AUTORADIOGRAPHIC STUDY OF THE DISTRIBUTION OF 5-HT RE-UPTAKE SITES IN RAT AND HUMAN BRAIN

By

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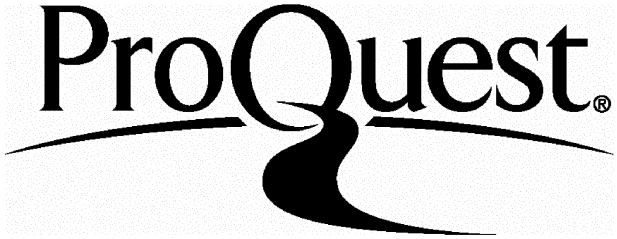

THIS THESIS IS DEDICATED TO MY MOTHER
PAMELA GIBSON TAYLOR
(1939 - 1995)

I have had worse partings, but none that so
Gnaws at my mind still. Perhaps it is roughly
Saying what God alone could perfectly show -
How selfhood begins with a walking away,
And love is proved in the letting go.

C. Day Lewis

ABSTRACT

The selective serotonin re-uptake inhibitors (SSRI's) are a novel group of antidepressants which are rapidly becoming a popular choice of drug for the successful treatment of depression. The hypothesis of their mode of action, however, in conjunction with the cause of depression, remains subject to many inconsistencies. By using autoradiography to determine the distribution of the recognition sites of these drugs in the rat brain, it may be possible to detect discrete alterations in the serotonin re-uptake site which are not revealed in membrane binding studies. In the human brain, isolated areas of tissue have been used to provide some details of the sites of recognition of the SSRI's, however these studies have been limited by technical problems.

Experimental protocols were established for the use of the SSRI ^{3}H-paroxetine with autoradiography. The distribution of ^{3}H-paroxetine binding sites in the rat brain revealed a heterogeneity of binding site densities, with the highest binding levels observed in the raphe nuclei in the brainstem. Direct injection of 5,7-dihydroxytryptamine (a selective serotonergic neurotoxin) into the dorsal raphe produced a reduction in ^{3}H-paroxetine binding levels throughout the brain, thereby confirming the selectivity of ^{3}H-paroxetine for the serotonin nerve terminal transporter site.

A novel method of performing autoradiography in human whole brain sections was developed. By modifying a technique originally used for cutting whole body animal sections, it was possible to cryosection whole brain coronal slices (40μm) for use in autoradiography, whilst preserving the integrity of the brain tissue. The distribution of ^{3}H-paroxetine binding sites within the human brain was determined and the symmetry of these binding sites established. A species difference in the localisation of the serotonin re-uptake site in rat and human brain was detected in a comparative study using ^{3}H-paroxetine.

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GLOSSARY

5-HIA	5-hydroxyindoleacetaldehyde
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
5,7-DHT	5,7-dihydroxytryptamine
8-OH-DPAT	8-hydroxy-2(N-dipropylamino)tertralin
BSA	Bovine serum albumin
CMC	Carboxymethylcellulose
CS	Crude synaptic membrane fraction
CDR	Dorsal raphe, caudal portion
DMSO	Dimethylsulphoxide
HIV	Human immunodeficiency virus
i.c.v.	Intracerebroventriclar
i.p.	Intraperitoneal
i.v.	Intraventricular
LSD	d-Lysergic Acid Diethylamide
MAO	Monoamine oxidase
MDA	Methamphetamine
MDMA	Methylendioxymethamphetamine
MLDs	Mouse minimum lethal doses
MM	Mitochondrial-myelin fraction
nu	Nucleus/nuclei
P_1	Crude nuclear fraction
P_2	Crude membrane fraction
PCA	p-chloroamphetamine
PCPA	p-chlorophenylalanine
PI	Polyethylenimine
RDR	Dorsal raphe, rostral portion
s.e.m.	Standard error of the mean
SBP	Serotonin binding protein

SSRI	Selective serotonin re-uptake inhibitor
WB	Whole brain homogenate

Abbreviations of rat and human brain structures

Am	Amygdala
Aq	Aqueduct
ATh	Anterior thalamic nucleus
BP	Basal pons
BLA	Basolateral amygdala
CA1,2,3,	Fields of Ammon's horn
CDR	Dorsal raphe, caudal portion
Ce	Cerebellum
cg	cingulum
CG	Central grey
Cl	Claustrum
CM	Centromedial thalamic nuclei
CN	Caudate nuclear body
CPu	Caudate putamen
DG	Dentate gyrus
DMH	Dorsomedial hypothalamus
DR	Dorsal raphe nucleus
Ent	Entorhinal cortex
Fac	Facial nuclei
fi	Fimbria
Fr	Frontal cortex
GP	Globus pallidus
Hi	Hippocampus
Hy	Hypothalamus
IC	Inferior colliculus
ICj	Islands of Calleja
IMD	Intermediodorsal thalamic nuclei

IP	Interpeduncular nuclei
LaA	Lateral amygdala
LC	Locus coeruleus
LG	Lateral geniculate
LH	Lateral hypothalamus
LHb	Lateral habenula
LP	Lateroposterior thalamic nuclei
LPB	Lateral parabrachial nuclei
LV	Lateral ventricle
mfb	Medial forebrain bundle
MG	Medial geniculate
MHb	Medial habenula
MnR	Median raphe nuclei
Oc	Occipital cortex
Par	Parietal cortex
Pu	Putamen
PVP	Paraventricular thalamic nuclei
py	Pyramidal tract
R	Red nucleus
RDR	Dorsal raphe, rostral portion
Re	Reuniens nucleus
Rh	Rhomboid nucleus
RMg	Raphe magnus
SCN	Superior central raphe nuclei
SN	Substantia nigra
Su	Superficial layer of the superior colliculus
Te	Temporal cortex
Tr	Trigeminal tract
VL	Venterolateral thalamic nuclei
VMH	Ventromedial hypothalamus
VP	Venteroposterior thalamic nuclei
VTA	Ventral tegmental area

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PRESENTATIONS ARISING FROM THIS THESIS

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Miller J., Bowery N.G. and Tulloch I. (1994). Autoradiographic comparison of the distribution of ^{3}H-paroxetine binding sites in rat and human brain. Neuropsychopharmacology **10,** 223S.

Miller J., Bowery N.G. and Tulloch I. (1995). An autoradiographic comparison of ^{3}H-paroxetine and ^{3}H-imipramine in human whole brain sections. European Congress of Neuropsychopharmacology (ECNP), Venice, Italy. (Poster to be presented)

CHAPTER ONE

GENERAL INTRODUCTION

INTRODUCTION

In 1942, a Swiss chemist, Albert Hofmann, accidently ingested a small quantity of d-Lysergic Acid Diethylamide (LSD) and experienced a feeling of intoxication with optical distortions, vertigo, restlessness and vivid imaginings. Determined to prove his suspicions that it was LSD he had ingested, he purposely swallowed a 0.25mg dose (far higher than necessary for a repetition of his previous experience). This time he experienced an extravaganza of psychedelic reactions. Further research into the effects of LSD suggested that this may be related to schizophrenic symptoms. When it was subsequently noted that serotonin and LSD were chemically related, it became apparent that serotonin might have a profound effect on the psychological well-being of a person.

Evidence for the role of serotonin in mood disorders resulted in the hypothesis that a reduction in brain serotonergic activity was involved in the aetiology of depression (see review by Meltzer and Lowy, 1987; Meltzer, 1989). This theory was substantiated by evidence that: (1) reserpine which depletes serotonin content in storage vesicles causes depression; (2) levels of 5-hydroxyindoleacetic acid (5-HIAA) were reduced in the cerebrospinal fluid of depressed patients (Brown and Linnoila, 1990; Coppen and Doogan, 1988; Faustman et al, 1990; Grahame-Smith, 1988); (3) plasma tryptophan or the ratio of total or free plasma tryptophan to large neutral amino acids may be reduced in depression (De Meyer et al, 1981; Delgado et al, 1990; Møller et al, 1980; 1986 and 1990); (4) platelet serotonin content and uptake is reduced in depression (Butler and Leonard, 1990; Coppen et al, 1978; Goodnick et al, 1995; Sheline et al, 1995) and (5) the fact that drugs which act to increase serotonin levels (monoamine oxidase inhibitors, serotonin re-uptake inhibitors) are successful in treating depression (Åberg-Wistedt, 1989; Kasper et al, 1992; Tyrer and Marsden, 1985).

These are just a few examples of serotonergic involvement in depression, and while serotonin is only a team player in the complexities of depressive illness, it is central to gaining an understanding of the cause of depression (Meltzer and

Lowy, 1987). In particular, the development of selective serotonin re-uptake inhibitors (SSRI's) has provided a host of new information regarding serotonin and depression. It is these drugs which are the focus of the investigations carried out within this thesis, however, in order to understand how and where they act, a brief overview of serotonin in the central nervous system is necessary.

1.1. SEROTONIN IN THE CENTRAL NERVOUS SYSTEM.

5-hydroxytryptamine (5-HT, serotonin) was first identified in the mammalian system in the mid-twentieth century. In rapid succession, it was isolated in platelets (Rapport, Green and Page, 1948), enterochromaffin cells in the gut and in the central nervous system (Sjoerdsma and Palfreyman, 1990). The name "serotonin" (serum tonic factor) was derived from its vasoconstrictive action when released from platelets during blood clotting. Twarog and Page (1953) reported the widespread distribution of serotonin in mammalian tissue, not only was it found in blood platelets, the gastrointestinal tract and the central nervous system, it was also present in the liver, skin, lungs and spleen. It has been estimated that the proportion of serotonin present in the human body is 90% in the digestive tract, 8-10% in blood platelets and 1-2% in nervous tissue (Feldman and Quenzer, 1985).

There are 3 main factors which characterize 5-HT as a neurotransmitter; the synthesis, storage and inactivation of 5-HT within the neuron, the localization of 5-HT containing cell bodies and axon terminals in the brain, and the ability of 5-HT to co-exist and interact with other neurotransmitters while having an intrinsic activity of its own.

1.1.1. 5-HT synthesis.

5-HT is synthesized from L-tryptophan which is actively taken up from blood plasma. In the neuron, tryptophan is hydroxylated by the enzyme tryptophan hydroxylase to form 5-hydroxytryptophan (5-HTP) which is then decarboxylated

by 5-HTP-decarboxylase to 5-hydroxytryptamine. Intracellular storage of 5-HT is carried out by an energy and ion dependant uptake process into serotonin storing secretory vesicles, where the 5-HT is bound to a specific serotonin binding protein (SBP); (Tamir and Gershon, 1990). Tryptophan hydroxylase is the rate-limiting step enzyme in the synthesis of 5-HT, however under normal physiological conditions it is not saturated. Therefore, 5-HT synthesis can be augmented by increasing the amount of free L-tryptophan in the nerve terminal. Equally, by elevating the amount of 5-HTP available, it is possible to increase 5-HT synthesis. The synthesis of 5-HT is inhibited by the administration of *p*-cholorphenylalanine (PCPA) which irreversibly blocks the action of tryptophan hydroxylase; and α-methlydopa and α-methyl 5-HTP which interfere with the action of 5-HTP decarboxylase

Inactivation of 5-HT released into the synaptic cleft through electrical stimulus (action potential) or drug action (amphetamine, fenfluramine) occurs primarily by an uptake process in the pre-synaptic terminal (Kuhar and Aghajanian, 1973). Once the 5-HT is returned to the neurone, it is either bound into storage vesicles or is subjected to oxidative deamination by monamine oxidase (MAO) which forms 5-hydroxyindoleacetaldehyde (5-HIA). This is further oxidized by aldehyde dehydrogenase to form the major inactive metabolite, 5-hydroxyindoleacetic acid (5-HIAA), which is excreted via the cerebrospinal fluid and bloodstream. Storage of 5-HT is disrupted by reserpine or tetrabenazine which deplete intracellular 5-HT content by exposing it to catabolism by MAO. This enzymatic breakdown can be prevented by MAO inhibitors such as pargyline and clorgyline. Inactivation by means of re-uptake of 5-HT into the neuron is prevented by re-uptake inhibitors (imipramine, paroxetine) and neurotoxins (p-chloroamphetamine, 5,7-dihydroxytryptamine).

1.1.2. Distribution of 5-HT in the mammalian nervous system.

The distribution of 5-HT cell bodies and terminals was first described by Dahlström and Fuxe (1964) using histochemical fluorescence. They developed the now commonly used "B group" annotation of the 5-HT containing cell

bodies in the brainstem, which give rise to axons that project throughout the brain (Fuxe, 1965). The use of histofluorescence techniques, however, was limited by low yields of fluorescence and photodecomposition resulting in incomplete visualization of fine serotonin-containing axons (Fuxe et al, 1968). The use of autoradiography to detect the distribution of ^{3}H-5HT binding sites in rat brain (Aghajanian and Bloom, 1967; Descarries et al, 1975; Parent et al, 1981) contributed to the organizational map of serotonergic innervation. Parent et al (1981) described 3 major pathways of serotonergic innervation. The descending serotonin system and 2 ascending pathways; the periventricular and transtegmental system. The origins of the descending pathway are found in the raphe magnus, raphe obscurus and raphe pallidus. These projections terminate in the dorsal horn, intermediate lateral columns and the motorneurons of the ventral horn of the spinal cord (Bowker et al, 1982). The major source of the ascending pathways are the dorsal and median raphe. The periventricular system originates in the rostral pole of the dorsal raphe, and extends upwards through the central grey. From here a distinct collateral emerges and ascends to the superior colliculi where it terminates in the superficial layer. The rest of the pathway follows the ventral surface of the brain with terminals in the hypothalamus. The transtegmental system originates in the dorsal and medial raphe and moves down through the raphe pontis to the interpeduncular nuclei. From here, most of the fibres turn and progress in a rostral direction through the ventral tegmental area and substantia nigra along the ventral surface of the brain. The two ascending pathways merge in the medial forebrain bundle to innervate the limbic system, diencephalon and forebrain with a heterogeneous pattern of fibre densities.

