either imipramine, mianserin or paroxetine, all of which have different mechanisms of action. Imipramine, whilst inhibiting the uptake of both NA and 5-HT, has a greater selectivity for the noradrenergic system (Iversen & Mackay, 1979) whereas paroxetine is a specific inhibitor of 5-HT uptake (Thomas et al, 1987). These latter authors observed that both paroxetine ($5mgkg^{-1}$ p.o.) and imipramine ($10mgkg^{-1}$ p.o.) selectively inhibited the uptake of either [^{3}H]5-HT or [^{3}H]noradrenaline, respectively, into rat hypothalamic synaptosomes 2 hours after the last dose of a 14 day administration period. Mianserin is an antagonist at α_2 autoreceptors (Baumann & Maitre, 1977) and also at serotonin$_2$ (5-HT$_2$) receptors (Maj et al, 1978). Thus, although the dose, route and duration of administration appeared to satisfy the conditions necessary to effect the neurochemical actions of these antidepressants, in this preliminary study, neither exerted any significant effect on $GABA_B$ receptors in the frontal cortex.

In their report, Thomas et al (1987) highlighted the fact that when [^{3}H]5-HT uptake was measured 24 hours following the last dose, no significant difference was observed between control and drug-treated groups. Since animals in this study were sacrificed after a similar 24 hour period, it would appear that a neurochemical marker need not necessarily

correlate with an antidepressant action and that receptor modulations may be more appropriate. To this end, paroxetine (5mgkg^{-1} ip. for 21 days) significantly reduced cortical 5-HT$_2$ receptors (Nelson et al, 1989).

The failure of the treatment regimes used in this study to influence GABA$_B$ receptor binding may therefore be attributable to inappropriate selections of dose or route and duration of drug administration. Subcutaneous infusions of mianserin (10mgkg^{-1}) and desipramine (5mgkg^{-1}), a metabolite of imipramine, in addition to intraperitoneal injection of fluoxetine (10mgkg^{-1}, also a selective 5-HT uptake inhibitor) for 18 days had previously been shown to increase [^{3}H]GABA (10nM) binding to GABA$_B$ receptors in rat frontal cortical membranes (Lloyd et al, 1985) by 33%, 51% and 83% above control values, respectively.

Chronic antidepressant administration via osmotic minipumps

In their study, Lloyd et al (1985) demonstrated increased GABA$_B$ receptor binding following the administration of a variety of antidepressants via Alzet 2002 minipumps, which were implanted for a period of 18 days. According to the manufacturer's specification (based on an *in vitro* qualification test), this particular minipump model operates a pumping rate that reaches steady state levels after 4 hours on day 1 continuing through to day 14. *In vivo*, the pumping rate is reduced to 90% of the *in vitro* value and taking these factors into consideration, an implantation period of 14 days was implemented for the second investigation.

Although infusion and intraperitoneal injections of imipramine (10mgkg^{-1}) appeared to increase the B_{max} of GABA$_B$ receptor binding, most notably in the outer lamina (I) of the frontal cortex, significance levels were not achieved. The ability of imipramine to up-regulate GABA$_B$ receptors has been demonstrated in cortical synaptic membranes by both Suzdak & Gianutsos (1986) and Szekely et al (1987) and indeed its demethylated metabolite, desipramine, also produces a similar response (Pilc & Lloyd,

1984; Lloyd et al, 1985; Szekely et al, 1987).

The K_D value derived by non-linear regression analysis in this study compares favourably with that found in frozen and thawed whole rat brain homogenates (K_D = 77nM, Hill & Bowery, 1981; Bowery et al, 1983). Furthermore, if a tissue:protein ratio of approximately 1:15 is assumed, then an average B_{max} value of 200fmol/mg tissue throughout the frontal cortex is in close agreement with that found in membrane preparations (1.22pmol/mg protein, Hill & Bowery, 1981). Nonetheless, since these parameters originate from transformed competitive binding data, experimental error is increased as independent variables are not taken into consideration. For this reason standard errors of the B_{max} values are relatively high (up to 33%) especially in the deeper laminae of the cortex. Such a methodological approach would therefore require the use of a larger animal population in order to unmask any possible drug-induced changes in $GABA_B$ receptor populations. A further point for future consideration using displacement analysis to derive kinetic binding parameters would be the selection of a lower GABA concentration range than that used in this study.

Discrete $GABA_B$ receptor up-regulation induced by desipramine

The antidepressants employed in the third investigation (desipramine, paroxetine and amitriptyline) were selected on the basis of their ability to selectively inhibit the uptake of NA, 5-HT or both monoamines. By administering these drugs chronically via the drinking water, it was anticipated that as with osmotic minipumps, more consistent plasma drug concentrations would be achieved (depending upon the rats' drinking behaviour) and thus allow similar $GABA_B$ receptor up-regulations to those reported by Lloyd et al (1985). Previous biochemical studies had validated this method of drug administration (Nelson et al, 1990) in that paroxetine inhibited [^{3}H]5-HT uptake into cortical synaptosomes (ED_{50} = 3mgkg^{-1} p.o.).

Moreover, following a similar dosing schedule, paroxetine (8.6mgkg^{-1}) and amitriptyline (9.2 & 27mgkg^{-1}) significantly reduced cortical 5-HT$_2$ receptors. Furthermore, the reduction in bodyweight gain by desipramine has also been reported by Cross & Horton (1988).

From the localised increase in GABA$_B$ receptor binding induced by desipramine in lamina I of the frontal cortex, it could be interpreted that the modulation of GABA$_B$ receptors by this particular antidepressant may have occurred as a consequence of the selective inhibition of the uptake of noradrenaline since both amitriptyline and paroxetine were without effect under the conditions employed in this study. This hypothesis would contradict the findings of Lloyd et al (1985) since both amitriptyline and fluoxetine as well as desipramine, increased GABA$_B$ receptor binding densities in rat cortical membranes by between 51% and 83% after chronic subcutaneous infusion.

Beta-adrenoceptor modulation by antidepressants

In addition to the possible modulation of GABA$_B$ receptors, the effects of the antidepressants described on beta-adrenoceptor binding were also examined with the aim of correlating potentially up-regulated GABA$_B$ receptors with down-regulated beta-adrenoceptors. In light of this, desipramine (but not amitriptyline or paroxetine) significantly reduced the beta-adrenoceptor population in all laminae of the frontal cortex being attributable to the beta$_1$-adrenoceptor sub-type in laminae V and VI, although an apparent reduction in this sub-type was also evident in the outer laminae. These findings are in agreement with the autoradiographical studies of Ordway et al (1988) who, by using (-)-[^{125}I]iodopindolol observed a reduction in beta$_1$- but not beta$_2$-adrenoceptors throughout the somatosensory cortex (and indeed in many thalamic nuclei and hippocampal regions) following treatment with desipramine (10mgkg^{-1} i.p., twice daily for 10 days). Selective beta$_1$-adrenoceptor modulation by

desipramine has also been demonstrated in cortical membranes preparations (Minneman et al, 1979; Beer et al, 1987; Heal et al, 1989). In these studies, $beta_2$-adrenoceptor binding was represented by subtracting the $beta_1$-adrenoceptor component from the total beta-adrenoceptor population. It was found that such an approach to calculate kinetic binding parameters using receptor autoradiography, resulted in too much variation between animals to allow accurate construction of Scatchard plots, thus making statistical comparisons impossible. It would therefore be necessary to define $beta_2$-adrenoceptors autoradiographically in the presence of a $beta_1$-adrenoceptor antagonist, for example, CGP 20712A.

In parallel with this investigation, desipramine also reduced the accumulation of cAMP in cortical slices in response to both noradrenaline- and isoprenaline-stimulated adenylyl cyclase activity (Nelson et al, 1990).

Surprisingly perhaps, in view of the mixed NA/5-HT uptake inhibiting properties of amitriptyline that no beta-adrenoceptor down-regulation was observed after repeated administration. To date, there have been no autoradiographical studies in which the effect of chronically administered amitriptyline on central beta-adrenoceptors has been examined. Heal et al (1989) showed that $beta_1$-adrenoceptors were reduced by 28% in whole cerebral cortical membranes by amitriptyline ($10mgkg^{-1}$ p.o. for 10 days) and indeed, under the same conditions and dose employed in this study, amitriptyline selectively reduced the B_{max} of $beta_1$-adrenoceptors in cortical membranes (Nelson et al, 1990).

In view of the fact that amitriptyline failed to show any reduction in either NA- or isoprenaline-stimulated adenylyl cyclase activity (Nelson et al, 1991), it is possible that brain levels achieved were insufficient to inhibit NA uptake. In support of this, not all researchers have found amitriptyline to be effective at modulating beta-adrenoceptors; for example, following 21 day intraperitoneal administration, amitriptyline did not influence cortical beta-adrenoceptors (Tang et al, 1981) whereas Sellinger-Barnette et al (1980)

and Wise & Halliday (1985) found amitriptyline to be much weaker than desipramine in reducing the cortical beta-adrenoceptor population.

The failure of paroxetine, to down-regulate beta-adrenoceptors, at a concentration known to inhibit 5-HT uptake, provided confirmation of its lack of effect in membrane binding studies (Nelson et al, 1990). By way of contrast, receptor autoradiography has shown that the 5-HT uptake inhibitors, fluoxetine and sertraline both decrease beta-adrenoceptors in specific laminae of rat frontoparietal cortex (Byerley et al, 1987, 1988). One possible explanation for this apparent discrepancy could reside in the fact that these drugs are less selective in their ability to inhibit 5-HT uptake.

Selective $GABA_B$ receptor-modulation by desipramine and a novel, putative $GABA_B$ receptor antagonist

The effects of amitriptyline, desipramine and paroxetine on $GABA_B$ receptor binding were subsequently confirmed in a fourth study, however, instead of dosing via the drinking water, in this instance, drugs were given at the same dose once daily via oral gavage. The localised up-regulation of $GABA_B$ sites induced by desipramine, observed formerly in lamina I of the frontal cortex, was again evident and although the K_D value was increased above control, unlike the former study, the values were not significantly different. Desipramine-induced beta-adrenoceptor down-regulation already discussed, was confirmed in this study and throughout all laminal regions, the effect was attributable to the $beta_1$-adrenoceptor sub-type. Whilst paroxetine still lacked any modulatory action on beta-adrenoceptors, in contrast to the previous study, amitriptyline effected a significant reduction of these sites, perhaps suggesting that the dosing regime employed in this study produced plasma concentrations high enough to inhibit noradrenaline uptake.

Repeated administration of an agonist would be expected to desensitise a specific receptor resulting in its eventual down-regulation.

Suzdak & Gianutsos (1986) reported a significant reduction in the B_{max} of high affinity $GABA_B$ receptors in mouse cortical membranes after 14 days of treatment with baclofen ($10mgkg^{-1}$). Moreover, in parallel with the $GABA_B$ receptor binding assay, such treatment also significantly reduced the ability of baclofen to potentiate NA-stimulated cAMP accumulation - an index of down-regulated beta-adrenoceptors (Suzdak & Gianutsos, 1985a). Such a reduction in $GABA_B$ receptors was not apparent after chronic baclofen treatment which may reflect the differing routes of administration used in this study and that of Suzdak & Gianutsos (1986), or, that after a period of 21 days had elapsed, tolerance to the effects of this agonist may have ensued. This treatment protocol did not, however, preclude a significant reduction of both total and resolved $beta_1$-adrenoceptor populations.

In parallel with the effects of orally administered desipramine, when injected intraperitoneally, a marked up-regulation of $GABA_B$ sites and down-regulation of beta-adrenoceptors was observed, the former effect again being restricted to lamina I.

The recent emergence of the centrally-active $GABA_B$ receptor antagonist, CGP 35348 (Olpe et al, 1990), should enable a significant advancement in our understanding of the possible physiological roles for central $GABA_B$ receptors. In their study, Olpe et al (1990) reported that when given *in vivo*, at doses of up to $300mgkg^{-1}$, CGP 35348 antagonised baclofen-induced impairment of rat rotarod performance 30 minutes after its injection. However, if given one hour prior to baclofen, only a 50% inhibition at $300mgkg^{-1}$ was seen. This would indicate that although CGP 35348 enters the CNS, it does so rather rapidly and that its effects are short lasting which could perhaps provide an explanation as to why after chronic administration of this antagonist, no alteration in $GABA_B$ receptor populations in the frontal cortex was observed.

Conversely, Compound X ($100mgkg^{-1}$ i.p.), increased the B_{max} of

$GABA_B$ receptors to a similar extent as desipramine ($10mgkg^{-1}$ i.p.) although again, the change was restricted to lamina I. This may indicate that it has a longer duration of action compared to CGP 35348. Compound X also produced a significant increase in the K_D value which may suggest that as $GABA_B$ receptor numbers increased, a compensatory reduction in the affinity of the ligand for the receptor ensued. Perhaps one of the most intriguing findings of this study was that although both CGP 35348 and Compound X reduced the total beta-adrenoceptor population, in contrast to those antidepressant effects already described, this modulation was not attributable to the $beta_1$-adrenoceptor sub-type. This prompts the suggestion of a possible involvement of $beta_2$-adrenoceptors in this response, however, in cortical regions, the $beta_2$-adrenoceptor sub-type contributes only 20-25% of the total beta-adrenoceptor population (Rainbow et al, 1984; Beer et al, 1987; Ordway et al, 1988; De Paermentier et al, 1989) and which have been suggested to be associated with non-nerve cell components such as glia and blood vessels (Minneman et al, 1979). Clearly, clarification of such a functional link between $GABA_B$ receptor antagonists and $beta_2$-adrenoceptors would require further experimental investigations.

CONCLUSIONS — An hypothesis of the possible role of $GABA_B$ receptors in antidepressant action

Following on from the discussion of the studies previously described, two themes appear to reoccur. Firstly, of the antidepressants tested, only desipramine was able to consistently mediate an up-regulatory signal for $GABA_B$ receptors. Additionally, the $GABA_B$ receptor antagonist, Compound X, also produced changes of a similar magnitude.

Secondly, where drug-induced increases in the $GABA_B$ receptor population were evident in the frontal cortex, such changes were restricted

to the outer lamina (I) of this region. Since desipramine specifically inhibits the uptake of noradrenaline, some speculation regarding the influence of this antidepressant (as well as Compound X) on $GABA_B$ receptors in this discrete anatomical location would seem most appropriately interpreted in relation to both the noradrenergic system and known markers of GABAergic activity.

Activation of $GABA_B$ receptors has been shown to inhibit noradrenaline release in slices of rat frontal cortex (Bowery et al, 1980) and in mouse cerebral cortex (Suzdak & Gianutsos, 1985b), suggesting an inhibitory action located presynaptically on noradrenergic terminals. Karbon et al (1983) proposed that low affinity $GABA_B$ sites are associated with cortical terminals since following a unilateral lesion of the dorsal noradrenergic bundle, the number of these sites in rat forebrain was significantly reduced, whilst high affinity sites remained unaffected by the lesion. A further modulatory link between these two neurotransmitter systems arises from the fact that preincubation of frontal cortical slices with baclofen and GABA increased the affinities of both high and low affinity components for isoprenaline binding to beta-adrenoceptors, in addition to increasing the proportion of low affinity sites (Scherer et al, 1989).

It had been hypothesised that following chronic antidepressant treatment, an animal showing the largest increase in $GABA_B$ receptors would also exhibit the greatest down-regulation of beta-adrenoceptors. By ranking the respective B_{max} values for both receptor classes after treatment with desipramine and Compound X, a positive correlation between these parameters was not apparent. However, if low affinity sites are coupled with noradrenergic terminals, the changes with respect to $GABA_B$ sites reported in this chapter, correspond to a high affinity component and would imply that these $GABA_B$ receptors may not necessarily be linked with the noradrenergic system in depression. Such a hypothesis, however, would contradict the findings of Lloyd et al (1985) and Suzdak & Gianutsos

(1986) where both high and low affinity sites were increased by antidepressants.

Interpretation of drug-induced $GABA_B$ receptor changes with respect to known morphology

Consideration of the drug-induced changes in $GABA_B$ receptors in lamina I of the frontal cortex requires examining in relation to the neuronal innervation of this particular region. Whilst the literature on the detailed analysis of GABAergic neurones projecting specifically to the rat frontal cortex appears to be relatively sparse, more general descriptions of such morphology are available for the neocortex as a whole. In a meticulous study of the distribution of GABAergic neurones and their terminals in the CNS of the rat, defined by GAD immunohistochemistry (Mugnaini & Oertel, 1985), lamina I (the molecular layer) of the neocortex was described as being rich in GABAergic cell bodies whereas cortical areas generally have a low to medium density of GAD-positive terminals. Within the motor area of the frontoparietal cortex, however, a medium to high density of axon terminals is evident in laminae I, II and III.

GAD-immunoreactive interneurones also exist within the neocortex and in lamina I, these local circuit neurones contribute to between 90% and 95% of the total neuronal population representing many different morphological categories (Schmechel et al, 1984). Furthermore, there is also a rich network of noradrenergic innervation throughout all laminae of the cerebral cortex, characterised in lamina I by tangential fibres which ramify other parts of this region (Levitt & Moore, 1978). A role for GABAergic interneurones in cortical inhibition was demonstrated by Iversen & Mitchell (1971) whereby stimulation of the lateral geniculate thalamic nucleus evoked Ca^{2+}-dependent release of GABA in the cat visual cortex. GABAergic cell bodies, however, appear to be concentrated in the reticular thalamic nucleus rather than being distributed uniformly throughout the thalamus

(Houser et al, 1980; Mugnaini & Oertel, 1985). Moreover, this particular thalamic nucleus may play an important role in the integration of thalamocortical processing (Scheibel & Scheibel, 1967) via the influence of GABAergic neurones on neuronal transmission (Houser et al, 1980).

Although desipramine has been shown to enhance the release of GABA from the rat thalamus (Korf & Venema, 1983), the mechanism(s) through which $GABA_B$ receptors in the frontal cortex are up-regulated by chronic treatment with antidepressants still remain to be elucidated. If indeed, a hypothesis relating $GABA_B$ receptors to the noradrenergic system in depression is valid, it could be that desipramine enhances the release of GABA from thalamic GABAergic neuronal projections to lamina I of the frontal cortex that may impinge upon noradrenergic terminals. In this situation, $GABA_B$ receptors would represent terminal autoreceptors on the thalamocortical projections which are up-regulated to provide an enhanced negative feedback mechanism compensating for an increased release of GABA. In turn, this would prevent endogenous GABA from reaching $GABA_B$ receptors situated presynaptically on noradrenergic terminals, thus enhancing the release of noradrenaline and leading ultimately to beta-adrenoceptor down-regulation together with a putative 'antidepressant' effect.