With the development of immunohistochemical techniques, the distribution of 5-HT innervation in the brain was further elucidated (Lidov et al, 1980, Azmitia and Gannon, 1986). Two principal types of axons are present in the forebrain of rats (Kosofsky and Molliver, 1978) and primates (Wilson et al, 1989) which originate from different regions in the brainstem. The axons which contain numerous thin, fusiform varicosities arise from the dorsal raphe and the thinner axons which contain larger, rounder "beaded" varicosities originate in the

median raphe. A third type of axon has been observed in the primate which is thick, non-varicose and can only be found in small numbers around the major fibre systems (Törk, 1990). A further distinction in serotonergic innervation was noted in the dorsal raphe (Baker et al, 1991), where projections from the rostral dorsal raphe innervate the cortex, septum, striatum, hypothalamus, thalamus and hippocampal gyrus. Projections from the caudal dorsal raphe innervate the hippocampus, locus coeruleus and entorhinal cortex. Fig 1.1 shows a schematic diagram of the serotonergic innervation of the rat brain. Although the serotonergic projections arising from the dorsal and median raphe appear to innervate different areas in forebrain (Imai et al, 1986; Jacobs et al, 1974; Kosofsky and Molliver, 1987) many of the serotonergic projections overlap with a greater contribution from either of the raphe nuclei influencing the morphological, neuroanatomical and pharmacological characteristics of the innervation in those areas (Bonvento et al, 1992; Hensler et al, 1994)

While the localization of 5-HT cell bodies is limited to the raphe nuclei in the brainstem, the terminal distribution of 5-HT fibres is detected in virtually every brain region, with the exception of the major fibre tracts such as the corpus callosum. In order to gain some idea of the extent of serotonergic innervation in the rat brain, quantification of 5-HT terminals estimated an average of 4.4×10^6, 2.6×10^6 and 2.7×10^6 5-HT varicosities per mm^3 of cerebral cortex (Doucet et al, 1988), neostriatum (Soghomonian et al, 1987) and hippocampus (Oleskavich and Descarries, 1990) respectively. Based on calculations of an estimated number of neurons per area and varicosities per cell body of origin, it could be inferred that each neuron issues an average number of axonal varicosities of at least 500 000 in the cerebral cortex, 60 000 in the neostriatum and 150 000 in the hippocampus (Descarries et al, 1990).

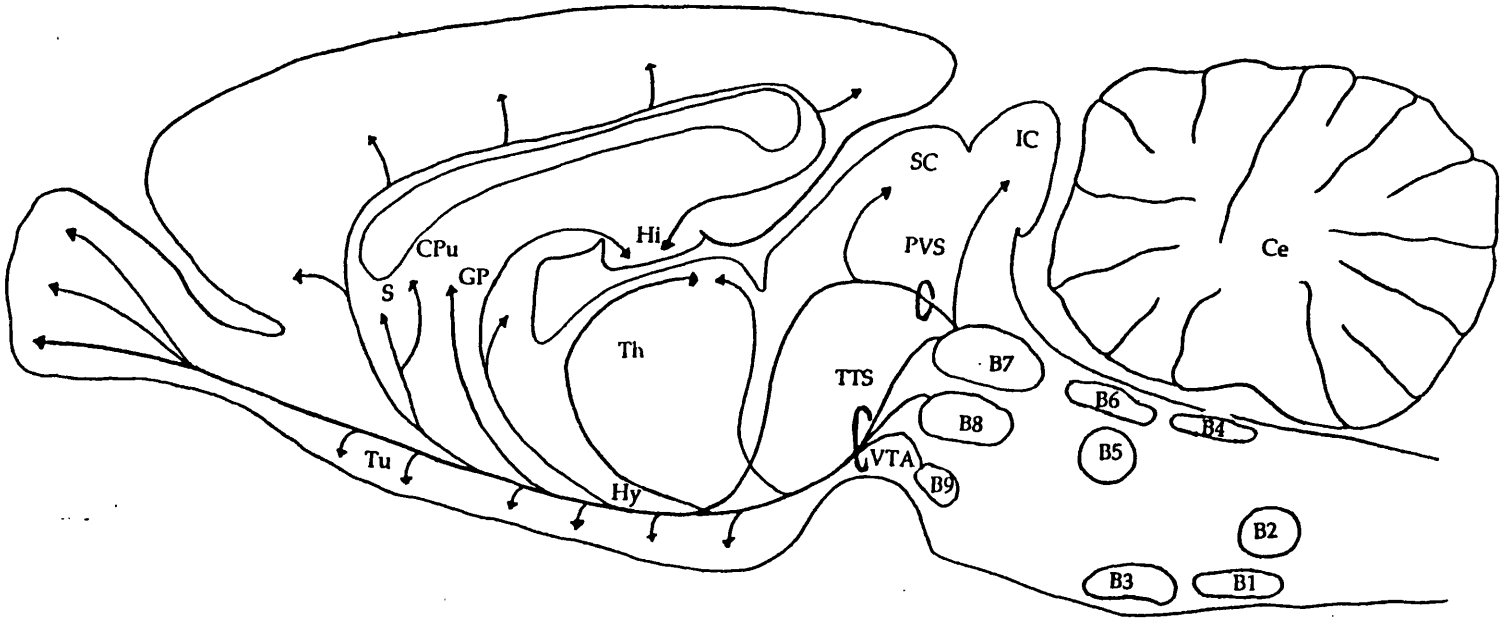


Fig. 1.1. Schematic representation of the major ascending 5-HT sytems in rat brain. PVS - periventricular system, TTS - transtegmental system; B1 - raphe pallidus, B2 - raphe obscurus, B3 - raphe magnus, B4 - raphe obscurus, dorsolateral, B5 - median raphe, caudal part, B6 - dorsal raphe, caudal part, B7 - dorsal raphe, rostral part, B8 - median raphe, rostral part, B9 - nucleus pontis, Ce - cerebellum, CPu - caudate putamen, GP - globus pallidus, Hi - hippocampus, Hy - hypothalamus, IC - inferior colliculus, S - septum, SC - superior colliculus, Th - Thalamus, Tu - olfactory tubercle. Adapted from Parent et al, 1981.

1.1.3. 5-HT receptors.

The first suggestion that there was more than one 5-HT receptor came from Gaddum and Picarelli (1957) who classified the receptor subtypes as 5-HTM (due to morphine blockage of acetylcholine release at nerve terminal by 5-HT) and 5-HTD (competitive inhibition of 5-HT-induced contractions of intestinal smooth muscle by dibenyline). Peroutka and Snyder (1979) subsequently identified two subtypes of the 5-HT receptor in membranes of the rat brain. ^{3}H-5HT was found to label the 5-HT_1 receptor with high affinity whereas ^{3}H-spiperone recognised the 5-HT_2 receptor and ^{3}H-LSD bound to both receptors with equal affinity. This secured the foundation for the classification of subtypes of 5-HT_1 and 5-HT_2 (5-HTD) receptors, with the 5-HTM receptor re-named as 5-HT_3 (Bradley et al, 1986). This classification was based on functional data obtained from radioligand binding studies, however, with the advancement of techniques in molecular biology, a host of new receptors (5-HT_4, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6 and 5-HT_7) have been discovered based on their recognition and transducer properties. This led to the proposal for a re-classification of the 5-HT receptors based on operational, transductional and structural characteristics (Humphrey et al, 1993). Table 1.1. shows a general classification of the current 5-HT receptors in the brain. Table 1.2. lists the distribution of 5-HT receptors in the human brain as determined by autoradiography.

For the purpose of this study details regarding each receptor subtype will be limited to the receptors relevant to the investigation. For a full review of the classification and molecular biology of 5-HT receptors see Martin and Humphrey (1994) and Boess and Martin (1994).

1.1.3.1. 5-HT_{1A} receptor.

5-HT_{1A} receptors can be found either as somatodendritic autoreceptors on the cell bodies of the raphe nuclei or as post-synaptic receptors in the terminal fields (Hall et al, 1985, Gozlan et al, 1993). Pre-synaptic 5-HT_{1A} receptors in

the dorsal and median raphe regulate 5-HT release via a negative feedback mechanism. The neuronal firing activity of serotonergic neurons is characterized by a spontaneous, slow, rhythmic discharge pattern (for review see: Jacobs and Azmitia, 1992). This highly regular discharge pattern appeared to be mediated via the 5-HT_{1A} receptors, as microiontophoretic or systemic administration of 5-HT_{1A} selective compounds were found to suppress the spontaneously firing dorsal raphe 5-HT neurons (Blier and De Montigny, 1983; Sprouse and Aghajanian, 1987; Vander Maelen and Wilderman, 1984). Confirmation of the 5-HT_{1A} dorsal raphe autoreceptor activity was shown when the synthesis and release of 5-HT in the hippocampus was reduced by administration of 8-OH-DPAT into the dorsal raphe (Hjorth and Magnusson, 1988; Hutson et al, 1989; Sharp et al, 1989).In addition, subcutaneous (s.c.) administration of 8-OH-DPAT reduced serotonin release in the frontal cortex and hippocampus (Carboni and Di Chiara, 1989; Hjorth and Auerbach, 1994a).

The inhibition of 5-HT release is mediated in a regional manner (Invernizzi et al, 1991a), where 5-HT release in the striatum is regulated by dorsal raphe autoreceptors and 5-HT release in the hippocampus is regulated predominantly by median raphe autoreceptors (Bonvento et al, 1992; Kreiss and Lucki, 1994). Lesion studies in rat brain revealed that the 5-HT_{1A} receptors elsewhere in the brain are located post-synaptically (Crino et al, 1990; Gozlan et al, 1983; Hall et al, 1985; Hensler et al, 1991; Lawrence et al, 1993a; Verge et al, 1985).

The 5-HT_{1A} receptor has been implicated in anxiety, where buspirone (Rickels et al, 1982) and 8-OH-DPAT (Higgens et al, 1988; Hogg et al, 1994) have shown an anxiolytic profile. This appears to be the result of stimulation of the autoreceptors in the raphe nuclei as post-synaptic 5-HT_{1A} receptors in the hippocampus play no role in alleviating anxiety.

Table 1.1. General classification of 5-HT receptors.

Receptor		Subtype	Radioligand	Agonist	Antagonist
5-HT_1	G-protein linked 7 transmembrane intronless ↓ cAMP	5-HT_{1A}	^{3}H-8-OH-DPAT	8-OH-DPAT	WAY100135
		5-HT_{1B}	^{125}I-GTI	CP93129	-
		5-HT_{1D}	^{125}I-GTI	sumatriptan	GR127935
		5-HT_{1E}	^{3}H-5HT	-	-
		5-HT_{1F}	^{125}I-LSD (+ mesulergine)	-	-
5-HT_2	G-protein linked 7 transmembrane introns and exons ↑ IP_3	5-HT_{2A}	^{3}H-ketanserin	α-methyl-5-HT	ketanserin
		5-HT_{2B}	^{3}H-5HT	α-methyl-5-HT	LY53857
		5-HT_{2C}	^{3}H-mesulergine	α-methyl-5-HT	mesulergine LY53857
5-HT_3	Ligand gated cation channel 4 hydrophobic regions	5-HT_3	^{3}H-zacopride	2-methyl-5-HT	ondansetron
5-HT_4	↑ cAMP	5-HT_4	^{3}H-GR113808	5-methoxy- tryptamine	GR113808
5-$ht_{5A,5B,6,7}$			^{125}I-LSD	-	-

Adaptation of information given in Martin and Humphrey (1994); Boess and Martin(1994).

Table 1.2. Regional distribution of 5-HT receptors in the human central nervous sytem.

	Receptor Subtype				
Binding site density	**5-HT$_{1A}$**[a]	**5-HT$_{1D}$**[b]	**5-HT$_{2A}$**[c]	**5-HT$_{2C}$**[d]	**5-HT$_3$**[e]
High	Hippocampus (CA1) Raphe nuclei Cortex (layer I, II)	Basal ganglia Substantia nigra Substantia gelatinosa	Cortex Hypothalamus	Choroid plexus	Substantia Gelatinosa Area postrema
Moderate	Amygdala Locus ceuruleus Central Grey	Thalamus Hypothalamus Striatum	Amygdala Claustrum Hippocampus	Basal ganglia Hippocampus Substantia nigra	Hippocampus Amygdala
Low	Basal ganglia Thalamus Hypothalamus Cerebellum	Amygdala Hippocampus Raphe nuclei Frontal Cortex	Raphe nuclei Substantia nigra Thalamus	Thalamus Amygdala Cerebellum	Cortex

[a] - Hoyer et al (1986a); Pazos et al (1987a), [b] -Waeber et al (1988); Palacios et al (1992); Bruinvels et al (1994), [c] -Hoyer et al (1986b), Pazos et al (1987b); Waeber et al (1994), [d] - Hoyer et al (1986b); Pazos et al (1987a), [e] - Abi-Dargham et al (1993), Waeber et al (1989).

1.1.3.2. 5-$HT_{1B/1D}$ receptor.

The 5-HT_{1B} receptor was initially identified in the rat brain and then in the brains of mouse, hamster and opossum (Deshmukh et al, 1982; Heuring et al, 1986; Pedigo et al, 1981). It was described as negatively coupled to adenylate cyclase and revealed a high density of binding sites in the basal ganglia and substantia nigra. While a receptor with identical features was identified in human, guinea pig, cat, cow and pig brains, it was found to exhibit pharmacologically distinct properties and was therefore called the 5-HT_{1D} receptors. This was considered to be the human version of the 5-HT_{1B} receptor (Hoyer and Middlemiss, 1989; Martial et al, 1989; Waeber et al, 1988a). Cloning of the receptors revealed two human 5-HT_{1D} receptors, 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$. The 5-$HT_{1D\beta}$ receptor displays a 92% homology with the rat 5-HT_{1B} receptor and a 63% amino acid identity with the human 5-$HT_{1D\alpha}$ receptor (Boess and Martin, 1994; Hartig et al, 1992). It is generally presumed that 5-HT_{1B} receptors are located pre-synaptically in the cortex, however 5,7-DHT lesions were found to increase binding to 5-HT_{1B} receptors, thereby suggesting the presence of post-synaptic 5-HT_{1B} receptors (Crino et al, 1990; Manrique et al, 1993).

Evidence that the 5-HT terminal autoreceptor is the 5-HT_{1B} subtype was proposed by Middlemiss (1984a) when it was shown that 8-OH-DPAT was inactive at the pre-synaptic 5-HT autoreceptor in rat brain frontal cortex slices (Middlemiss, 1984b). The 5-HT_{1B} pre-synaptic autoreceptor was confirmed by Engel et al (1986) and Maura et al (1986). Furthermore, the 5-HT_{1D} receptor in the guinea-pig was found to act as an autoreceptor in frontal cortex slices (Schipper et al, 1988). The concept that the 5-HT_{1B} and 5-HT_{1D} receptors are the 5-HT terminal autoreceptors and the 5-HT_{1A} receptors are the 5-HT somatodendritic autoreceptors is not entirely clear-cut however, as recent evidence has shown that the 5-HT_{1D} receptor in the guinea-pig also acts as a functional autoreceptor in the dorsal raphe nucleus (Starkey and Skingle, 1994)

1.1.3.3. 5-HT_2 receptor.