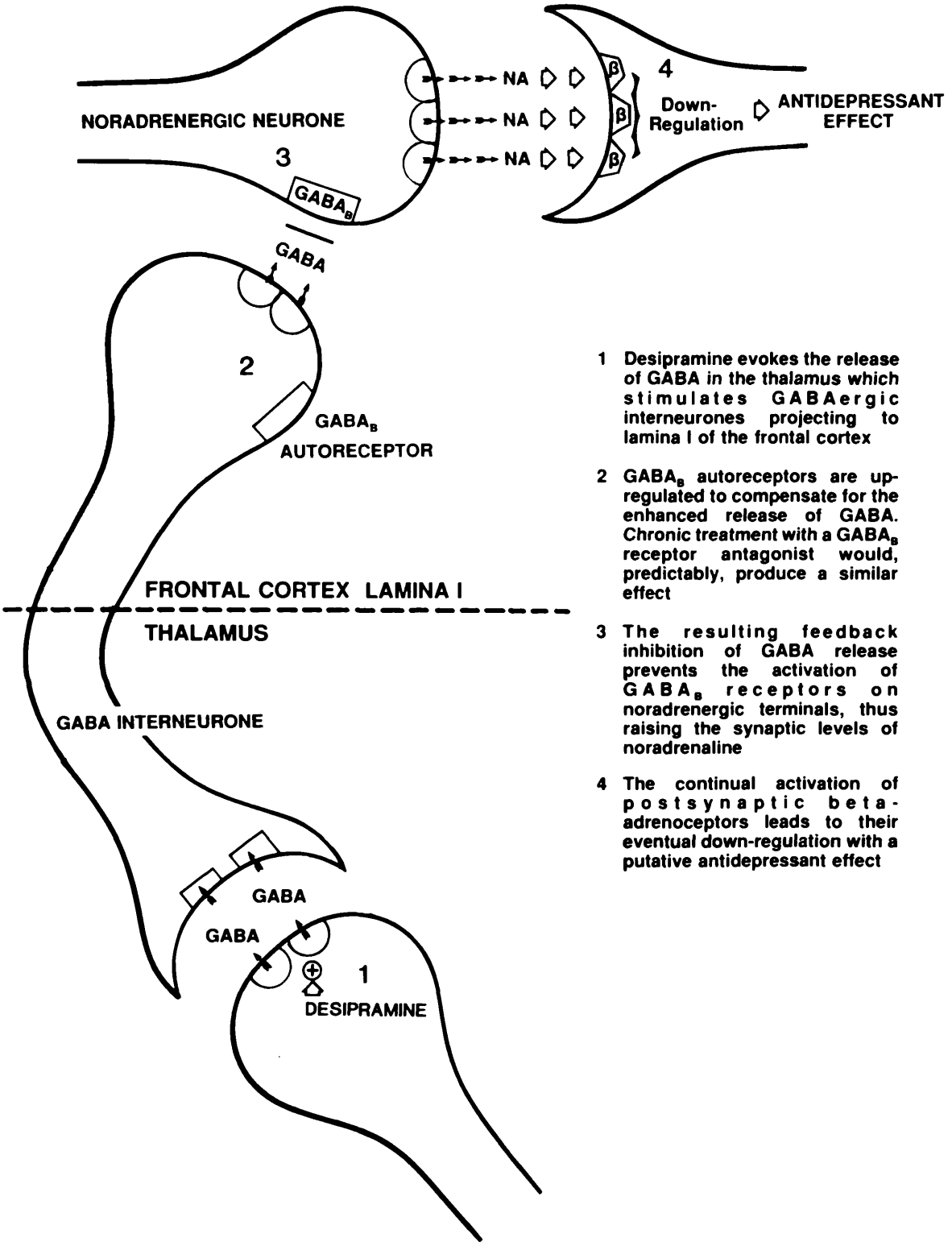
However, since GABA is an inhibitory neurotransmitter, desipramine-induced GABA efflux in the thalamus is unlikely to facilitate further release in the frontal cortex through *direct* thalamocortical projections. This may, however, be achieved indirectly, possibly through GABAergic disinhibition, via multiple interneuronal connections. Alternately, since desipramine also enhances the efflux of glutamate and aspartate from the thalamus (Korf & Venema, 1983), these excitatory neurotransmitters could activate their respective receptors, possibly situated on the cell bodies of GABAergic interneurones, thereby effecting the direct release of GABA in the frontal cortex.

In relation to this hypothesis, one of the predicted effects of a $GABA_B$ receptor antagonist would be to inhibit the suppression of noradrenaline release induced by GABA acting at presynaptic $GABA_B$ receptors on noradrenergic terminals. Although there is no experimental evidence in support of this at present, CGP 35348 has been shown inhibit the release of [^{3}H]GABA from cortical slices (Waldmeier & Baumann, 1990). The $GABA_B$ receptors increased following chronic treatment with Compound X, again, are likely to represent presynaptic $GABA_B$ autoreceptors. Persistent occupation of a receptor by an antagonist would eventually lead to a compensatory 'supersensitisation', blocking the release of GABA by negative feedback to these autoreceptors. If, on the other hand, this phenomenon was applied to those $GABA_B$ receptors on noradrenergic nerve terminals, their increased sensitivity would still enable endogenous GABA to inhibit the release of noradrenaline, thus preventing an 'antidepressant' action (Figure 5.11). Such a scenario may also indicate the presence of separate $GABA_B$ receptor sub-types for which a $GABA_B$ receptor antagonist would exhibit selectivity.

It should be stressed that these hypotheses are purely speculative at this stage and do not account for any possible modulations of post-synaptic $GABA_B$ receptors or for that matter, changes in other brain regions. Nonetheless, it is hoped that these findings will provide a significant step forward in our understanding of the role of $GABA_B$ receptors in depression.

Figure 5.11 opposite: page 225

A hypothetical schematic representation of the possible role of $GABA_B$ receptors in antidepressant action.



1 Desipramine evokes the release of GABA in the thalamus which stimulates GABAergic interneurones projecting to lamina I of the frontal cortex

2 $GABA_B$ autoreceptors are up-regulated to compensate for the enhanced release of GABA. Chronic treatment with a $GABA_B$ receptor antagonist would, predictably, produce a similar effect

3 The resulting feedback inhibition of GABA release prevents the activation of $GABA_B$ receptors on noradrenergic terminals, thus raising the synaptic levels of noradrenaline

4 The continual activation of postsynaptic beta-adrenoceptors leads to their eventual down-regulation with a putative antidepressant effect

CHAPTER 6

$GABA_B$ RECEPTOR-MODULATION OF ADENYLYL CYCLASE ACTIVITY

INTRODUCTION

The functional consequences of $GABA_B$ receptor stimulation with respect to the production of cAMP, mediated through the activation of adenylyl cyclase, have been well-characterised over recent years. In fact, $GABA_B$ receptors are coupled to this effector system through both negative and positive transduction mechanisms. Baclofen inhibits the direct activation of adenylyl cyclase activity by forskolin (Wojcik & Neff, 1984; Karbon & Enna, 1985; Hill, 1985) and potentiates the accumulation of cAMP produced in response to noradrenaline in brain slices (Karbon & Enna, 1985; Hill, 1985).

In Chapter 3, the $GABA_B$ receptor agonist, 3-aminopropylphosphinic acid (3-APA), was shown to be more potent than (-)-baclofen in its ability to displace [^{3}H]GABA binding from $GABA_B$ receptor sites, assessed both autoradiographically and in whole brain synaptic membranes. The aim of ths chapter will be to firstly characterise the action of 3-APA on both forskolin- and noradrenaline-stimulated adenylyl cyclase systems comparing its potency with (-)-baclofen. Secondly, the preceding chapter demonstrated that the modulation of $GABA_B$ receptor binding sites following repeated administration of desipramine and the putative $GABA_B$ receptor antagonist, Compound X, was discretely localised in the outer lamina (I) of the frontal cortex. It had been hypothesised that such an up-regulation might be detected through the $GABA_B$ receptor-linked adenylyl cyclase transduction mechanisms whereby an increase in the number of $GABA_B$ binding sites should confer a greater respective inhibition or enhancement of forskolin- and noradrenaline-stimulated cAMP production by (-)-baclofen. The effects of the compounds administered in the previous chapter (Chapter 5) were therefore examined concomitantly on these $GABA_B$ receptor-linked second messenger responses.

RESULTS

Characteristics of $GABA_B$ receptor-modulated adenylyl cyclase activity

Direct activation of the catalytic subunit of the adenylyl cyclase enzyme with forskolin produced a large increase in the amount of cAMP accumulated by slices of rat cerebral cortex (Figure 6.0A). The dose response relationship was linear over a range of approximately 3-20μM and although concentrations above 30μM were not tested, the levels of cAMP produced appeared to begin to plateau at this concentration. An EC_{50} value was approximated to be in the range of 10μM (2.90 $\pm$ 0.36 pmol cAMP/min/mg protein) and this concentration was subsequently employed in future experiments. In the presence of (-)-baclofen (100μM), the dose response to forskolin was markedly suppressed which at a concentration of 10μM, amounted to 38% inhibition of the control response ($P< 0.05$, Student's 't' test; n=3).

By comparison, the dose response to noradrenaline-stimulated cAMP formation was much shallower than that to forskolin, beginning to plateau at around 100μM (Figure 6.0B). Although the amount of accumulated cAMP only afforded two-thirds of the levels produced in response to 10μM forskolin (2.00 $\pm$ 0.18 pmol cAMP/min/mg protein), this concentration was employed in subsequent experiments. The ability of (-)-baclofen to potentiate noradrenaline-stimulated adenylyl cyclase activity was clearly evident, amounting to a 100% increase over the control levels ($P< 0.01$, Student's 't' test; n=3).