5-HT_{2A} receptors are located post-synaptically on 5-HT neurons and are predominantly labelled with 5-HT_2 receptor antagonists such as ^{3}H-ketanserin, ^{3}H-spiperone and ^{3}H-mianserin (Martin and Humphrey, 1994). Chronic administration of the antidepressants, mianserin and imipramine, in the rat were found to downregulate the 5-HT_2 receptor with a simultaneous reduction of 5-HT stimulated phosphoinositide (PI) hydrolysis. Therefore it was suggested that 5-HT_2 receptors play some role in the pathogenesis of depression (Conn and Sanders-Bush, 1986; Kendall and Nahorski, 1985). The effects of long-term treatment with the newer antidepressants (SSRI's), however, revealed that PI hydrolysis could be altered without affecting the 5-HT_2 receptor site (Cadogan et al, 1993; Sanders-Bush et al, 1989). Equally so, 5-HT_2 receptors were found to be reduced (Gross-Isserhof et al, 1990, Cheetham et al, 1988), increased (Arango et al, 1990; Hrdina et al, 1993; Mann et al, 1986) or unchanged (Crow et al, 1984; Lowther et al, 1994; Owen et al, 1986) in different brain regions of suicide victims. Additional evidence of 5-HT_2 receptor involvement with Alzheimer's disease (Crow et al. 1984) and schizophrenia (Arora and Meltzer, 1991; Joyce et al, 1993; Laruelle et al, 1993) make it clear that 5-HT_2 receptors are implicated in a modulatory role in neurological disorders.

1.2. THE SEROTONIN RE-UPTAKE SYSTEM.

The successful use of selective serotonin re-uptake inhibitors has shown that the serotonin transporter plays a major role in defining the aetiology and treatment of depression. By investigating the drugs which act at the serotonin re-uptake site, it may be possible to gain a further understanding of serotonin and its role in affective disorders.

There are two distinct mechanisms for the uptake of serotonin in neuronal tissue. The serotonin transporter present on vesicle membranes is

characterised by its sensitivity to reserpine and tetrabenazine and the fact that vesicular uptake in unaffected by neurotoxic lesions of serotonergic neurons (Ross, 1982; Slotkin et al, 1978). The second transport mechanism is found on neuronal membranes and is distinguished by its sensitivity to cocaine, imipramine and the SSRI's, and the inhibition of binding by these drugs following lesions of the 5-HT terminal (Marcusson and Ross, 1990; Ross, 1982). In this thesis, reference to the serotonin transporter will be limited to the 5-HT neuron terminal and platelet transport site.

1.2.1. Molecular biology of the serotonin transporter.

The complementary DNA encoding the serotonin transporter has been isolated in neuronal and non-neuronal tissue of both rat and human. The DNA sequence of the serotonin transporter in the rat tissue predicted a protein of between 607 and 653 amino acids with a molecular weight of 68 000 to 73 000 Da. Analysis identified 11-13 putative transmembrane domains with two potential sites for N-glycosylation on a large, extracellular loop between the 3rd and 4th hydrophillic domains (Blakely et al, 1991; Hoffman et al, 1991). An identical human serotonin transporter site was identified by Lesch et al (1993a) in neuronal tissue and platelets containing 630 amino acids with 12 transmembrane segments and a molecular weight of 70 320 Da. In addition to the two glycosylation sites indicated in the rat, two phosphorylation sites for cAMP-dependant protein kinase recognition and three potential phosphorylation sites for protein kinase C recognition were identified. The human transporter site was found to be 92% analogous to the rat serotonin transporter (Lesch et al, 1993a; Lesch et al, 1993b), and identical to the human platelet 5-HT transporter (Lesch et al, 1995).

1.2.2. Serotonin uptake by astrocytes.

The high affinity uptake of serotonin into astrocytes has been demonstrated in primary cultures and *in situ* (Katz and Kimelberg, 1985; Kimelberg, 1986;

Anderson et al, 1992). This uptake mechanism appears to be Na^+ and Cl^- dependent and sensitive to fluoxetine (Dave and Kimelberg, 1994; Katz and Kimelberg, 1985). The absence of pargyline reduced the Na^+ dependant uptake of ^{3}H-5-HT in neonatal rat cerebral cortical primary astrocyte cultures suggesting that the process of monoamine oxidase metabolism of 5-HT to 5-HIAA is also present in astrocytes (Katz and Kimelberg, 1985). Glial uptake in different brain regions revealed that there are differences in the regional distribution of ^{3}H-5-HT uptake (Amundson et al, 1992), however this did not compare well with the distribution of serotonin re-uptake sites in neuronal tissue *in situ*. The brainstem shows a very high density of 5-HT re-uptake binding sites as labelled with ^{3}H-paroxetine ,^{3}H-citalopram and ^{3}H-imipramine (D'Amato et al, 1987; De Souza and Kuyatt, 1987; Hrdina et al, 1990), whereas it was ranked second lowest in the glial uptake distribution. Glial ^{3}H-5-HT uptake was highest in the hippocampus and then the cerebral cortex, areas which contain only a moderate to low number of 5-HT re-uptake sites. Both astrocytes and neuronal tissue showed a low distribution of binding sites in the cerebellum (Amundson et al, 1992). The pharmacological action of serotonin uptake inhibitors on ^{3}H-imipramine binding sites in brain astroglial cells did not correlate with the inhibition of ^{3}H-serotonin uptake in the same cells (Whitaker et al, 1983), and therefore it can be assumed that ^{3}H-imipramine binding sites in astrocytes do not match the recognition sites on the serotonin transporter in nerve terminals.

The fact that the serotonin uptake sites in glial and neuronal tissue do not show identical pharmacological or distribution profiles suggests that the astrocyte serotonin transport mechanism may not be related to the neuronal transporter. Until such time as the role of the astrocyte serotonin transporter has been clarified, it is not possible to determine whether it is the same as the neuronal transporter. It would be prudent, however, to bear in mind this glial binding capacity when performing radioligand binding experiments with serotonin re-uptake inhibitors in brain tissue.

1.2.3. The serotonin re-uptake inhibitors.

The noradrenaline hypothesis of depression was proposed by Schildkraut in 1969, however with increasing evidence that the tricyclic antidepressants also exerted a serotonin re-uptake blocking action, the serotonin hypothesis was also proposed (Carlsson et al, 1969a and 1969b; Lapin and Oxenkrug, 1969). The serotonin hypothesis gained popularity with the development of a new group of antidepressants, the selective serotonin re-uptake inhibitors which include: paroxetine (Buus Lassen 1978a and 1978b); citalopram (Hyttel, 1977); fluoxetine (Wong et al, 1974a and 1975); sertraline (Koe et al, 1983); and fluvoxamine (Claasen, 1977). These drugs were found to selectively block the re-uptake of serotonin with very little activity at other neurotransmitter uptake systems or receptor sites (Claasen, 1983; Hyttel, 1982; Koe et al, 1990; Magnusson et al, 1982; Thomas et al, 1987; Wong et al, 1982).

1.2.3.1. Binding sites of the serotonin re-uptake inhibitors.

While the binding sites for drugs acting at the 5-HT re-uptake site are thought to be located on the same protein molecule as the serotonin transporter, it is becoming apparent that these binding sites are situated at different positions within the transport protein (Briley et al, 1981a; Plenge et al, 1990; Marcusson and Ross, 1990). Characterization of a single site model of radioligand binding (^{3}H-paroxetine, ^{3}H-citalopram) to the 5-HT re-uptake site indicates that there is a common binding site for the 5-HT re-uptake inhibitors and 5-HT itself (Arranz and Marcusson, 1994; Marcusson et al, 1989; D'Amato et al, 1987; Plenge and Mellerup; 1991). The differences in dissociation rates of ^{3}H-paroxetine, ^{3}H-citalopram and ^{3}H-imipramine, however, suggest that in addition to the common site, the 5-HT uptake inhibitors also bind simultaneously to individual allosteric sites thereby inducing a conformational change in the serotonin transporter (Plenge et al, 1991; Erreboe et al, 1995).

1.2.3.2. Ion dependence

The translocation of serotonin into the neuron involves a 4 step process. Na^+, serotonin and Cl^- bind to the transport site which instigates a conformational change in the carrier to allow the dissociation of Na^+, 5-HT and Cl^- inside the membrane. Intracellular K^+ then binds to the carrier which reverses the conformational change to relocate back to the external surface of the neuron (Ross, 1982; Marcusson and Ross, 1990).

The impetus required for the translocation of the serotonin carrier is the presence of a Na^+ gradient. Sodium dependence of both serotonin and ligand binding to the transport site has been shown with ^{3}H-5HT (Talvenheimo et al, 1983; Wood et al, 1987), ^{3}H-imipramine (Briley et al, 1981b; Bäckström and Marcusson, 1987) and ^{3}H-paroxetine (Cool et al, 1990; Mann and Hrdina, 1992) although the nature of the sodium requirement is not clear. ^{3}H-5HT and ^{3}H-paroxetine binding requires a 1:1 ratio to Na^+ (Wood 1987; Mann and Hrdina, 1992), whereas some studies using ^{3}H-imipramine have shown a 1:2 ratio of binding to sodium ions (Humphreys et al, 1994; Talvenheimo et al, 1983). It is possible that the second Na^+ ion is required for binding to an additional site close to, but separate from, the transporter. As the low affinity component of ^{3}H-imipramine binding (displaced by desipramine) is not dependent on the presence of NaCl, it is unlikely that this component is related to the serotonin transporter (Bäckström and Marcusson, 1987).

The role of Cl^- in the serotonin transporter is unclear. Cl^- is not necessary for serotonin or ligand binding to the transport site, however the presence of Cl^- is required for the translocation process (Ross, 1982). While ^{3}H-imipramine binding occurs in the absence of Cl^-, it does increase the affinity of ^{3}H-imipramine binding (Talvenheimo et al, 1983; Humphreys et al, 1994). It is thought to achieve this by inhibiting the Na^+ and ^{3}H-imipramine dissociation, thereby slowing ^{3}H-imipramine dissociation from the binding site (Humphreys et al, 1994). ^{3}H-paroxetine binding, on the other hand, is unaffected by the

presence or absence of Cl^- (Cool et al, 1990). Therefore it is possible that Cl^- acts in conjunction with the additional Na^+ to implement ^{3}H-imipramine binding to a putative allosteric site.

1.2.3.3. Temperature Dependence.

The effect of increasing incubation temperature (0°C to 20°C) reduces the affinity of ^{3}H-imipramine binding to the 5-HT re-uptake site but does not alter the B_{max} of ^{3}H-imipramine binding (Plenge and Mellerup, 1984; Segonzac et al, 1987). The rapid dissociation rate of ^{3}H-imipramine from the transporter at 20°C ($t_{½}$ = 5 minutes as opposed to 60 minutes at 37°C) suggests that this may be the reason for the decreased affinity (Segonzac et al, 1987). In contrast, the affinity of ^{3}H-paroxetine and ^{3}H-citalopram binding increases with an increase in incubation temperature (Plenge and Mellerup, 1991; Plenge and Mellerup, 1984). At 0°C the association and dissociation rates for these ligands were very slow (> 10 hours), however, at 20°C ^{3}H-paroxetine reached equilibrium within 3 hours and ^{3}H-citalopram within 1 hour. At 20°C and 37°C, the B_{max} of ^{3}H-paroxetine remained the same as did the B_{max} for ^{3}H-citalopram at 0°C and 20°C (Plenge and Mellerup, 1991; Segonzac et al, 1987). From these results it can be assumed that while the affinity of these radioligands binding to the 5-HT re-uptake site is temperature dependent and this may reflect a conformational change in the binding site. The stability of B_{max} values indicates that the number of available binding sites on the transport proteins is independent of temperature changes.

1.2.4. Consequences of serotonin re-uptake inhibition by SSRI's.

1.2.4.1. 5-HT and 5-HIAA.

a) Acute effects of inhibition.

The acute effect of serotonin re-uptake inhibition is to increase the amount of

extracellular 5-HT in the synaptic gap (fluvoxamine, sertraline - Cacci et al, 1993; fluoxetine, sertraline - Rutter and Auerbach, 1993; citalopram - Hjorth, 1993; fluoxetine - Guan and McBride, 1988; Daily et al, 1992; Perry and Fuller, 1992; Fuller et al, 1988) and plasma (fluoxetine, fluvoxamine, sertraline, paroxetine - Ortiz and Artigas, 1992). However, not all the serotonin re-uptake inhibitors produce a consistent response (paroxetine, fluoxetine, sertraline - Cacci et al, 1993; citalopram - Invernizzi et al, 1992; sertraline - Invernizzi et al, 1991b). In some cases the response was dose-dependent, where an increase in 5-HT was only observed after an increase in dosage (citalopram, sertraline - Invernizzi et al, 1991a and 1992; paroxetine - Ortiz and Artigas, 1992; fluoxetine, sertraline - Rutter and Auerbach, 1993; fluoxetine - Fuller et al, 1988). 5-HT levels also appeared to be affected by the route of administration (chlomipramine i.p - Adell and Artigas, 1991; s.c - Carboni and DiChiara, 1989), and region specificity (hippocampus, cortex - Invernizzi et al, 1991a and 1994; cortex - Cacci et al, 1993; diencephalon, striatum - Rutter and Auerbach, 1993, raphe nuclei - Adell and Artigas, 1991; Bel and Artigas, 1992,).

By blocking the re-uptake of 5-HT, the reduction of intracellular 5-HT would be expected to lead to a reduction in levels of its metabolite 5-HIAA. This was observed following acute administration of serotonin re-uptake inhibitors (paroxetine, fluoxetine, fluvoxamine - Cacci et al, 1993; sertraline - Manfridi et al, 1992; fluoxetine - Baldessarini et al, 1992; Reinhard and Wurtman, 1977, Hwang and Woert, 1980, Baron et al, 1988, Fuller et al, 1988).

In addition to blocking 5-HT re-uptake, fluoxetine was found to facilitate the release of ^{3}H-5-HT from synaptosomes *in vivo* (Cacci et al, 1992; Garattini et al, 1991; Gobbi et al, 1992). In contrast, 3 day pre-treatment of rats with high doses of fluoxetine decreased basal and potassium-evoked 5-HT and 5-HIAA release. 7 days after the last dose, basal 5-HT and 5-HIAA release returned to normal but the potassium-stimulated serotonin release was still suppressed (Gardier and Wurtman, 1991). Dexfenfluramine-evoked serotonin release was also suppressed by chronic treatment with fluoxetine (Sarkissian et al, 1990).

Electrical stimulation of ^{3}H-5-HT release in the hypothalamus was not enhanced by the action of citalopram (Langer and Moret, 1982).

The mechanism whereby fluoxetine increases 5-HT release is unclear. Gobbi et al (1992), suggested that fluoxetine blocks both the serotonin transporter and diffuses into the synaptosome to 'displace' ^{3}H-5-HT from the storage vesicles. Since the carrier is blocked, the ^{3}H-5-HT is exposed to deamination by MAO. As the radioactivity released consists of only 20% ^{3}H-5-HT, the remainder is probably the metabolite ^{3}H-5-HIAA which then diffuses out of the synaptosome.

b) Long term effects on 5-HT and 5-HIAA levels by re-uptake inhibition.

The effects of chronic administration of serotonin re-uptake inhibitors on 5-HT and 5-HIAA levels are less variable than the effects of acute administration. In general, extracellular 5-HT and 5-HIAA levels are decreased following long-term (> 10 days) administration of fluoxetine (Baldessarini et al, 1992; Caccia et al, 1992 and 1993; Hwang and Van Woert, 1980); paroxetine (Caccia et al, 1993) and clomipramine (Caccia et al, 1993).

Inconsistencies arise with the observation that chronic administration of citalopram increases 5-HT levels in the cortex and dorsal raphe (Invernizzi et al, 1992 and 1994). This phenomenon was also seen with fluvoxamine which increased 5-HT in the frontal cortex but not in the raphe nuclei following a 14 day treatment schedule (Bel and Artigas, 1993). Caccia et al (1993), however noted that the same length of administration of fluvoxamine reduced 5-HT levels in the cortex without a concomitant decrease in 5-HIAA levels. 5-HT levels were found to remain constant following treatment with clomipramine (Ortiz and Artigas, 1992) and fluoxetine (Sarkissian et al, 1990).

1.2.4.2. 5-HT$_1$ Autoreceptors.

If 5-HT re-uptake is inhibited and the amount of extracellular 5-HT is increased

as a consequence, then the 5-HT autoreceptors will be activated to inhibit 5-HT neuronal firing and the release of 5-HT from the nerve terminal.

The role of the 5-HT_{1A} autoreceptor in the action of serotonin re-uptake inhibitors was demonstrated *in vitro* by Baumann and Waldemeier (1981), where LSD-evoked release of ^{3}H-5-HT was inhibited by citalopram. This was confirmed when Langer and Moret (1982) and Galzin et al (1985) showed that the inhibition of electrically-induced release of ^{3}H-5-HT by 5-HT_{1A} agonists; LSD or 5-methoxytryptamine, was opposed by citalopram and paroxetine. The *in vivo* effect of 5-HT_{1A} autoreceptors on serotonin re-uptake inhibition was demonstrated by Hjorth in 1993. An increase in extracellular 5-HT caused by systemic administration of citalopram was significantly potentiated by the s.c. administration of 5-HT_{1A} antagonists (Hjorth, 1993). When citalopram or paroxetine were administered systemically in the presence of a local reverse-dialysis perfusion of citalopram into the hippocampus, 5-HT release was reduced by more than 50%. Pre-treatment with a 5-HT_{1A} antagonist (racemic pindolol or (+)-WAY100135 completely blocked the inhibition of 5-HT release by citalopram or paroxetine (Hjorth and Auerbach, 1994b). A similar 5-HT_{1A} effect was seen with fluoxetine and sertraline, where the decrease in fluoxetine-induced 5-HT release was reversed by the administration of 8-OH-DPAT (Rutter and Auerbach, 1993).

In contrast to the acute autoreceptor-mediated decrease in 5-HT release following serotonin re-uptake blockade, chronic administration of serotonin re-uptake inhibitors seem to enhance 5-HT neurotransmission by increasing the effect of electrically stimulated 5-HT release. This occurs with a resumption in normal 5-HT neuronal discharge patterns as seen with 2, 7 and 14 day zimeldine (Blier and De Montigny, 1983) and citalopram (Chaput et al, 1986) treatment. The reason for this appears to be two-fold; (1) desensitization of the 5-HT somatodendritic autoreceptors as shown by a reduction in the inhibition of 5-HT release by LSD or 8-OH-DPAT (somatodendritic autoreceptor agonists), following chronic citalopram treatment (Chaput et al, 1986; Invernizzi et al,

1994; Moret and Briley, 1990) and/or (2) desensitization of the 5-HT terminal autoreceptors indicated by the attenuation or complete inhibition of electrically stimulated 5-HT release by a methiothepin challenge (a 5-HT autoreceptor antagonist) following chronic treatment with citalopram (Chaput et al, 1986) or fluoxetine (Blier et al, 1988; Kreiss and Lucki, 1993). Hjorth and Auerbach (1994a) failed to detect any inhibition of an 8-OH-DPAT challenge on 5-HT release following a 14 day treatment schedule with citalopram, however the dose used in these experiments was 5mg/kg, as opposed to the higher doses (10 - 50mg/kg) administered in experiments exhibiting desensitization of the 5-HT autoreceptors.

Despite the long-term effect of chronic treatment with serotonin re-uptake inhibitors on the functioning of the 5-HT_{1A} autoreceptors, the actual number of available receptors throughout the brain remains constant. In a review of the effects of subchronic effects of fluoxetine on 5-HT_1 receptors, Beasley et al (1992) found that fluoxetine downregulated 5-HT_1 receptors in 9 experiments, with 6 reports showing no effect on the 5-HT_1 receptors, although in most cases these did not specify whether they were 5-HT_{1A} receptors. Welner et al (1989) did find a 20% reduction in ^{3}H-8-OH-DPAT binding in the dorsal raphe by fluoxetine, but no change in the cortex or hippocampus was detected. Citalopram and sertraline did not alter the B_{max} of ^{3}H-8-OH-DPAT binding in the dorsal or median raphe, or in the hippocampus, hypothalamus, cortex or amygdala (Frazer and Hensler, 1990; Hensler et al, 1991). Furthermore, chronic administration of paroxetine had no effect on ^{3}H-8-OH-DPAT binding in rat cortical membranes (Nelson et al, 1989).

1.2.4.3. 5-HT_2 receptors.

The role of the 5-HT_2 receptor in the action of the serotonin re-uptake inhibitors is unclear. Not only have inconsistent results been found amongst the SSRI's but also with each drug itself. The most widely examined SSRI with regards to the 5-HT_2 receptor is fluoxetine, where the 5-HT_2 receptors have been found

to downregulate (Hrdina et al, 1993; Stoltz et al, 1983), upregulate (Dumbrille-Ross and Tang, 1983), remain unchanged (Peroutka and Snyder, 1980; Cadogan et al, 1993; Fuxe et al, 1983a) or change according to region specificity (Wamsley et al, 1987). A reduction in ^{3}H-ketanserin binding following chronic treatment with paroxetine was observed in the rat (Cadogan et al, 1992; Nelson et al, 1989), whereas Cadogan et al (1993) showed no change in either the K_d or B_{max} of ^{3}H-ketanserin binding in the guinea pig.

Repeated administration of sertraline did not alter the 5-HT_2 binding site, however, it did desensitize the 5-HT_2 mediated hydrolysis of phosphoinositide, suggesting that alterations in receptor function may be due to a change in the receptor signalling pathways (Sanders-Bush et al, 1989). This was supported by evidence that fluoxetine and paroxetine increased the 5-HT_2 stimulated phosphoinositide hydrolysis, without any apparent change in binding site parameters (Cadogan et al, 1993). Recent evidence of a reduction in protein kinase C (PKC) distribution and activity in rat brain cortex and hippocampus following repeated administration of fluoxetine (Mann et al, 1995) indicates that the functional biochemical correlates, as opposed to the actual receptor sites, may be the target for antidepressant action.

1.2.4.4. β-adrenoreceptors.

A frequent effect of long-term antidepressant treatment is a downregulation of central β-adrenoreceptors, and the delay in onset of antidepressant effect is thought to be related to the two to three week time course required for β-adrenoreceptor downregulation. This effect, however, is not inherent in the SSRI's. In most reports, long-term administration of fluoxetine does not downregulate β-adrenoreceptors (Maggi et al, 1980; Mishra et al, 1979; Peroutka and Snyder, 1980; Stoltz et al, 1983; Wong et al, 1985), however a decrease in β-adrenoreceptors has been observed in some cases (Byerley et al, 1988; Wamsley et al, 1987). Only one account of upregulation by fluoxetine has been described (Pälvimäki et al, 1994) and in this report chronic

administration of citalopram was also found to up-regulate β-adrenoreceptors in the frontal cortex and caudate putamen. Paroxetine does not have any effect on the β_1 or β_2 adrenergic receptors (Nelson et al, 1989, 1990 and 1991), whereas sertraline was found to decrease the number of β-adrenoreceptors with no change in affinity (Byerley et al, 1988; Koe et al, 1983 and 1987).

A consequence of antidepressant downregulation of β-adrenoreceptors is a reduction in the sensitivity of the norepinephrine receptor-coupled adenylate cyclase system. The lack of β-adrenoreceptor downregulation seen with the SSRI's reflects the inability of the SSRI's to decrease the cAMP coupled response (Fuxe et al, 1983a; Mishra et al, 1979; Nelson et al, 1991). Of interest, however, was the ability of fluoxetine and sertraline to diminish the norepinephrine or isoproterenol-stimulated cAMP accumulation *in vitro* after subacute administration (4 days), coupled with a downregulation of the β-adrenoreceptors (Baron et al, 1988; Koe et al, 1987).

1.2.4.5. The 5-HT transporter site.

The effect of chronic administration of SSRI's on the 5-HT transporter is variable. The majority of studies have shown that repeated administration of antidepressant treatment, in particular citalopram or electroconvulsive shock, has no effect on the 5-HT re-uptake site (Cheetham et al, 1993; Gleiter and Nutt, 1988; Graham et al, 1987; Marcusson and Ross, 1990) or the mRNA encoding the 5-HT transporter (Spurlock et al, 1994). Fluoxetine, on the other hand, has been found to upregulate 5-HT re-uptake sites in the frontal cortex and hippocampus (Hrdina and Vu, 1993), decrease the affinity but not the density of ^{3}H-imipramine binding sites (Hrdina, 1987) and decrease steady-state concentrations of the 5-HT transporter mRNA in the midbrain raphe nuclei (Lesch et al, 1993c). Recent evidence has also shown that paroxetine downregulates ^{3}H-paroxetine binding in the hippocampus and cortex (Piñyero et al, 1994).

1.2.5. Suicide and the serotonin transporter.

The common appearance of suicidal thoughts and the rarer occurrence of suicide attempts during depressive episodes have shown that depression and suicide are closely interlinked. Biological variables, such as CSF 5-HIAA content and platelet or brain 5-HT receptors, which have been used in research on suicide (See reviews: Åsberg et al, 1987; Mann et al, 1989; Stanley and Stanley, 1990), show similar characteristics to those used in depression studies. The clinical use of SSRI's in depression has shown that they are effective in reducing suicidal ideation and suicide attempts (Montgomery, 1992; Beasley et al, 1991; Muijen et al, 1988), and therefore it is possible that alterations in the serotonin transporter could be a potential marker for pre-disposition to suicide.

The first indications that the serotonin transporter was associated with aspects of suicide appeared with the findings that ^{3}H-imipramine binding sites were reduced in postmortem brain samples of frontal cortex of suicides as compared to controls (Crow et al, 1984; Stanley et al, 1982). Subsequent investigations showed that ^{3}H-imipramine binding sites were also increased (Meyerson et al, 1982) or remained unchanged (Arora and Meltzer, 1989; Owen et al, 1986) in the brains of suicide victims. An autoradiographic study in postmortem brains of suicides revealed a region-specific increase, decrease and lack of change in the density of ^{3}H-imipramine binding sites (Gross-Isserhof, 1989). Taking into account the heterogeneity of imipramine binding in the brain, it became apparent that the use of ^{3}H-imipramine binding to 5-HT re-uptake sites in brains from suicides is unlikely to reveal a consistent pattern of serotonin transport involvement.

The use of more selective 5-HT re-uptake inhibitors as markers of the serotonin transporter in suicide is limited. Lawrence et al (1990) found that ^{3}H-paroxetine binding was no different in the brains of suicides compared to controls in 10 brain regions studied. The only significant difference noted was a decrease in the B_{max} of ^{3}H-paroxetine binding in the putamen of non-violent suicides as

compared to controls (Lawrence et al, 1990a). Subsequent evidence from Andersson et al (1992), indicated a lack of significant alterations in the K_d and B_{max} of ^{3}H-paroxetine binding sites in the frontal cortex, cingulate cortex and hypothalamus of violent and non-violent suicide victims. A more recent study showed that ^{3}H-paroxetine binding site densities were unchanged in the frontal cortex and amygdala of postmortem brain tissue from suicides, although they showed some variability, with a slight increase in frontal cortex and a decrease in the amygdala of suicide victims. However, these values were not significant even when the outlying value of each group was omitted (Hrdina et al, 1993).

1.3. *IN VITRO* AUTORADIOGRAPHY

The technique of *in vitro* autoradiography was first described by Young and Kuhar (1979) and has subsequently become a successful and widely utilized tool for mapping the localization of receptors, with radiolabeled compounds, in a broad range of tissues. It provides a means of detecting receptor sites with much greater sensitivity than biochemical techniques, describes the neuronal pathways which contain receptors, and reflects receptor changes which occur in some types of neuropathology.

1.3.1. Basic principles of *in vitro* autoradiography.

The process of autoradiography is based on photographic principles, whereby exposure to radiation activates a silver bromide crystal suspension in a gelatin matrix. Latent images of the source of radiation are formed when "sensitivity specks" (deliberately introduced structural defects in the silver halide; Ag^+) trap the activated electrons from the bromide ion (Br^-) to form a silver nucleus (Ag atom). During development, the reducing agent in the developer converts the silver bromide to metallic silver which is deposited on the silver nuclei to form enlarged grains. The fixative which contains sodium thiosulphate then complexes with any unchanged silver bromide and can be washed away

leaving the developed silver grains on a transparent background (Flitney 1990; Rogers: Chapter 2, 1979).

The source of radiation comes from unstable radioisotopes which are incorporated into the molecular structure of the drug or neurotransmitter by direct synthesis or by a catalytically induced isotope exchange with either a hydrogen atom in the case of tritium (^{3}H), or hydroxyl group in the case of iodination (^{125}I). The nuclear configuration of these isotopes is unstable so that they spontaneously transform to a more stable form by emission of β- or γ-radiation (Bennett and Yamamura, 1985) .

The majority of *in vitro* autoradiography is carried out using tritiated compounds for a number of reasons: (1) inclusion of the tritium isotope does not alter the molecular structure sufficiently to affect the biological activity of the compound; (2) it is possible to synthesise compounds with a high specific activity which allows greater resolution; (3) tritium has a long half-life ($t_{½}$ approximately 12.5 years) and it remains stable under proper storage conditions; (4) it is relatively safe to use due to the poor penetrability of β particles. Disadvantages to using tritium include: long exposure times (weeks, maybe months); variable specific activity and radiochemical purity. ^{125}I offers the advantage of rapid exposure time and lower labelling costs, however this is at the expense of high resolution, stable molecular structures and true biological activity of the compounds (Kuhar, 1985; Bennett and Yamamura, 1985).