A representative time course for cAMP accumulation in response to adenylyl cyclase activation by either forskolin (10μM) or noradrenaline (100μM) is shown in Figure 6.1 A,B). In both cases the formation of cAMP continued to increase after an incubation period of up to 30 minutes. In the presence of (-)-baclofen (100μM) cAMP levels produced in response to forskolin were lower at all time points, whereas conversely, the levels

obtained in the presence of noradrenaline were augmented, again at all time points. An incubation period of 10 minutes for both assay systems was selected for future experiments.

The relationship between protein concentration and cAMP generated in response to forskolin and noradrenaline is shown in Figure 6.2. Increasing the amounts of tissue by 25µl aliquots produced similar incremental increases in both basal cAMP concentrations and also following adenylyl cyclase activation. The amounts of cAMP generated were corrected for protein concentrations of 400, 800 and 1200µg, determined from 25, 50 and 75µl aliquots of packed tissue slices by the method of Bradford et al (1976). For future experiments, 50µl aliquots of cross-chopped cortical slices were employed.

Characterisation of $GABA_B$ receptor agonists on transmitter-coupled adenylyl cyclase activation

The modulatory effects of (-)-baclofen on forskolin and noradrenaline-stimulated adenylyl cyclase activity were further characterised to establish EC_{50} values in each respective system, in parallel with the $GABA_B$ receptor agonist 3-APA.

Figure 6.3A shows that inhibition of the forskolin-induced formation of cAMP by (-)-baclofen was concentration-dependent with an EC_{50} value of 2.23 $\pm$ 0.89µM (n=5). Such a dose relationship was also evident in the noradrenaline-stimulated system (Figure 6.3B) where (-)-baclofen (100µM) potentiated control responses by 76% (EC_{50} = 6.01 $\pm$ 1.47µM; n=5). (-)-Baclofen was without effect on basal cAMP levels. The stereoselectivity of these $GABA_B$ receptor-mediated actions was demonstrated by the inability of the (+)-enantiomer of baclofen (100µM) to significantly alter forskolin or noradrenaline responses.

3-APA was equieffective with (-)-baclofen behaving as a full agonist to inhibit forskolin-stimulated adenylyl cyclase (EC_{50} = 3.09 $\pm$ 1.18µM; n=5).

By contrast, 3-APA afforded only a 60% potentiation (EC_{50} = 25.4 $\pm$ 7.3) of the maximal effect produced by (-)-baclofen indicating that this compound appeared to act as a partial agonist.

A partial agonist would be expected to reduce the response to a full agonist and this could be demonstrated with 3-APA. In the presence of a maximal concentration of 3-APA (100μM), the potentiation of noradrenaline by a submaximal concentration of (-)-baclofen (20μM) was significantly reduced ($P < 0.05$, Student's 't' test; n=5; Figure 6.4).

Effects of repeated antidepressant treatment on $GABA_B$ receptor transduction

This study was performed in parallel with the fourth investigation described in Chapter 5 with the omission of paroxetine.

Table 6.0 provides a summary of the effects of orally administered desipramine, amitriptyline and baclofen in addition to intraperitoneally injected desipramine, CGP 35348 and Compound X on $GABA_B$ receptor-modulation of forskolin and noradrenaline-stimulated adenylyl cyclase.

The responses to both forskolin- and noradrenaline-stimulated adenylyl cyclase in control tissue did not significantly differ after either oral or intraperitoneal vehicle administration. With the exception of tissue from desipramine-treated (i.p.) rats, the forskolin-induced increase in cAMP formation was significantly inhibited by (-)-baclofen (100μM) after all of the above treatments. The reason for the apparent insensitivity of desipramine-treated tissue to forskolin was unclear, but may have been a consequence of a gross, general reduction in the populations of adenylyl cyclase enzymes.

Similarly, the augmentation of noradrenaline-stimulated cAMP production induced by (-)-baclofen amounted in most instances to around 100%, except in the case of desipramine (i.p.) where cAMP levels were enhanced by only 66%. Moreover, the responsiveness of adenylyl cyclase to

noradrenaline stimulation alone, was significantly reduced following repeated administration of amitriptyline (p.o.) and desipramine (i.p.).

Although protracted treatment with the $GABA_B$ receptor antagonist, CGP 35348, failed to influence the forskolin-stimulated system, in contrast, a significant increase in the accumulation of cAMP produced in response to forskolin was seen after treatment with Compound X. Since the response to (-)-baclofen did not significantly differ from control tissue, this might suggest an increase in the $GABA_B$ receptor-mediated effect.

Figure 6.0

Characteristics of $GABA_B$ receptor-modulated adenylyl cyclase activity. (A) Activation of adenylyl cyclase with forskolin (O) produced a concentration-dependent increase in the amount of cAMP accumulated. Although concentrations above 10μM forskolin were not tested, an EC_{50} was approximated to be in the range of 10μM (2.90 ± 0.36pmol cAMP/min/mg protein). In the presence of (-)-baclofen (100μM) (●) the dose response to forskolin was suppressed, amounting to 38% inhibition of the control response at a concentration of 10μM. (B) Noradrenaline produced a concentration-dependent increase in the amount of accumulated cAMP which in the presence of 100μM (-)-baclofen was augmented by around 100% above control levels. Data points represent the mean ± s.e.m. of three separate experiments for which triplicate determinations were made. The amounts of accumulated cAMP were corrected for basal levels of 0.38 ± 0.02 pmol/min/mg protein.

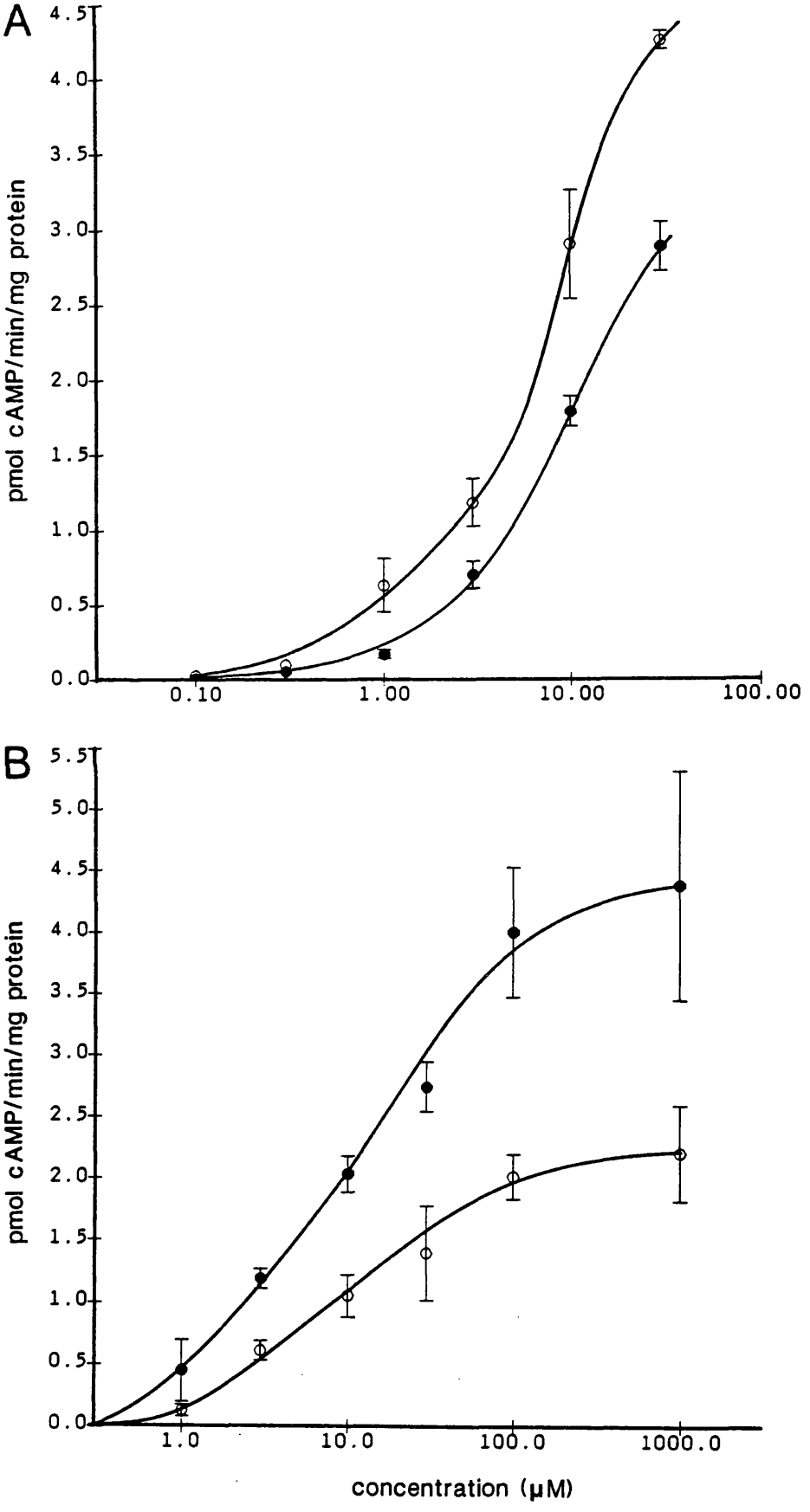

A
B
pmol cAMP/min/mg protein
pmol cAMP/min/mg protein
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
0.10
1.00
10.00
100.00
1.0
10.0
100.0
1000.0
concentration (μM)

Figure 6.1

Characteristics of $GABA_B$ receptor-modulated adenylyl cyclase activity. Time course for the accumulation of cAMP produced through the activation of adenylyl cyclase in response to (A) forskolin (10μM) and (B) noradrenaline (100μM) (O). In both instances the formation of cAMP continued to increase up to 30 minutes after the onset of the incubation. In the presence of (100μM) (-)-baclofen (●), the action of forskolin was suppressed, whilst conversely, an augmentation of the noradrenergic response was observed. The amounts of cAMP shown were corrected for basal levels of 0.53 $\pm$ 0.07 pmol/mg protein which did not significantly differ over the 30 minute incubation period.