Initially autoradiography was performed using the coverslip method, where a coverslip is coated with emulsion and apposed to the slide-mounted tissue section for the duration of exposure. The coverslip is then bent away from the section and developed. Once the tissue section is stained, the coverslip is re-attached to the section for grain density counting. The development of commercially available tritium-sensitive sheets of film coated with emulsion (Ultrofilm, LKB; Hyperfilm, Amersham) facilitated the process of autoradiography by doing away with the complicated process of coating and developing

coverslips. This allows for the densitometric evaluation of autoradiographic images by computerised image analysis systems. Coverslip autoradiography is still carried out today, however the popularity of film autoradiography means that the predominant method of autoradiogram generation is carried out using these tritium sensitive films (Kuhar, 1985; Flitney, 1990).

Densitometric evaluation of the autoradiograms involves passing a light through the autoradiogram and measuring the optical density of the grains produced on the autoradiogram. This is performed by measuring the proportion of light transmitted through a specimen to the total incident light, and then the optical density can be calculated using the formula:

$$\text{Optical Density (OD)} = -\log_{10} \text{Transmittance}$$

In order to relate the optical density measure to the quantity of radioactivity present in a given area, it is necessary to include appropriate standards when exposing sections. In the past, brain paste standards, incorporated with known quantities of radioactivity and tissue content, have been used, however the development of commercially available radioactive embedded polymer strips (^{3}H-microscales, Amersham) has proven to be just as accurate and therefore these are most commonly used today. The optical density of the area measured is compared to a calibration curve and a conversion factor derived from the standards curve is then used to calculate the amount of radioactivity (nCi) per mg of tissue in each area measured (Kuhar, 1985; Stewart and Bourne, 1992; Dashwood, 1992).

Resolution of the source of radioactivity is dependent on distance between the source and the photographic emulsion, and the thickness of the source. β particles from tritium do not travel very far in biological specimens (approximately 1.5μm, maximum 2μm) and so the probability of activating the emulsion at a distance from the source is low. This also means that the majority of the silver grains are generated from the radioligand binding to

receptors at the surface of the tissue specimen. Therefore, the thickness of the section is no longer crucial to quantification once it is greater than 1.5μm. The thickness of any section greater than 2μm is known as "infinite thickness". If a section is too thick, however, irregularities at the surface of the section, caused by shrinkage during thaw-mounting and drying, can result in an uneven development of silver grains as the β particles are not in uniform contact with the emulsion (Rogers: Chapter 4 and 11, 1979; Flitney, 1990).

1.3.2. Limitations of *in vitro* autoradiography.

As with any technique, there are limitations associated with autoradiography which have to be considered and where possible, precautions taken to avoid unnecessary inaccuracies in the analysis process.

The relationship between the optical densities in film and the radioactive content of tissue is not linear because the optical density does not increase as rapidly as the increase in radioactivity present in the tissue. Increasingly high concentrations of radioactivity are misrepresented by a levelling out of the optical density measurements, and receptor binding appears to be saturated before it actually happens. The introduction of standards has resolved this problem, however their use is limited in terms of time of exposure and development. With an increase in exposure time, the probability of a silver crystal being hit by more than one β particle is increased. Therefore a true reflection of the radioactive density of the region examined is masked as the silver crystal can only produce one developed grain. If developing time is too long, the individual grains become so enlarged that they overlap and appear as a single, dense grain and this can alter the optical density measurements (Rogers, Chapter 4, 1979; Kuhar, 1986; Kuhar and Unnerstall, 1985).

Latent images can also be produced by pressure, heat, exposure to light and certain chemicals, especially reducing agents. These form the 'background' grains found on every autoradiographic film and can interfere with the

densitometric quantification of the autoradiograms by masking the true value of areas with a low radioactive content. In order to avoid artifacts generated by very high or very low radioactivity, measurements of optical density should only be taken from those which fall within the linear scale of the standard curve.

Precautions should be taken to prevent gross insult to the film by handling them carefully and only at the extreme edges. Appropriate safelighting conditions prevent undue exposure to light and inadvertent mishandling by processing in total darkness. In order to allow as much exposure to β radiation as possible, tritium-sensitive films do not have a protective film covering the emulsion, therefore even when the films are dry following development, they should be stored and handled with care. Chemography can produce latent image artifacts (reducing agents) or fading of the latent image (oxidizing agents). By including a section which has not been incubated with the radiolabel for exposure to film, it is possible to determine whether any chemical interference is present in the section (Rogers: Chapter 6, 1979; Flitney, 1990). This is especially important when working with human postmortem tissue from patients who were undergoing drug treatment at the time of death

The second major problem associated with autoradiography is exclusive to the use of tritiated ligands. Because of the low energy of the β radiation, tissue quenching of the radioactive signal occurs. This however, is not a uniform process as the white matter of brain tissue absorbs the β-rays to a much greater degree than the grey matter, and therefore an equal amount of radioactivity will be represented by different optical densities depending on the tissue being measured. In addition to this regional quenching, standards do not necessarily exhibit the same amount of quenching as seen in the tissue. Quenching can be minimized by defatting the tissue with ethanol and xylene, however this may interfere with the radioligand binding to receptor sites. An alternative to this, is to include correction factors for quenching when calculating the radioactive content from measured optical densities (Geary et al, 1985; Kuhar and Unnerstall, 1985; Rainbow et al, 1984). In autoradiography which

compares radioligand binding in the same region under different conditions, eg.different radioligands, saturation or inhibition studies or disease states, quenching artifacts do not present a problem. It should be taken into account, however, when the tissue density of a particular region is altered during surgical lesions or neuropathological conditions.

1.3.3. *In vitro* autoradiography in human brain tissue.

Animal models of disease states have traditionally been used to investigate neuropathological changes, under controlled circumstances, which enable viable statistical analyses to be performed. Early experiments using human brain tissue were hampered by a lack of sufficient sample sizes and uncertainty of the effect of variables such as age at death, postmortem delay, gender and drug history. As technology has developed and systematic collection of matched brain samples has increased, extensive information regarding basic biochemical and neuroanatomical patterns in the human brain has been gathered. As a result of these developments, rapid progress has been made in the field of human neuropharmacology and the use of human postmortem brain tissue is now a fundamental part of research into disease states of the human brain. It also provides vital information about the similarities and differences in receptors in laboratory animals and humans.

The use of *in vitro* receptor autoradiography has proved to be a valuable tool for studying receptor alterations in diseased human brains. A review of autoradiographic ligand binding characteristics to dopamine D_1 and D_2 receptors, α_1 and α_2-adrenoreceptors and histamine H_1 recognition sites showed that there is a strong similarity in kinetic constants in rat and human brain (Fowler, 1986). A similarity was also seen in human brain autopsy samples and *in vivo* experiments in living human brain using positron emission tomography (PET scan).

Palacios and colleagues (1988) investigated the use of receptor autoradiography as a means of determining receptor modifications in brain autopsy samples taken from patients with Parkinson's disease and senile dementia. They found that site specific neuronal loss will not only result in receptor loss at a local level but can also induce receptor modification at distant locations. Thus, any receptor modification observed does not necessarily relate directly to neuronal loss. These observations indicate that *in vitro* receptor autoradiography provides important information about diseases in the human brain.

1.4. Aims of this thesis.

From the introduction above, it is clear that the role of the SSRI's in the treatment of depression is not an easy one to decipher. Studies of acute and long-term treatment show conflicting results in receptor site alterations and at a functional level. These inconsistencies arise not only between the SSRI's but also within different studies using the same compound. This is not surprising given the heterogeneity of the molecular structures of the SSRI's and therefore care should be taken not to assume that each drug has the same secondary or tertiary mode of action. It is necessary, however, to first establish whether the SSRI's all act at the same receptor sites in different regions in the brain, in order to exert their primary effect of inhibiting 5-HT re-uptake. Membrane binding studies have revealed that the SSRI's bind to the same serotonin transporter protein, however this technique is unable to detect any regional differences which may only be apparent on a microscopic level.

The distribution of ^{3}H-paroxetine and ^{3}H-citalopram binding sites has been examined using autoradiography in predominantly rat brain as well as some studies in the human brain and those of other laboratory animals. Therefore the aim of the present study was to provide a detailed map of ^{3}H-paroxetine binding sites in rat brain and compare this to the distribution of binding sites of other radiolabeled 5-HT re-uptake inhibitors such as ^{3}H-sertraline and ^{3}H-fluoxetine

which have not yet been used in autoradiographical studies. In order to characterize the binding properties of these ligands, the effect of inhibition by other SSRI's as well as serotonergic nerve terminal neurotoxins were compared on their similarities and differences in binding to the 5-HT re-uptake site.

Mapping the distribution of ^{3}H-paroxetine binding sites in rat brain using autoradiography has shown that the 5-HT re-uptake site is located in discrete brain regions which, on the whole, match the pattern of serotonergic innervation throughout the brain (De Souza and Kuyatt, 1987; Hrdina et al, 1990). While the gross anatomy of structures in rat and human brain are similar, and the pharmacological character of the 5-HT re-uptake sites is considered to be the same in both rat and human, an increasing number of discrepancies in the distribution of these binding sites are emerging (Duncan et al, 1992). Since only one detailed description of ^{3}H-paroxetine binding sites in the human brain has been carried out using autoradiography (Cortés et al, 1988), it was the intention of the present study to map the distribution of binding sites of ^{3}H-paroxetine as well as ^{3}H-fluoxetine and ^{3}H-sertraline binding sites in human brain. By doing so the details gained from rat brain studies could be compared to human brains, in order to determine the regional specificity of the species differences.

Much of the autoradiography performed in human brain involves cutting sections from blocks of tissue containing relevant areas and thaw-mounting them onto large microscope slides (Arango et al, 1992; Hoyer et al, 1986; Palacios et al, 1992).The resultant images are restricted to the particular region being studied and do not allow an overview of the plane in which the region is situated, therefore it is not possible to make a direct comparison of binding in regions from different areas of the brain in the same image. A number of studies using whole hemisphere coronal sections for *in vitro* autoradiography were reported by Biegon et al (1986) and Gross-Isserhof et al (1989, 1990), however no mention was made of the method used to collect such large

sections. They simply reported that 40µm sections were cut in a whole body cryostat and mounted onto glass slides. While the use of single hemispheres allows a direct visual comparison of regions within the hemisphere, it does not provide for a symmetrical analysis of regions in both the left and right hemisphere. Since it is possible that neuronal loss in one region can result in receptor modification in distant regions or even in the adjacent hemisphere, this study investigated the use of human whole brain (both hemispheres) coronal sections with *in vitro* autoradiography. The aim was to set up a technique for human whole brain section autoradiography not only for mapping the distribution of tritiated SSRI's, but also as a method which could subsequently be used with a variety of different radioligands in both healthy and diseased human brains.

CHAPTER TWO

METHODS AND MATERIALS

2.1. CHARACTERIZATION OF ^{3}H-PAROXETINE BINDING SITES IN THE RAT BRAIN.

2.1.1. Preparation of brain sections for autoradiography.

Male, Wistar rats (150-200g), housed under standard laboratory conditions, were stunned and decapitated. The brains were carefully removed and mounted on cork slices with "Tissue Tek" (Miles Scientific), frozen in iso-pentane cooled to approximately -40^{0}C in liquid nitrogen and stored at -80^{0}C until required. Coronal or sagittal sections (10µm) were cut at -20^{0}C using a Frigostat 2800 cryostat (Reichert-Jung Ltd.), thaw mounted onto gelatine/alum subbed glass microscope slides and air-dried for 1 hour. The slides were stored at -20^{0}C until use or for a maximum of 2 weeks.

2.1.2. Autoradiographic distribution of binding sites.

Frozen brain sections were thawed for one hour and pre-incubated at room temperature for 15 minutes in Tris-HCl buffer (50mM, pH 7.7) containing 120mM NaCl and 5mM KCl. After thorough drying, brain sections were then incubated in plastic coplin jars for 2 hours in 10ml Tris-HCl buffer containing ^{3}H-paroxetine (NEN Du Pont, specific activity: 21.1 Ci/mmol, 16.6 Ci/mmol and 15 Ci/mmol of consecutive batches). Non-specific binding was determined by the addition of citalopram, made up to 4µM in distilled water, to the incubation medium. Specific binding was calculated by subtracting the non-specific ^{3}H-paroxetine binding (in the presence of 4µM citalopram) from the total ^{3}H-paroxetine binding (in the absence of 4µM citalopram).

To determine optimum binding parameters, the sections were subjected to different wash or incubation times and then broken up and placed in β vials with 10ml "Optiphase Safe" liquid scintillation fluid (LKB Scintillation Products) overnight. Radioactivity was measured by counting the samples in an LKB 1219 "Rackbeta" scintillation counter.

Saturation binding values (K_d and B_{max}) were measured using increasing concentrations (0.02 to 3.0nM) of ^{3}H-paroxetine. For displacement studies; specific binding of 0.25nM ^{3}H-paroxetine was determined in the presence of each concentration of displacing ligand. Paroxetine and citalopram dilutions were prepared using distilled water. Fluoxetine and sertraline were first dissolved in 100µl absolute alcohol and then made up to the required concentration with distilled water.

Following incubation, sections were washed for 2 x 60 minute periods at room temperature in glass coplin jars containing 25ml Tris-HCl buffer. Sections were then dipped in distilled water for 3 seconds and air-dried under a stream of cool air. Once thoroughly dried, the sections and calibrated ^{3}H-micro-scales (Amersham International Ltd.) were apposed to Hyperfilm-^{3}H (Amersham, U.K. Ltd.) and stored in X-ray cassettes at room temperature for 8 weeks. Preliminary experiments to determine the initial saturation and displacement binding values were carried out by counting adjacent sections in the β counter.

2.1.3. Local injection of 5,7-dihydroxytryptamine in the dorsal raphe.

Male Sprague-Dawley rats (200-250g), housed under standard laboratory conditions, were pre-treated with desipramine (25mg/kg; i.p.) 30 minutes prior to injection. The rats were then anaesthetized in a halothane chamber and transferred into a Kopf stereotaxic frame with an attached anaesthetic mask. Anaesthesia was maintained with 2% halothane at 1.5L/min throughout the course of the procedure. An incision was made in the scalp to expose the skull, and a hole was drilled into the skull using a trepan drillbit. 2µl of 5,7-dihydroxytryptamine (5,7-DHT, Sigma, U.K.), dissolved in 0.1% ascorbic acid to a concentration of 2µg/µl, was injected over 2 minutes into the dorsal raphe nucleus using an SGE 10µl syringe and a Carnegie Medicin CMA/100 pump. The site of injection (Fig. 2.1) was positioned according to the following co-ordinates of the rat brain atlas of Paxinos and Watson (1986):

Anterior from bregma = -5.2mm
Lateral from midline = 4.8mm
Below the dura mater = 6.5mm

The volume of injection was 1µl for all animals, at a rate of 1µl/min by means of a Hamilton syringe (5µl) mounted on a stereotaxic micromanipulator. After injection the syringe was left in place for 5 minutes and then withdrawn slowly to reduce the possibility of tetanus toxin reflux along the injection tract. Using the same procedure, phosphate buffer (vehicle) was injected into the contralateral ventral hippocampus. Vehicle was also injected unilaterally into the ipsilateral ventral hippocampus of control rats to determine the effects of the injection procedure.