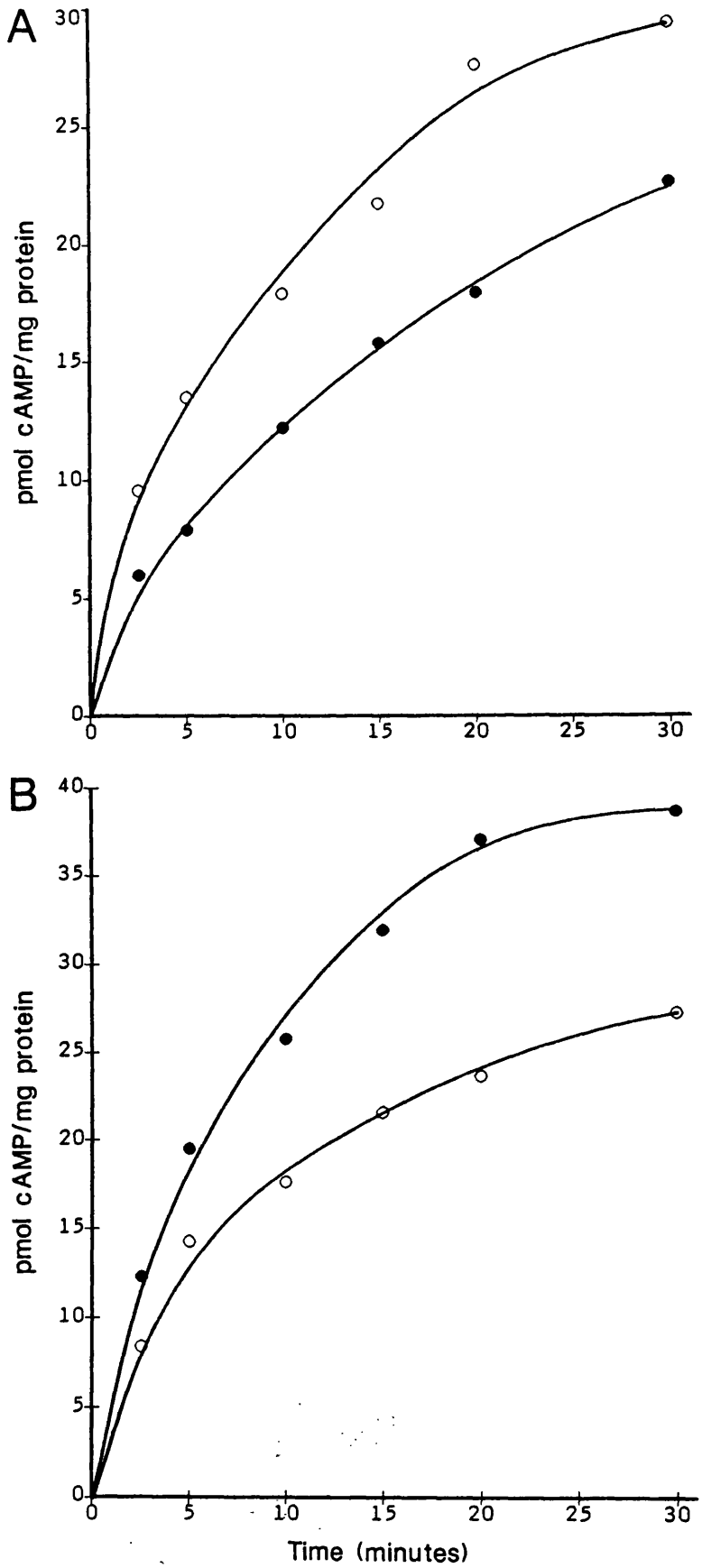

A
B
pmol cAMP/mg protein
pmol cAMP/mg protein
Time (minutes)
0
5
10
15
20
25
30
35
40

Figure 6.2

Characteristics of $GABA_B$ receptor-modulated adenylyl cyclase activity. The relationship between protein concentration and amount of cAMP produced in response to forskolin and noradrenaline. The amounts of cAMP produced from 25, 50 and 75µl aliquots of packed cross-chopped slices of rat cerebral cortex were corrected to respective protein concentrations of 400 (open bars), 800 (hatched bars) and 1200µg (cross-hatched bars). Accumulated cAMP was clearly a function of protein concentration, for which increasing the amounts of tissue by 25µl aliquots produced incremental increases in both basal cAMP concentrations and under conditions of adenylyl cyclase activation. Data shown represent the mean $\pm$ s.e.m. of three separate experiments.

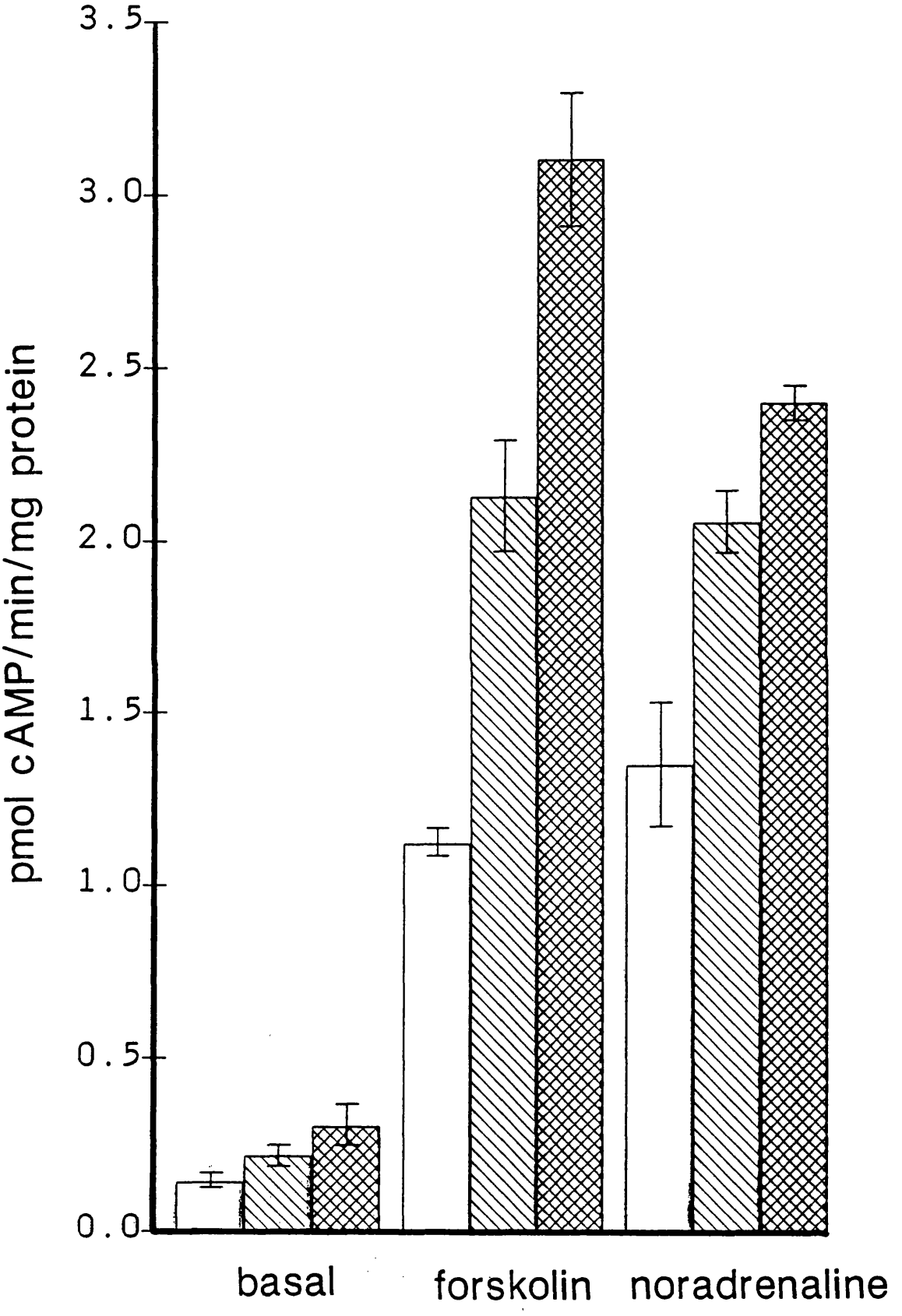

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
pmol cAMP/min/mg protein
basal
forskolin
noradrenaline

Figure 6.3

Characterisation of $GABA_B$ receptor agonists on forskolin- and noradrenaline stimulated adenylyl cyclase activity. (A) The inhibitory effects of (-)-baclofen (○) and 3-aminopropylphosphinic acid (3-APA; ●) on forskolin-stimulated adenylyl cyclase. With respective EC_{50} values of 2.23 ± 0.89μM and 3.09 ± 1.18μM, these $GABA_B$ receptor agonists were clearly equipotent. By contrast, on the noradrenaline-activated system (B), whilst (-)-baclofen appeared to act as a full agonist to potentiate the control response (EC_{50} = 6.01 ± 1.47μM), the augmentation attributable to 3-APA was only partial by comparison (EC_{50} = 25.4 ± 7.3μM). Although the effects of these agonists are expressed in terms of either percentage inhibition or potentiation of forskolin and noradrenaline responses, the EC_{50} values were calculated from the actual concentrations of cAMP produced, corrected for basal levels of 0.22 ± 0.02 pmol/min/mg protein. Data points represent the mean ± s.e.m. of five separate experiments.