The rats were allowed to recover and housed under standard laboratory conditions for 7 days following injection before being sacrificed by stunning and decapitation. The brains were removed, mounted onto cork slices, frozen in iso-pentane cooled in liquid nitrogen and stored at -80°C. Coronal brain sections (10µm) were cut sequentially from 600µm caudal to 600µm rostral of the injection site at -20°C and autoradiography was performed on the sections, using 0.25nM ^{3}H-paroxetine, as described in section 2.1.2.

2.1.5. Determination of the effects of postmortem delay on ^{3}H-paroxetine binding in rat brain.

Male, Wistar rats (200g) were sacrificed by stunning and decapitation. The method of Procter et al (1991) was used to simulate human postmortem conditions of body cooling and cold storage. The rat heads were placed in sealed beakers in a water bath at 37°C which was switched off at time 0 hours and allowed to cool to room temperature. After 4 hours the heads were removed from the water bath and placed in a cold room. At time 0, 4, 24, 48, 72 and 96 hours from death, the brains were dissected out, frozen in iso-pentane cooled in liquid nitrogen and stored at -80°C.

Anterior from bregma = -7.5mm
Lateral from midline = -2mm
Below the dura mater = 6.3mm
Angle from the vertical midline = 18.4°

After the injection the syringe was left in place for 2 minutes and then withdrawn slowly. Using the same procedure, 0.1% ascorbic acid (vehicle) was injected into the dorsal raphe of control rats.

The wound was closed with 1 stitch and 1 staple and the animals allowed to recover. They were housed under standard laboratory conditions, keeping the controls separate from the treated rats. 14 days following injection, the rats were sacrificed by stunning and decapitation, the brains removed and mounted onto cork slices, frozen in iso-pentane cooled in liquid nitrogen and stored at -80°C. Coronal brain sections (10µm) were cut sequentially, at -20°C, from the brainstem in a caudal to rostral direction at 8 different positions along the brain according to plates 10, 19, 33, 41, 43, 48, 53 and 56 of Paxinos and Watson (1986). Autoradiography was performed under the conditions described in Section 2.1.2. using 0.25nM ^{3}H-paroxetine with 4µM citalopram included to determine non-specific binding.

2.1.4. Local injection of Tetanus Toxin in the ventral hippocampus.

Rats were anaesthetized by intraperitoneal (i.p.) injection of chloral hydrate (400mg/kg; dissolved in 0.9% saline) and positioned into a Kopf stereotaxic frame. The scalp was incised and the skull exposed. A burr hole was drilled in the skull with a microdrill. Tetanus toxin, specific toxicity of 2.5×10^{7} mouse minimum lethal doses (MLDs) was dissolved in phosphate buffer (pH 7.4). A 1000 MLDs dose of tetanus toxin was microinjected unilaterally into the ventral hippocampus (Fig. 2.2.) using the following co-ordinates according to the rat brain atlas of Paxinos and Watson (1986):

The binding assay was carried out following the method of Plenge and Mellerup (1985). For the membrane preparation, the whole brains were brought up to room temperature and homogenised in 15ml Tris-HCl buffer with a Potter-Elvehjem homogeniser at high speed for 30 seconds. The homogenate was centrifuged at 30 000 x g for 10 minutes and the pellet was resuspended with buffer (15ml/g tissue) and centrifuged again at 30 000 x g for 10 minutes. This procedure was repeated twice and the final pellet resuspended in buffer to a concentration of approximately 70mg wet weight tissue/ml. The membranes were stored at -80^{0}C.

For the binding assay; the membranes were resuspended in buffer to a concentration of 0.7mg protein/ml. 800µl aliquots of homogenate were incubated for 2 hours with increasing concentrations of 100µl aliquots of ^{3}H-paroxetine to make a final incubation concentration of 0.1, 0.25, 0.5 and 1nM ^{3}H-paroxetine. 4µM citalopram was included to determine non-specific binding. To terminate the reaction the samples were diluted with 5ml chilled Tris-HCl buffer and filtered through Whatman GF/B glass fibre filters pre-soaked with 0.3% polyethylenimine (PI) using a Brandell Cell Harvester. The filters were dried and placed in β vials with 10ml liquid scintillation fluid overnight prior to counting in the β counter.

2.2. CHARACTERISATION OF ^{3}H-PAROXETINE BINDING SITES IN HUMAN WHOLE BRAIN SECTIONS.

Details of the development of this method for human whole brain autoradiography are described in Chapter Four: Development of novel method of human whole brain autoradiography using ^{3}H-paroxetine. Human brain structures were identifed from a number of different human brain atlases and references (Carpenter, 1978; De Armond et al, 1989; England and Wakely, 1991; Everett et al, 1971; Ford et al, 1978; Heimer, 1983; Nauta and Feirtag, 1986; Niewenhuys et al, 1981).

2.2.1. Tissue preparation.

Human whole brains were obtained from control subjects at postmortem. The criteria used to determine their control value included: no record of depression or other psychological illness, no indication of psychologically active drugs taken at time of death, and the cause of death was unrelated to any central nervous system disease. The demographic details for the selected brains are shown in Table 2.1. To avoid the risk of infection from Hepatitis B and HIV, brains from patients with a history of drug abuse, haemophilia or homosexuality were also excluded.

At postmortem, the whole brains of subjects no 1, 5 and 6 were removed and frozen to -80^{0}C for storage. Brains from subjects no 2, 3 and 4 were sliced at postmortem into 1-3cm coronal sections, placed on card, and sealed in plastic wrapping before freezing. When required, the temperature of the frozen whole brain (Fig. 2.3.) was brought up to -20^{0}C and the brains were cut into 1.5-2cm whole coronal slices using a commercially available meat slicer. Each slice was placed on a tray of dry ice and then stored between two aluminum plates (Fig. 2.4.) in dry ice during transport from the hospital to the laboratory. On arrival at the laboratory, the slices were removed from the plates and stored individually in airtight plastic bags at -80^{0}C.

2.2.2. Embedding brain slices.

Brain slices were placed in a wooden frame and embedded in a 2% carboxymethylcellulose (CMC) solution (Fig. 2.5), which was then frozen to provide structural support during sectioning, and stored overnight at -20^{0}C. The frames were coated with high-pressure vacuum grease to facilitate their removal once the block was frozen. The following day, the block was stuck to an aluminum stage, using the semi-liquid CMC as an adhesive (Fig. 2.6.), which was then screwed down onto the platform of the cryostat. The block was left to equilibrate to -16^{0}C in the cryostat for 2 - 3 hours.

2.2.3. Cutting whole brain coronal sections.

All sectioning was performed in a LKB PMV 2500 whole body cryostat (Fig. 2.7.) which has been modified for cutting human tissue. Attempts were made to cut sections with the knife blade at angles of 20^0 25^0, 30^0 and 40^0, and at varying temperatures of -25°C, -20°C, -16°C and -12°C. Optimum conditions were set at a knife angle of 20^0 and a temperature of -16°C.

The block was shaved down to expose the brain tissue by cutting thick (90µm) sections until the area of interest was reached. A piece of positively-charged, nylon membrane (Hybond N+, Amersham, U.K., Ltd or Electran, Merck, U.K.) was cut to size (15 x 13cm) and placed over the block. It was gently smoothed down with a plastic holder to ensure uniform adhesion to the brain tissue. The knife level was set to 40µm below the top surface of the block and sections were cut by moving the platform very slowly past the blade, while gently pressing the plastic holder down on the membrane at the knife-edge. As the membrane, with the 40µm section adhering to it, appeared between the knife and plastic holder, it was gently pulled up and away from the knife (Fig. 2.8.). The sections were then allowed to thaw-mount for 5 minutes and secured to perspex frames with adhesive tape for storage at -20°C.

2.2.4. Autoradiographic distribution of ^{3}H-paroxetine binding sites.

Brain sections were equilibrated to room temperature for 1 hour and pre-incubated for 15 minutes in Tris-HCl buffer (50mM, pH 7.4), containing 120mM NaCl and 5mM KCl, in a 5L perspex wash chamber designed to hold the perspex frames in an upright position. After pre-incubation, the sections were allowed to dry at room temperature, removed from the perspex frames and placed onto glass plates (20 x 20cm). A border of vacuum grease was drawn around each section to prevent the incubation medium from spreading away from the section. Incubation was performed using the "drop" method whereby 10-15ml ^{3}H-paroxetine in Tris-HCl buffer was poured drop by drop to form a pool of incubation medium over the section. Pieces of rolled up tissue paper

saturated with buffer were placed around the glass plates and a perspex lid was placed over all of this to prevent evaporation of the incubation medium.

Following 2 hours incubation, the radioligand was aspirated and the sections were rinsed for a few seconds in Tris-HCl buffer to remove the excess incubation liquid. The sections were then re-attached to the adhesive tape and frames. Because the sections do not adhere to the tape when they are wet, they were attached by stapling the sections to the tape. The frames were placed in the wash chambers containing 5 litres Tris-HCl buffer for 2 x 60 minute washes. To rinse off the excess buffer, the sections were dipped in distilled water for 4 seconds and then dried under a stream of cool air. To complete the drying process, the sections were placed in a freeze-dryer for 1 hour.

When thoroughly dry, the sections were removed from the frames, glued to white backing card using SprayMount (3M) and apposed with calibrated ^{3}H-microscales to Hyperfilm-^{3}H and stored in X-ray cassettes for 4 weeks.

For autoradiographic localisation of paroxetine binding sites, sections from consecutive blocks (in a caudal to rostral direction) of whole coronal brain slices were incubated with 5nM ^{3}H-paroxetine. Non-specific binding was determined by the inclusion of 20µm citalopram.

2.2.5. Saturation and Inhibition studies.

In order to perform saturation and inhibition studies, smaller sections of brain (an area 7cm x 7cm of frontal cortex) were used in order to reduce the amount of radioligand required for each experiment.
A concentration range of 0.005 to 6nM was used to determine the K_d and B_{max} of ^{3}H-paroxetine binding in the cortex. IC_{50} values of other SSRI's were determined from the specific binding of 5nM ^{3}H-paroxetine in the absence and presence of increasing concentrations (1nM, 10nM, 100nM, 1µM, 10µM and 100µM) of inhibitor.

The sections were subjected to procedures of autoradiography as described (Section 2.2.4.) and set down with Hyperfilm-^{3}H for 6 weeks in the case of saturation studies and 4 weeks for the inhibition experiments.

2.2.6. Autoradiography of ^{3}H-imipramine binding sites in human brain sections.

Autoradiography of human whole brain coronal sections (40µm) mounted on positively charged nylon membranes (Merck) was carried out as described by Cortés et al (1988). Sections were pre-incubated for 15 minutes in Tris-HCl buffer (50mM, pH 7.4) containing 120mM NaCl and 5mM KCl at room temperature, dried, and then incubated for 1 hour at 4^{0}C in a cooling chamber with 10nM ^{3}H-imipramine (New England Nuclear, 53.2 Ci/mmol). 100µM desipramine was included to assess non-specific binding.

Following incubation the sections were washed with chilled Tris-HCl buffer for 10 and 20 minutes consecutively and rinsed with distilled water for a few seconds before air-drying and then freeze-drying for an hour. The dried sections and calibrated ^{3}H-microscales were apposed to Hyperfilm-^{3}H and stored in X-ray cassettes for 2 weeks.

2.3. ^{3}H-FLUOXETINE BINDING IN RAT BRAIN.

Fluoxetine was custom-synthesized to ^{3}H-fluoxetine with a specific activity of 42.6Ci/mmol and stored in ethanol at 1mCi/ml. The second batch of ^{3}H-fluoxetine, following subsequent re-purification, was stored in ethanol at 0.25mCi/ml. All batches were kept in light protective containers at -20^{0}C.

2.3.1. Binding Assay.

2.3.1.1. Binding to different rat brain tissue fractions.

The brains from male, Wistar rats (200g) were removed and homogenised in

20ml ice-cold sucrose (0.32M) with glucose (10mM) using a Potter-Elvehjem homogeniser (high speed, 30 seconds). A 5ml sample from each brain homogenate was removed for the *whole brain homogenate (WB)* fraction.

The rest of the homogenate was centrifuged at 1000 x g for 10 minutes at 4°C and the supernatant was removed. The pellet was re-suspended in 5ml sucrose for the *crude nuclear fraction (P_1)*. The supernatant was centrifuged at 20 000 x g for 20 minutes at 4°C and half the resultant pellets were re-suspended in 5ml sucrose for the *crude membrane fraction (P_2).*

The other half of the pellets were washed with the supernatant to rinse the buffy coat off the pellet. This was done by collecting the supernatant and adding 10ml to the pellet, gently shaking and re-collecting. This was repeated 3 times. The washed pellet was removed and suspended with 5ml distilled water for the *mitochondrial - myelin fraction (MM).*

The supernatant and buffy coat were then centrifuged at 48 000 x g for 20 minutes at 4°C. The resultant supernatant was poured off and each pellet was washed twice by vortexing with 5ml ice-cold distilled water and then adding 20ml ice-cold distilled water, hand-homogenising and centrifuging at 48 000 x g for 20 minutes. The final pellet was re-suspended with 5ml distilled water as the *crude synaptic membrane fraction (CS).* The protein content (mg/ml) of each sample was determined from a protein standard calibration curve (Fig. 2.9.) using the Bradford method (Bradford, 1976). All fraction samples were stored at -20°C overnight.

To prepare the different tissue fractions for binding, the method of Wong et al (1985) was followed. Each sample was re-suspended by homogenisation in a total volume of 25ml Tris-HCl (50mM, pH 7.4) containing 120mM NaCl and 5mM KCl. The suspensions were incubated at 37°C for 10 minutes, centrifuged at 50 000 x g for 10 minutes, and the pellets re-suspended in 25ml Tris-HCl buffer as above. The incubation and centrifuging steps were repeated and the final pellet suspended in 15ml buffer.

1ml aliquots of fraction preparations were incubated for 90 minutes at 4°C with 1nM ^{3}H-fluoxetine and 10µM paroxetine for non-specific binding. A total volume of 2ml was made up with Tris-HCl buffer. The reaction was terminated with 4 x 5ml chilled Tris-HCl buffer washes filtered through Whatman GF/B glass fibre filters using a Brandell Cell Harvester. The filters were placed in β vials with 10ml liquid scintillation fluid overnight and measured for radioactivity in the β-counter.

2.3.1.2. Binding to P_2 membrane fractions.

P_2 membranes were prepared as described above and the pellets were suspended to final protein concentrations of 0.1, 0.3, 0.75, 1.0, 1.5 and 2.25mg/ml. 1ml aliquots were incubated for 90 minutes at 4°C with 1nM ^{3}H-fluoxetine and 10µM paroxetine was included to determine non-specific binding.

2.3.2. Autoradiography of ^{3}H-fluoxetine binding sites.

Brains from male, Wistar rats (200g) were prepared as described in Section 2.1.1. Sagittal brain sections (10µm) were brought up to room temperature for one hour prior to pre-incubation for 15 minutes at room temperature in Tris-HCl buffer (50mM, pH 7.4) containing 120mM NaCl and 5mM KCl. Incubation was carried out at room temperature for 90 minutes with ^{3}H-fluoxetine. 10µM paroxetine was included to determine non-specific binding. Following 2 x 60 minute washes in Tris-HCl buffer, the sections were dipped in distilled water for 3 seconds and dried under a stream of cool air.

All incubation conditions were carried out using 1nM ^{3}H-fluoxetine in Tris-HCl buffer unless otherwise specified in saturation studies. The concentration of 1nM was selected from binding studies carried out by Wong et al (1983, 1985).

2.3.2.1. Determination of optimum wash and incubation times.

To determine optimum binding parameters, the sections were subjected to

increasing wash (5 to 240 minutes) or incubation (5 to 300 minutes) times and then broken up and placed in β vials with 10ml liquid scintillation fluid overnight. Radioactivity was measured by counting the samples in the β counter.

2.3.2.2. Light and temperature sensitivity.

Rat brain sections were incubated with 1nM ^{3}H-fluoxetine for 90 minutes at room temperature in containers exposed to natural light or kept covered for the duration of the incubation. For temperature assessment experiments, the incubation step was carried out at 4^{0}C, room temperature or 37^{0}C. Sections were then washed for 2 x 60 minutes in Tris-HCl buffer at room temperature, dipped in distilled water and air-dried. The radioactivity bound to the sections was measured in the β counter.

2.3.2.3. Binding to glass and plastic.

Incubation of rat brain sections was carried out for 90 minutes in 7ml (plastic slide holders) and 25ml (glass Coplin jars) aliquots of 1nM ^{3}H-fluoxetine in Tris-HCl buffer. 10μM paroxetine was included to determine non-specific binding. Following 2 x 60 minute washes in Tris-HCl buffer, the sections were dipped in distilled water, air-dried, broken up, placed in vials containing 10 ml liquid scintillation fluid and measured for radioactivity in the β counter.

2.4. ^{3}H-SERTRALINE BINDING IN RAT BRAIN.

(+)Sertraline-HCl was custom-synthesized to ^{3}H-sertraline with a specific activity of 85Ci/mmol and stored in ethanol at 0.585 mCi/ml at -80^{0}C.
Rat brain membrane binding experiments were carried out according to Koe et al (1990). Autoradiographic procedures were modified from this binding methodology.

2.4.1. Membrane binding assay.

Male, Wistar rats (200-250g) were sacrificed by stunning and decapitation. The whole brains were rapidly removed, frozen in iso-pentane cooled to -40°C in liquid nitrogen and stored at -80°C until needed.

The brains were brought up to room temperature and homogenised in 20ml ice-cold Tris-HCl buffer (50mM, pH 7.4) containing 120mM NaCl and 5mM KCl. The homogenate was centrifuged at 30 000 x g for 10 minutes at 4°C. The pellet was suspended in 15ml buffer and re-centrifuged at 30 000 x g for 10 minutes at 4°C. This washing step was repeated twice. The final pellet was re-suspended in buffer to give a final protein concentration of 0.3mg/ml.

Aliquots (800µl) of membrane preparation were incubated for one hour at room temperature with a final incubation concentration of 1nM ^{3}H-sertraline (100µl) and 10µM paroxetine (100µl) to determine non-specific binding. The reaction was terminated with 4 x 5ml ice-cold Tris-HCl buffer washes through Whatman GF/C glass fibre filters using a Brandell Cell Harvester. The filters were placed in β vials with 10ml scintillation fluid overnight and were measured for radioactivity in the β-counter.

2.4.2. Autoradiography of ^{3}H-sertraline binding sites.

Sagittal rat brain sections (10µm) were pre-incubated for 15 minutes with Tris-HCl buffer (50mM, pH 7.4) containing 120mM NaCl and 5mM KCl. The sections were air-dried and incubated for one hour at room temperature in Tris-HCl buffer with ^{3}H-sertraline. 10µM paroxetine was added to determine non-specific binding. The sections were then washed in Tris-HCl buffer for 2 x 1 hour periods, dipped in distilled water for 3 seconds and dried under a stream of cool air. Sections were apposed to Hyperfilm-^{3}H with calibrated ^{3}H-microscales in X-ray cassettes for 4 weeks.

With the exception of saturation studies, all experiments were carried out using 1nM ^{3}H-sertraline. Optimum wash and binding time experiments, and light and temperature sensitivity experiments were carried out using the parameters described in Sections 2.3.2.1. and 2.3.2.2. respectively. For these experiments; sections were broken up and placed overnight in vials containing 10ml liquid scintillation fluid for counting in the β-counter.

2.5. AUTORADIOGRAM GENERATION AND DENSITOMETRIC ANALYSIS.

Hyperfilm-^{3}H films were removed from the X-ray cassettes following exposure to sections, at room temperature, for the required length of time. All procedures were carried out in a darkroom using a dark red Wratton No 2 filter for safelighting conditions. The films were developed in Kodak D-19 developer for 60 - 90 seconds, washed for 1 minute in water and then fixed for 3 minutes with Unifix (Kodak, 1 in 4 dilution). A final wash of 25 minutes in running tap water and air drying overnight completed the process.

The optical density of the photographic images generated on film was measured using a Quantimet 970 image analyzer (Cambridge Instruments, U.K., Ltd.) A calibration curve obtained from the ^{3}H-microscales was used to convert the optical density to radioactive tissue values of nCi/mg.

These values were then converted to fmol/mg tissue using the equation:

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

The amount of radiolabel specifically bound was defined as the difference between total and non-specific binding.

2.6. MATERIALS.

Absolute alcohol, glucose, HCl, KCL, NaCl and sucrose were obtained from BDH Chemicals U.K. Ltd. Trizma base was obtained from Sigma Chemicals U.K. Ltd. Paroxetine, citalopram, sertraline and fluoxetine were donated as a gift from Smithkline Beecham. The custom-synthesized radioligands ^{3}H-fluoxetine and ^{3}H-sertraline were also kindly donated by the radiochemistry department of Smithkline Beecham.

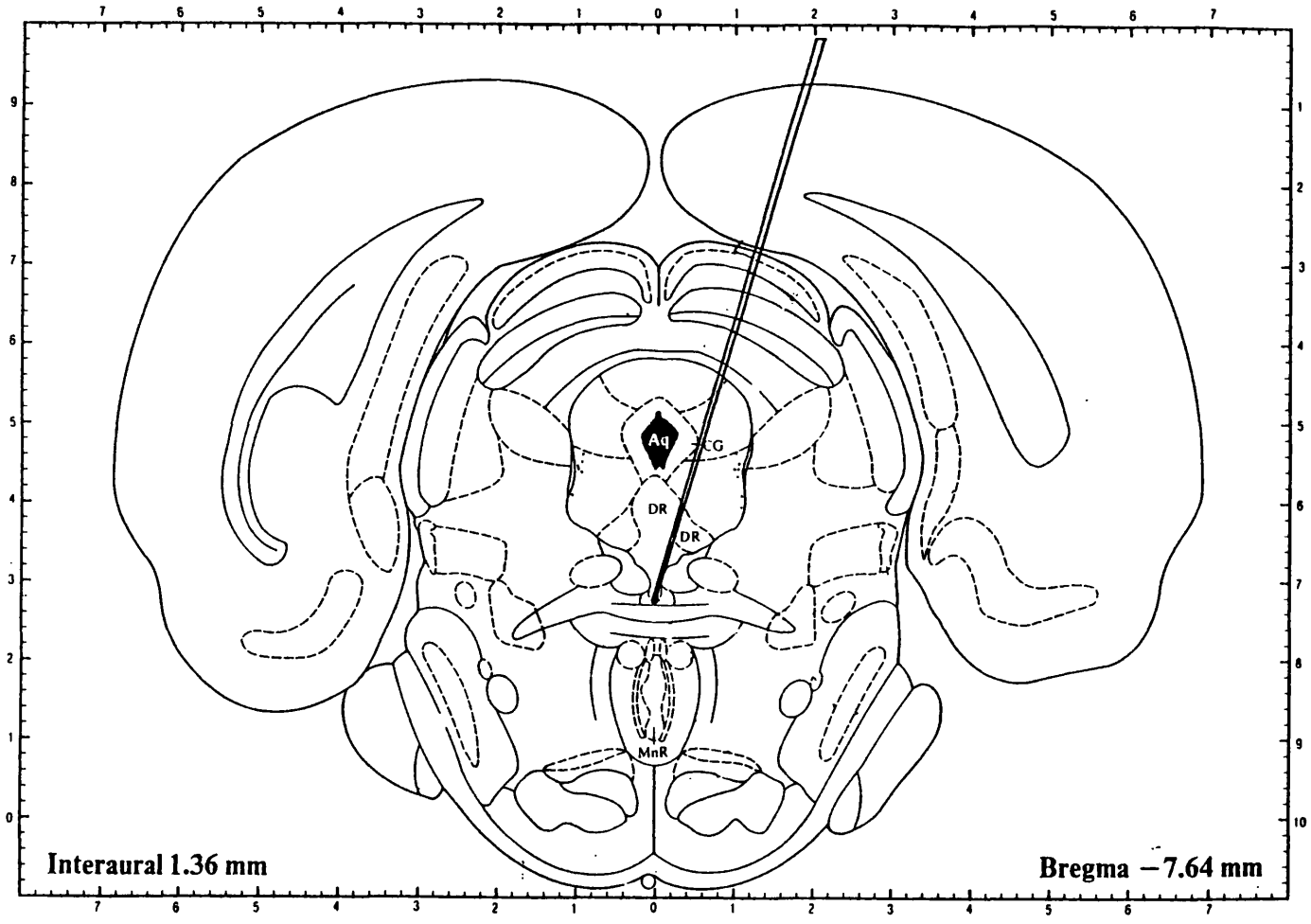


Fig. 2.1. Coronal section of rat brain indicating site of injection of 5,7-DHT or vehicle in the dorsal raphe nuclei. Aq:aqueduct, CG: central gray, DR: dorsal raphe nuclei, MnR: median raphe nuclei. Adapted from figure 48 of the rat brain atlas of Paxinos and Watson (1986).

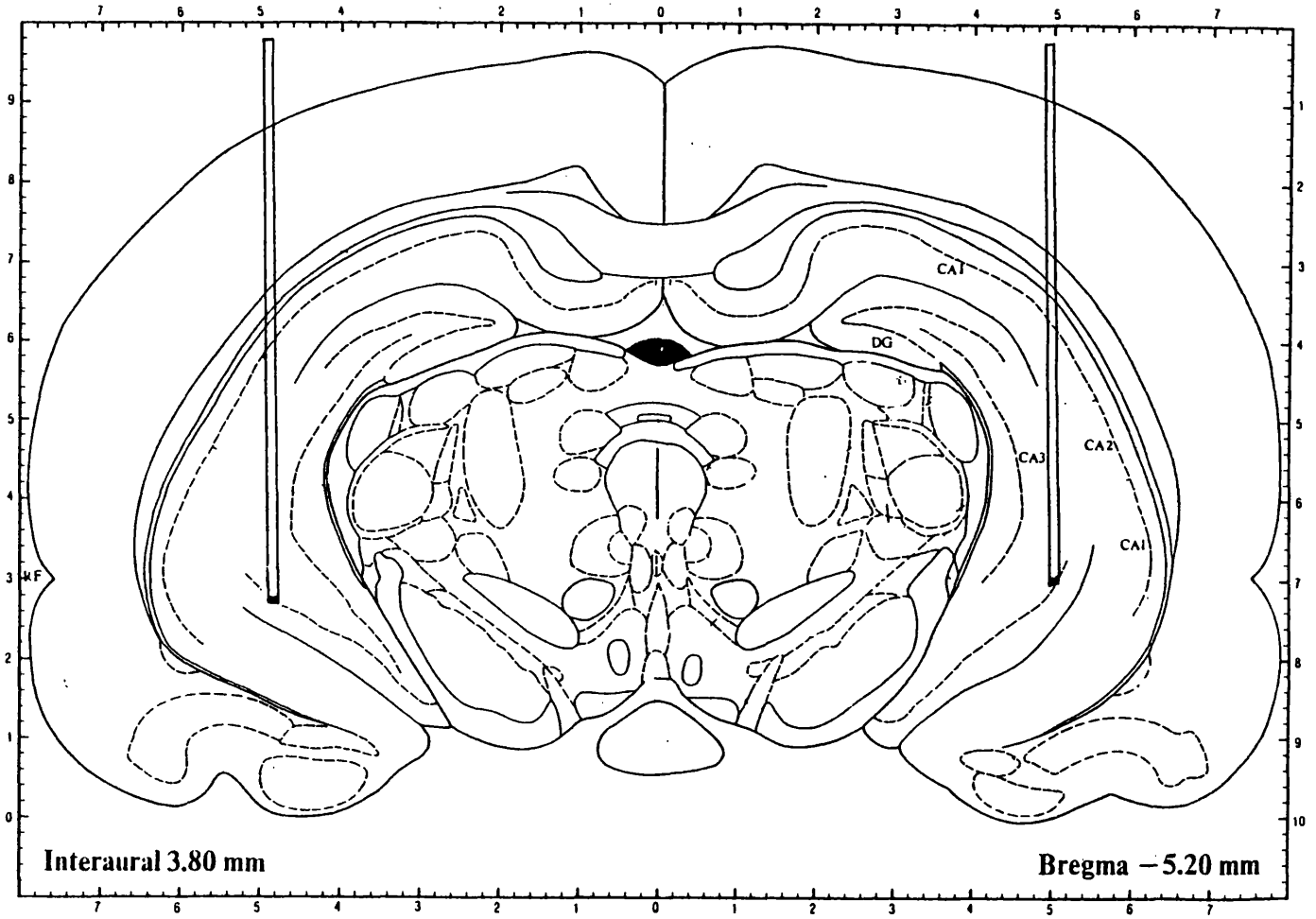


Fig. 2.2. Coronal section of rat brain indicating sites of injection of tetanus toxin and vehicle. CA1-CA3: fields CA1 - CA3 of Ammon's horn, DG: dentate gyrus. Adapted from figure 38 of the rat brain atlas of Paxinos and Watson (1986).

Patient	Sex	Age (years)	Postmortem delay (hours)	Cause of Death
1	M	41	57	Myocardial infarction
2	M	59	43	Pulmonary embolism
3	M	83	26	Coronary artery occlusion
4	M	70	<41	Gastro-intestinal haemorrhage
5	M	68	<48	Haemopericardium, cardiac infarct
6	F	66	<48	Haemopericardium, aneurysm of ascending aorta

Table 2.1. Demographic details of human brain tissue used in this study.