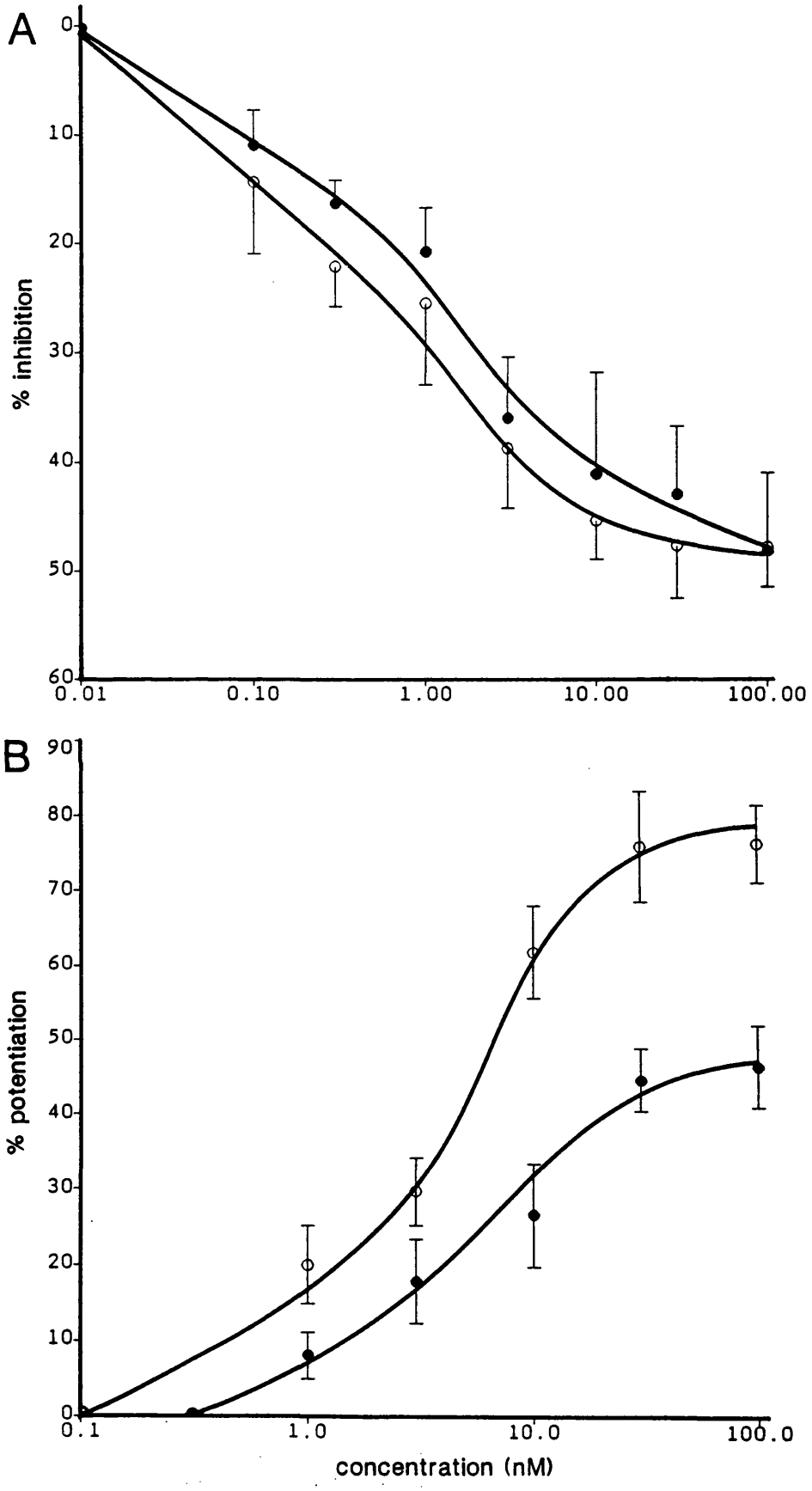

A
% inhibition
0
10
20
30
40
50
60
0.01
0.10
1.00
10.00
100.00
B
% potentiation
90
80
70
60
50
40
30
20
10
0
0.1
1.0
10.0
100.0
concentration (nM)

Figure 6.4

Demonstration of the partial agonism exerted by 3-aminopropylphosphinic acid (3-APA). In the presence of a maximal concentration of 3-APA (100μM), the potentiation of the effect of noradrenaline by a sub-maximal concentration of (-)-baclofen (20μM) was significantly reduced (* $P < 0.05$, Student's 't' test; $n = 5$).

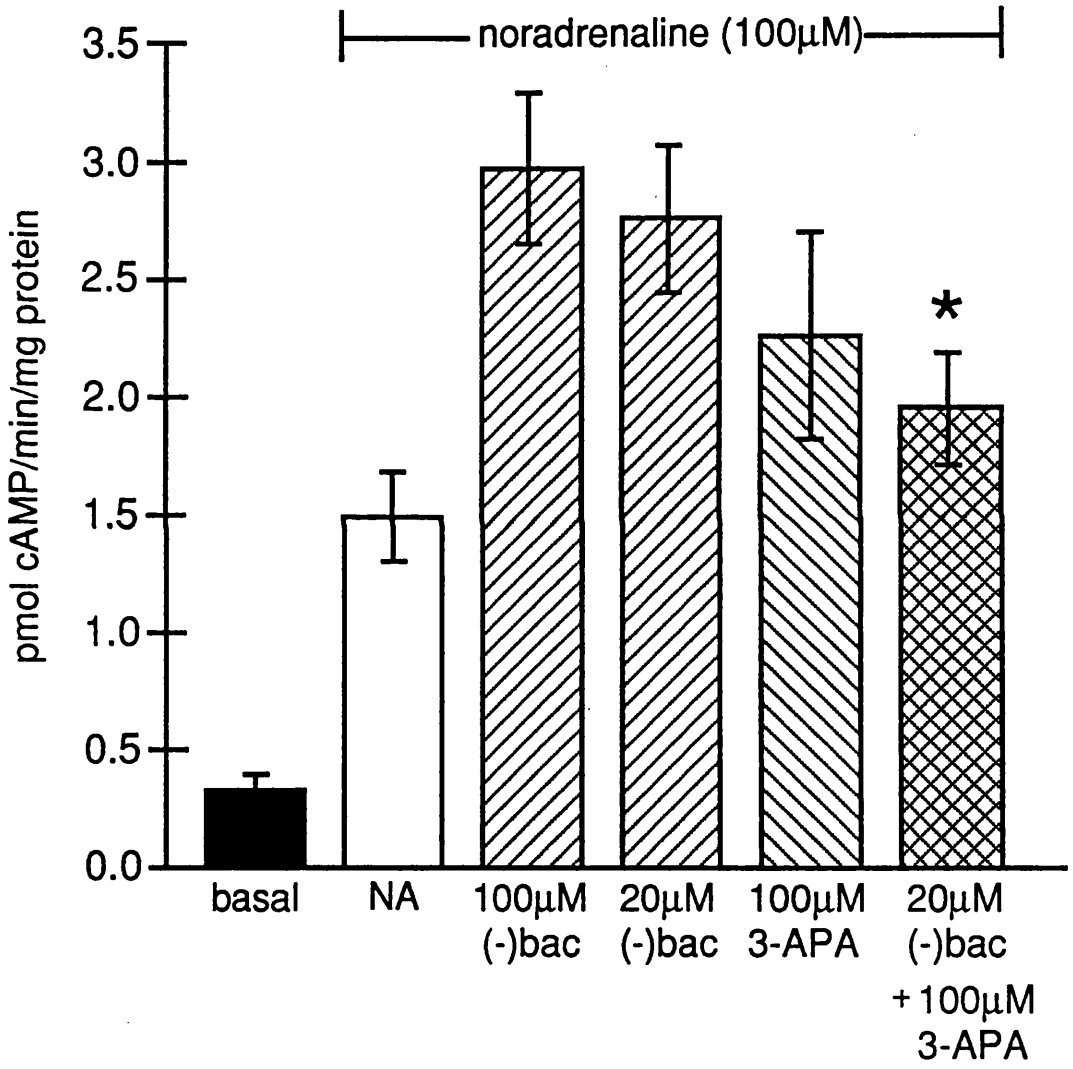

noradrenaline (100μM)
pmol cAMP/min/mg protein
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
basal
NA
100μM (-)bac
20μM (-)bac
100μM 3-APA
20μM (-)bac + 100μM 3-APA

Table 6.0 **The effects of protracted treatment with antidepressants and $GABA_B$ receptor antagonists on $GABA_B$ receptor-modulated forskolin- and noradrenaline-stimulated adenylyl cyclase activity**

TREATMENT	DOSE $mgkg^{-1}$	n	Forskolin		Noradrenaline	
			alone	+ (-)-baclofen	alone	+ (-)-baclofen
Control (p.o.)		5	3.77 ± 0.51	2.18 ± 0.33[b]	1.08 ± 0.19	2.04 ± 0.42[b]
Desipramine	20 p.o.	4	4.64 ± 0.11	3.82 ± 0.34[b,d]	0.94 ± 0.12	1.90 ± 0.31[c]
Amitriptyline	30 p.o.	4	2.67 ± 0.38	1.70 ± 0.23[c]	0.45 ± 0.05[a]	0.88 ± 0.11[c]
Baclofen	10 p.o.	5	4.68 ± 1.07	2.62 ± 0.42[b]	0.83 ± 0.14	1.50 ± 0.18[c]
Control (i.p.)		5	3.92 ± 0.47	2.63 ± 0.28[b]	1.18 ± 0.14	2.05 ± 0.24[c]
Desipramine	10 i.p.	3	1.50 ± 0.42[a]	1.80 ± 0.56	0.38 ± 0.07[a]	0.60 ± 0.07[b,d]
CGP 35348	100 i.p.	5	3.69 ± 0.36	2.79 ± 0.18[b]	1.06 ± 0.08	1.76 ± 0.21[c]
Compound X	100 i.p.	5	5.72 ± 0.29[a]	3.38 ± 0.43[c]	1.23 ± 0.06	2.38 ± 0.16[c]

values represent the mean (± s.e.m) amounts of cAMP accumulated (pmol/min/mg protein)

a $P< 0.01$ forskolin/noradrenaline stimulation alone vs. control
b $P< 0.05$ forskolin/noradrenaline stimulation vs. (-)-baclofen (100μM)
c $P< 0.01$ forskolin/noradrenaline stimulation vs. (-)-baclofen (100μM)
d $P< 0.01$ (-)-baclofen response (100μM), drug-treatment vs. control

DISCUSSION

Previous reports (Karbon & Enna, 1985; Hill, 1985; Robinson et al, 1989) demonstrating the ability of (-)-baclofen to modulate signal transduction through $GABA_B$ receptor-linked adenylyl cyclase systems either directly, in the case of forskolin, or indirectly, via the activation of adrenoceptors by noradrenaline, have been confirmed by the present study. The dose response profile to forskolin in terms of the activation of adenylyl cyclase agreed closely with the findings of Karbon & Enna (1985) and Hill (1985) where, at a concentration of 10μM (employed routinely in the present study), submaximal amounts of cAMP were generated. By including (-)-baclofen in the incubation solution, an attenuation of the amount of cAMP produced in response to forskolin (amounting to a 38% reduction) was produced. This consequence of $GABA_B$ receptor activation was clearly stereoselective since the degree of inhibition by (+)-baclofen was insignificant.

It was perhaps intriguing that a complete (-)-baclofen-induced inhibition of forskolin-activated adenylyl cyclase did not occur (up to a concentration of 100μM). Such an observation has also been reported by Hill (1985), Xu & Wojcik (1986) and Robinson et al (1989) where the maximal inhibition rarely exceeded 50%. Since the effect of baclofen on this system is pertussis toxin-sensitive (Xu & Wojcik, 1986), one plausible explanation could reside in the possible existence of $GABA_B$ receptor subtypes that are not necessarily linked through G_i proteins. However, it is more likely that since forskolin is not selective in its action solely for $GABA_B$ receptor-linked adenylyl cyclase enzyme subunits, cAMP levels formed in response to this agent are likely to arise through the activation of other GTP-linked receptors such as α_2-adrenergic, dopamine D-2 and adenosine-1 receptors (Rodbell, 1980). The implication of this is that high concentrations of (-)-baclofen *do* in fact completely block the effects of

forskolin through $GABA_B$ receptors but that this is masked by the cAMP produced in response to the activation of other receptor classes.

Although Robinson et al (1989) observed a concentration-dependent forskolin-induced production of cAMP, the response in their assay system saturated at a concentration of 1.5μM. The reasons for the lower efficacy of forskolin are not immediately apparent since the methodology employed was essentially similar to that of the present study. One obvious difference was that the phosphodiesterase III inhibitor, 3-isobutylmethylxanthine (IBMX) was included in the forskolin (but not the isoprenaline) assay to prevent the hydrolysis of accumulated cAMP. Hill (1985) had previously shown that (-)-baclofen possesses at least a 100-fold greater potency than its corresponding (+)-enantiomer at inhibiting forskolin-stimulated adenylyl cyclase, thus demonstrating the stereoselectivity of this agonist for $GABA_B$ receptors. However, cAMP levels generated in the presence of IBMX were only slightly higher than in its absence, with no significant differences being observed under these two conditions. Moreover, the degrees of inhibition of forskolin by (-)-baclofen and (±)-baclofen were the same. For these reasons, therefore, IBMX was not included in the assays of the current study.

This stereoselectivity of baclofen for $GABA_B$ receptors observed in rat brain slices has also been demonstrated in cerebellar synaptic membranes (Wojcik & Neff, 1984) and also in cultured cerebellar neurones (Xu & Wojcik, 1986). In both instances the assays were performed in the presence of IBMX. It was therefore surprising that Robinson et al (1989) found that both (-)-baclofen and (+)-baclofen were equipotent at inhibiting forskolin-stimulated adenylyl cyclase activity. These authors concluded that due to this lack of stereoselectivity, in addition to the inability of the $GABA_B$ receptor antagonist, phaclofen, to block the action of baclofen, forskolin-stimulated cAMP accumulation is not influenced by $GABA_B$ receptors. It is felt that such a statement is rather misleading since phaclofen (albeit

selective for $GABA_B$ receptors) is a very weak antagonist. Moreover, the forskolin concentration employed routinely (0.5μM) by Robinson et al (1989) produced less than a 2-fold increase in cAMP from basal levels. This concentration in the current study produced only a modest increase in cAMP accumulation which contrasted considerably with the 7.5-fold enhancement mediated by 10μM forskolin. It is feasible, therefore, that in order to observe a more substantial interaction between $GABA_B$ receptors and adenylyl cyclase activation, a much stronger stimulatory signal is required.

Noradrenaline enhanced cAMP production in rat brain slices in a concentration-dependent manner, although its potency was somewhat weaker than that of forskolin, producing only a 4-fold increase in accumulated cAMP at a concentration of 100μM. The dose response profile for noradrenaline closely resembled that reported by Karbon & Enna (1984) and Hill (1985). Whilst noradrenaline activates the production of cAMP by acting through both alpha- and beta-adrenoceptors (Daly et al, 1980), the lack of effect of baclofen on the stimulatory response to the α_1-adrenoceptor agonist, phenylephrine, would suggest that the augmentation of cAMP levels produced in response to noradrenaline in the presence of (-)-baclofen arise as a consequence of a transduction coupling mechanism between both $GABA_B$ receptors and beta-adrenoceptors. The magnitude of the (-)-baclofen-induced enhancement of cAMP amounted to a 100% increase above control levels. Furthermore, this response was stereoselective, the (+)-enantiomer of baclofen (100μM) producing less than a 10% potentiation. Again, these findings concur with previous observations (Karbon & Enna, 1985; Hill, 1985) and provide functional confirmation of receptor binding data (Bowery et al, 1983, 1985; Bittiger et al, 1988).

Since the amounts of cAMP produced in response to adenylyl cyclase activation are usually expressed as a function of protein concentration, it

was necessary to demonstrate that the protein concentrations of aliquotted tissue slices added to each assay were not excessive. The importance of not allowing the cAMP generating system to be saturated by the tissue substrate has particular relevance when comparing the effects of drug treatments on the sensitivities of adenylyl cyclase-linked receptors. If the tissue/protein concentrations are not optimised, such changes, if subtle, may pass undetected. The linearity of the relationship between protein concentration and the amounts of cAMP produced in response to both forskolin and noradrenaline was clearly apparent in the present study (Figure 6.2).

Characterisation of 3-APA as a $GABA_B$ receptor agonist

Incubation of forskolin with increasing amounts of (-)-baclofen, inhibited the accumulation of cAMP in a concentration-dependent manner reaching a maximal effect of 50% at 100μM. An EC_{50} value of 2..2μM was in close proximity to the reports by Xu & Wojcik (1986) (5μM) and Robinson et al (1989) (7.9μM). The dose response profile for 3-APA was remarkably similar to that of (-)-baclofen, signifying its interaction as a full agonist at $GABA_B$ receptors. Conversely, the maximum potentiation induced by (-)-baclofen on the noradrenaline-stimulated accumulation of cAMP, was not achieved with 3-APA. In fact, only a partial response was obtained. From this observation, it was concluded that 3-APA acts as a partial agonist on the noradrenaline-stimulated adenylyl cyclase system. Indeed, this pharmacological property was confirmed by the ability off 3-APA to antagonise the potentiation of a control noradrenaline response produced by a submaximal concentration of (-)-baclofen. Although no other reports exist as to the effects of 3-APA on $GABA_B$ receptor-mediated modulation of adenylyl cyclase activity, the electrophysiological studies of Seabrook et al (1990) add support to the findings of the present study in that compared with baclofen, 3-APA again exhibited a partial agonistic action in

depressing glutamate-mediated striatal EPSPs. Furthermore, the phosphonic acid derivative of GABA, 3-aminopropylphosphonic acid (3-APPA) mimicks (-)-baclofen at inhibiting the action of forskolin whilst having no effect on isoprenaline-stimulated cAMP formation (Scherer et al (1988). This evidence, together with that of the present study, confirms the notion that those $GABA_B$ receptors linked to the two adenylyl cyclase systems are likely to represent distinct heterogeneous populations. It should also be noted that although concentrations of 3-APA greater than 100μM were not examined, the possibility exists that 3-APA may, in fact, be a full agonist on the noradrenergic system. Even if this were to be true, this $GABA_B$ receptor agonist would nevertheless, possess a higher EC_{50} than (-)-baclofen, again providing a possible index of $GABA_B$ receptor subclasses. The potency of 3-APA to displace the binding of [^{3}H]GABA from $GABA_B$ binding sites has been reported to be approximately 10 times greater than that of (-)-baclofen (IC_{50} = 3.4nM, Chapter 3). The present study therefore highlights the importance of not relying solely on receptor binding data for the characterisation of a particular compound since in this functional $GABA_B$ receptor model, there was approximately a 1000-fold shift in potency. Thus, although a ligand may have a high affinity for a specific binding site, this does not necessarily represent a functional receptor.

Drug-induced changes in adenylyl cyclase sensitivity

In an attempt to provide a functional index of $GABA_B$ receptor modulation, the sensitivities of both forskolin- and noradrenaline-stimulated adenylyl cyclase systems were examined in brain slices following repeated oral administration of desipramine, amitriptyline and baclofen, in addition to intraperitoneally administered desipramine and the $GABA_B$ receptor antagonists, CGP 35348 and Compound X.

The sensitivity of adrenergic receptors to noradrenaline stimulation was significantly reduced after chronic treatment with both desipramine

(i.p.) and amitriptyline, which was likely to be a consequence of down-regulated beta-adrenoceptors (Minneman et al, 1979; Beer et al, 1987; Heal et al, 1989). Since the ability of (-)-baclofen to enhance the effect of noradrenaline was unaltered following these treatments, a drug-induced modulation of these particular $GABA_B$ receptors was not evident. Suzdak & Gianutsos (1986) reported that intraperitoneal treatment with baclofen for 14 days decreased the sensitivity of beta-adrenoceptors to noradrenaline activation in addition to suppressing the ability of baclofen to augment this response. Although there was some indication that such an effect may have occurred in the present study, significant differences from control values were not obtained. Furthermore, no additional drug-induced modulations of the $GABA_B$ receptor-mediated noradrenergic potentiation of beta-adrenoceptor activation were detected.

Focussing on the forskolin-activated adenylyl cyclase system, with the exception of desipramine (i.p.), the (-)-baclofen-induced inhibition of activated cAMP production was evident following all of the chronic treatments. The preceding chapter reported that protracted treatment with desipramine (p.o. and i.p.) as well as Compound X, significantly up-regulated $GABA_B$ receptor sites in the outer lamina (I) of the frontal cortex. It was anticipated that such an increase in the population of $GABA_B$ receptors would also be demonstrated by an enhancement of the ability of (-)-baclofen to inhibit the stimulatory action of forskolin. In the case of desipramine (p.o.), the levels of cAMP produced in response to forskolin alone were not significantly increased after this treatment although in the presence of (-)-baclofen, the amount of accumulated cAMP was significantly greater than the control value. This may reflect $GABA_B$ receptor up-regulation, although the percentage inhibition of the forskolin response was reduced by this treatment from 42% (control) to only 18%.

Conversely, protracted treatment with Compound X, significantly increased the levels of cAMP formed by forskolin alone. Moreover,

although the percentage inhibition of this effect by baclofen was enhanced to 41% from 33% in control tissue, unlike desipramine, the increased cAMP levels observed in the presence of (-)-baclofen after treatment with Compound X were not significantly different from the control response. This would be indicative of an enhancement of the $GABA_B$ receptor-mediated effect and provides a functional correlate of the $GABA_B$ receptor up-regulation induced by Compound X (described in the preceding chapter). Moreover, since treatment with Compound X failed to modulate those $GABA_B$ receptors associated functionally with the noradrenaline-stimulated adenylyl cyclase system, this further supports the contention that the $GABA_B$ receptors linked with these two transduction mechanisms are almost certainly heterogeneous.

One limitation of the methodology of the adenylyl cyclase assay was that tissue from the frontoparietal region of the cerebral cortex had been employed. This would have the likely effect of diluting any discretely localised $GABA_B$ receptor modulations which although are inferred by the current study, could perhaps be consolidated in future experiments from a more precise dissection of the appropriate brain region.

FUTURE DIRECTIONS

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The structural components of the dorsal vagal complex have formed a central feature of this thesis providing the neuroanatomical substrate in which the relative distributions of 5-HT_3, $GABA_B$ and $GABA_A$ receptor sites were described in Chapters 3 and 4. With regard to the latter GABAergic sites, although there was no apparent clear demarcation of the distribution of $GABA_B$ receptor sites between the nucleus tractus solitarius (NTS) and the underlying dorsal motor nucleus of the vagus nerve (DMNX), $GABA_A$ receptor sites, by contrast, appeared to be restricted to the DMNX. In order to obtain a more precise delineation between both $GABA_B$ and $GABA_A$ receptors in these hindbrain regions, one proposed future study would be to perform autoradiographical comparisons of [^{3}H]GABA (under conditions to label both $GABA_B$ and $GABA_A$ receptors) in conjunction with the receptor site-selective radioligands, [^{3}H]baclofen and [^{3}H]muscimol. Secondly, preliminary studies (performed by Dr. J.-M. Fritschy, University of Zürich) using the monoclonal antibody bd-24, have revealed high levels of antigenic recognition sites for the α_1 subunit of the $GABA_A$ receptor to be located especially in the DMNX, with comparatively low levels in the overlying NTS. Such immunohistochemical observations therefore add support to the autoradiographical findings reported in Chapter 4 and together with the technique of *in situ* hybridisation, the relative distributions of the various $GABA_A$ receptor subunits within the dorsal vagal complex could be elucidated. At the present time, since no antibodies exist specifically against $GABA_B$ recognition sites, their detailed localisation can only be confined to autoradiographical techniques.

The use of four 5-HT_3 receptor radioligands has enabled the precise characterisation of 5-HT_3 recognition sites in rat dorsal vagal complex, the highest concentrations of such sites being located in the NTS. With regard to the antiemetic properties of 5-HT_3 receptor antagonists, the central

locations of their recognition sites is of particular interest, especially in attempting to assess their functionality in preventing emesis. Although the rat does not exhibit a complete emetic response (unlike the ferret), their 5-HT_3 receptor sites present in the NTS could, nevertheless, represent functional receptors involved with the integration of vagal afferent stimuli. *In vivo* microdialysis would provide an appropriate means for measuring the release of 5-HT (and other neurotransmitters) within the NTS of the rat following vagal stimulation and indeed, subsequent to the intravenous administration of cisplatin to ferrets. By co-administering 5-HT_3 receptor antagonists in parallel with cytotoxic therapy, their effects on the central release of neurotransmitters could be assessed. Such an approach may therefore provide further insights into the possible central mechanisms of 5-HT_3 receptor function.

Of the antidepressants examined in the four investigations described in Chapter 5, only desipramine produced a consistent, discretely localised $GABA_B$ receptor up-regulation in lamina I of the frontal cortex, coupled with a concomitant down-regulation of $beta_1$-adrenoceptors. Similar observations were made after the repeated administration of the putative $GABA_B$ receptor antagonist, Compound X (although the beta-adrenoceptor down-regulation could not be attributed to the $beta_1$-adrenoceptor subtype. Since desipramine is selective for the inhibition of noradrenaline uptake, these findings substantiate a link between both noradrenergic and GABAergic systems. How such adaptive receptor changes interact to produce possible antidepressant activity is, at the present time, purely speculative. One such hypothesis (presented in Chapter 5) relies on the ability of desipramine to release GABA and/or glutamate/aspartate from the thalamus (Korf & Venema, 1983) which then mediate an enhanced release of GABA from thalamocortical interneurones impinging on noradrenergic neurones in lamina I of the frontal cortex. The increase in

$GABA_B$ receptors observed after such treatment (also after Compound X), are thought to represent terminal autoreceptors located on these interneurones which compensate for the enhanced release of GABA via negative feedback mechanisms. Whether or not such a hypothesis were true could be examined following surgical or chemical lesioning (with for example, kainic acid) of certain thalamic nuclei such as the reticular nucleus which should prevent the observed localised $GABA_B$ receptor changes. It would also seem appropriate to investigate the effects of Compound X in conjunction with desipramine on the turnover of both noradrenaline and GABA in these two brain regions.

Although the majority of published studies have examined the effects of antidepressants on $GABA_B$ receptors only in frontal cortical membranes, it could be that such receptor changes occur in other brain regions, for example of the limbic system. In light of this, Lloyd et al (1985) reported significant increases in $GABA_B$ receptor binding in hippocampal membranes following desipramine treatment (also after viloxazine and citalopram) and indeed, such measurements would form the basis of further autoradiographical studies.

Based on electrophysiological and biochemical observations, the evidence for $GABA_B$ receptor heterogeneity is becoming increasingly clear and will certainly form a central focus of the next phase in $GABA_B$ receptor pharmacology. From the adenylyl cyclase studies described in Chapter 6, experimental findings implicating the possible discrimination of $GABA_B$ receptor subtypes was seen to emerge. It would appear that the $GABA_B$ receptor agonist, 3-APA, is able to differentiate between those $GABA_B$ receptors linked with two separate adenylyl cyclase transduction mechanisms. Whilst it exhibits full agonist activity (in parallel with (-)-baclofen) on the forskolin-stimulated system, it only potentiates noradrenaline-activated adenylyl cyclase, submaximally, (again, compared

with (-)-baclofen, conferring upon itself, partial agonist character. Additional evidence supporting the contention that these second messenger-linked $GABA_B$ receptors are in fact heterogeneous, stems from preliminary observations that the methyl derivative of 3-APA (3-aminopropyl(methyl)phosphinic acid (referred to also as SKF 97541; see Seabrook et al, 1990) is equieffective with (-)-baclofen on the forskolin-activated system but apparently inactive at potentiating noradrenaline-stimulated cAMP accumulation. The situation is further complicated since the $GABA_B$ receptor antagonist, CGP 35348, again from preliminary studies, potently antagonises the action of (-)-baclofen on noradrenaline-stimulated adenylyl cyclase but is, in contrast, only a weak antagonist in the forskolin-activated system. Clearly, these findings require further consolidation by a future series of experiments.

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Read, not to contradict and confute,
nor to believe and take for granted,
nor to find talk and discourse
but to weigh and consider.

Francis Bacon (1561-1626)