Fig. 2.3. Male, human whole brain frozen at -80^{0}C. The lower brainstem and spinal cord were removed at postmortem and the remaining upper brainstem and cerebellum were folded along the ventral surface of the brain during the freezing process.

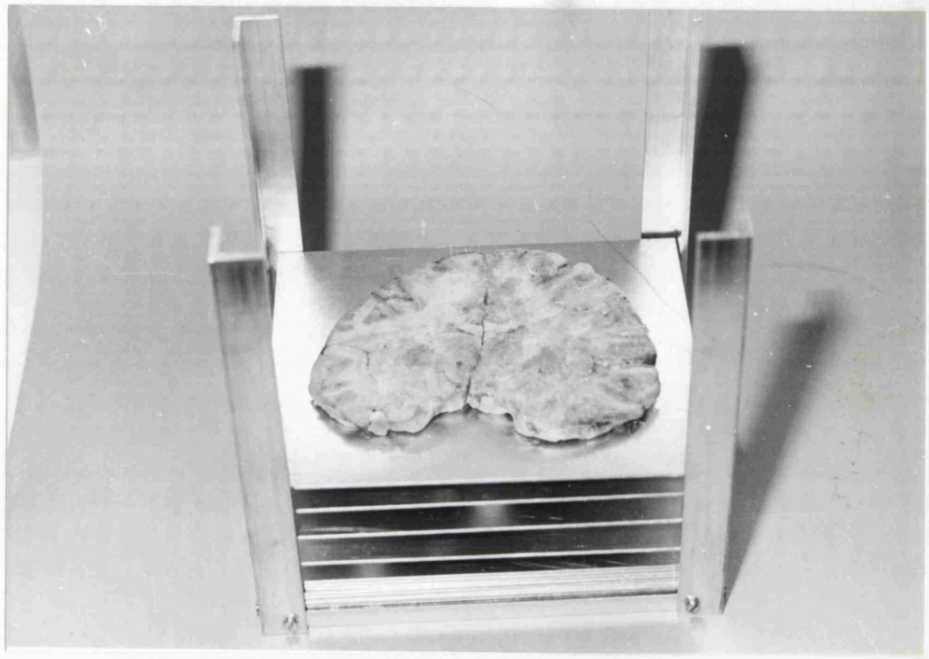


Fig. 2.4. A: 1.5-2cm coronal whole brain slices were transported between aluminum plates stored in a frame in dry ice from hospital to laboratory. B: Slices were stored at -80^{0}C in individual air-tight plastic bags.

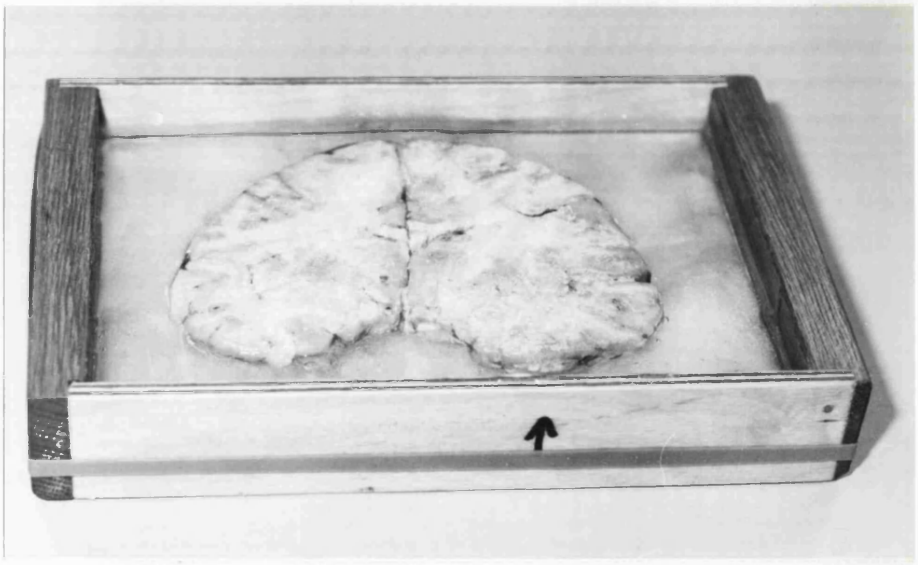


Fig. 2.5. Each whole brain slice was embedded in a 2% carboxymethylcellulose solution and frozen to a solid block in a wooden frame.

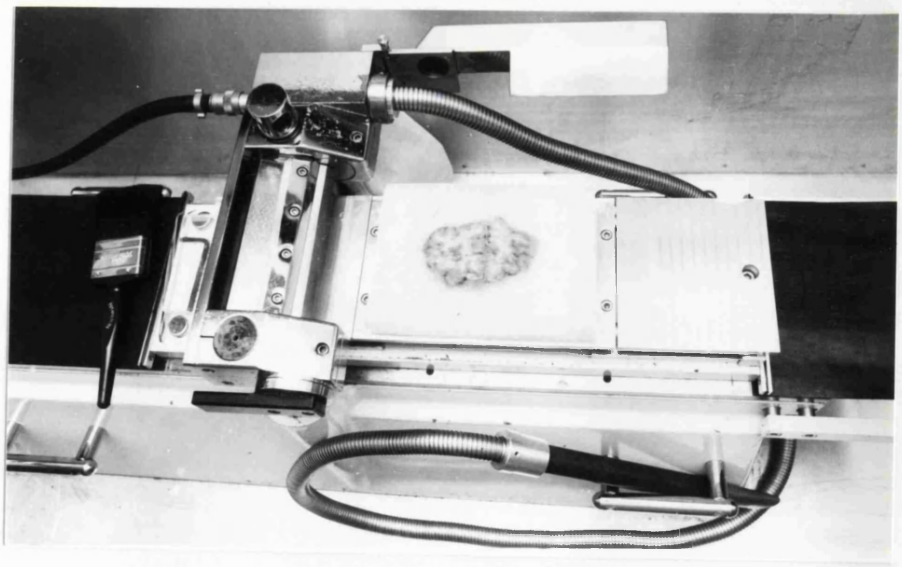


Fig. 2.6. The temperature of the tissue embedded in the frozen block of CMC was brought up to -20^{0}C overnight and then secured to an aluminum stage which was screwed down onto the moveable platform of the cryostat.

Fig. 2.7. An LKB PMV 2500 whole body cryostat modified for human tissue use.

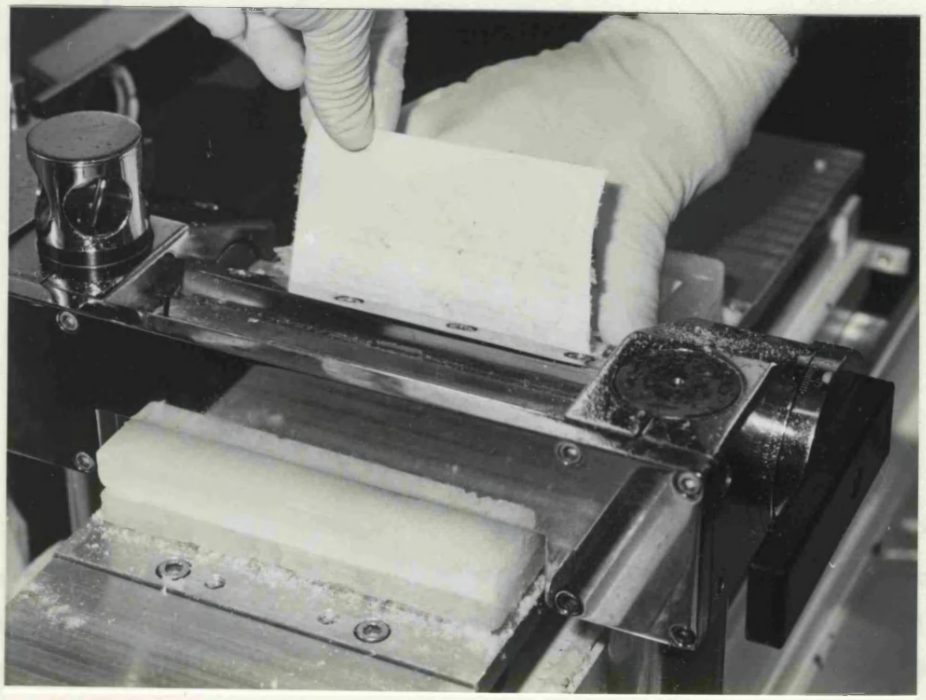


Fig. 2.8. As the 40μm human tissue section was cut, it adhered to the membrane and was pulled up and away from the knife edge. A plastic holder was pressed against the knife edge to ensure uniform adhesion to the membrane.

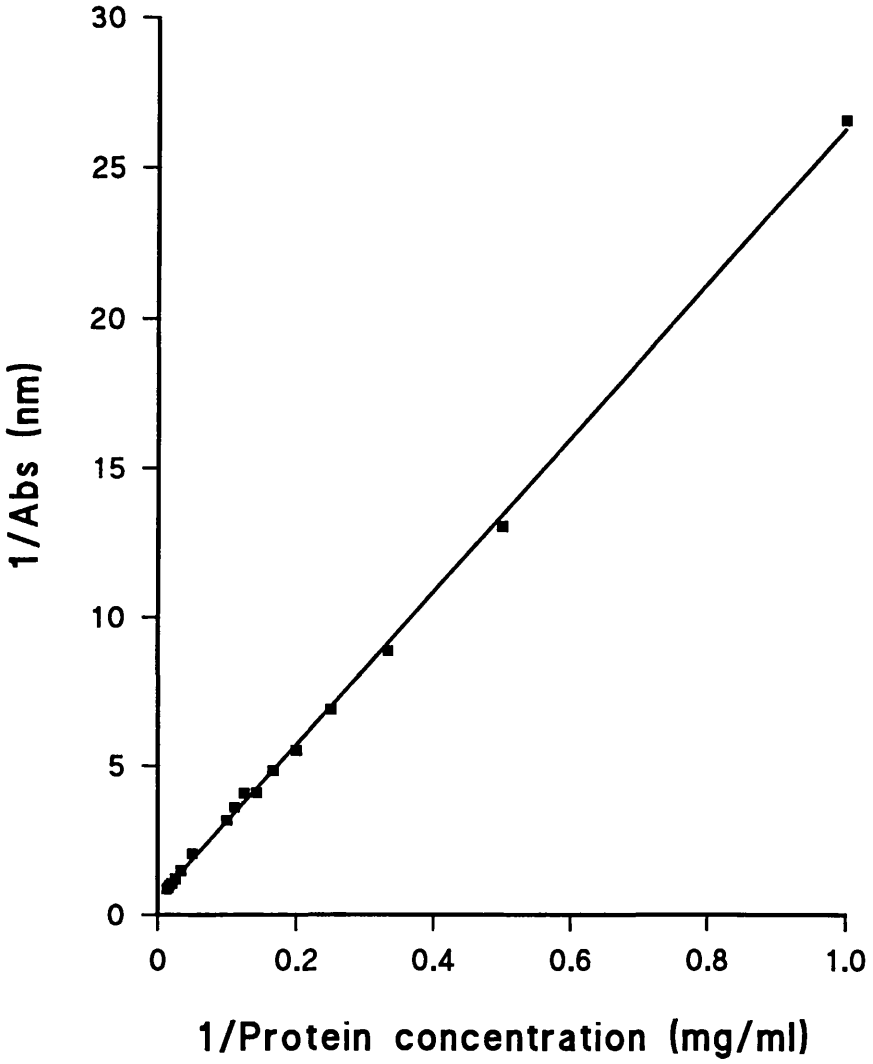


Fig. 2.9. A protein standard calibration curve determined by plotting 1/absorbence of Coomassie Brilliant Blue G-250 azo dye binding to protein vs 1/known concentrations of bovine serum albumin (BSA). 1ml Bradford reagent (100ml 85% (w/v) phosphoric acid added to 100mg Coomassie Brilliant Blue G-250 dissolved in 50ml 95% ethanol and made up to 1L distilled water) was incubated for 15-30 minutes with 100µl aliquots of BSA standards (0-1mg/ml) prepared with 0.15M NaCl. Absorbence was read at 595nm on a spectrophotometer (Cecil Instruments). The concentrations of 100µl protein samples (diluted 1:2, 1:10 and 1:100 with 0.15M NaCl) were calculated from the standard curve.

CHAPTER 3

CHARACTERIZATION OF ^{3}H-PAROXETINE BINDING IN RAT BRAIN SECTIONS USING RECEPTOR AUTORADIOGRAPHY.

INTRODUCTION.

Paroxetine, a phenylpiperidine derivative, is a member of a group of antidepressants known as the SSRI's (selective serotonin re-uptake inhibitors). It acts by inhibiting the re-uptake of 5-HT by the serotonin transporter back into the neuron (Cheetham et al, 1993; Johnson et al, 1989; Thomas et al, 1987) thereby increasing the effect of 5-HT in the brain (Buus Lassen, 1978a). ^{3}H-paroxetine has been proven as an effective scientific tool for studying the 5-HT re-uptake site because it binds selectively with very high affinity to a single site on the serotonin transporter (Habert et al, 1985; Marcusson et al, 1988; Mellerup and Plenge, 1986). ^{3}H-paroxetine binding shows a strong pharmacological correlation with ^{3}H-5HT uptake (Cheetham et al, 1993) and is inhibited by other SSRI's at low nanomolar concentrations (Habert et al, 1985; Marcusson et al, 1992; Mellerup and Plenge, 1986).

Autoradiographical studies of the distribution of ^{3}H-paroxetine binding sites have been performed by a number of groups (Chen et al, 1992; De Souza and Kuyatt, 1987; Gobbi et al, 1990; Hrdina et al, 1990). The regional distribution of serotonin re-uptake sites labelled by ^{3}H-paroxetine was found to correlate well with the pattern of 5-HT innervation in rat brain (De Souza and Kuyatt, 1987; Hrdina et al, 1990). No comparison has been made, however, of the distribution of radiolabelled SSRI's within the same study, and therefore the intention was to compare the distribution of ^{3}H-paroxetine, ^{3}H-sertraline and ^{3}H-fluoxetine binding sites in the rat brain using autoradiography in the present study. Unfortunately, it was found that ^{3}H-fluoxetine and ^{3}H-sertraline were not viable as radioligands (see Chapter 6). Therefore, the work carried out to establish a detailed map of the distribution of only ^{3}H-paroxetine binding sites is laid out in this chapter.

The specificity of ^{3}H-5-HT uptake for serotonergic nerve terminals was established by Kuhar and Aghajanian (1973) where electrolytic lesions of the midbrain raphe nuclei resulted in a marked reduction in the high affinity uptake

of ^{3}H-5HT by synaptosomes in rat forebrain homogenates accompanied by a reduction of forebrain 5-HT levels and tryptophan hydroxylase activity. Subsequently, serotonin selective neurotoxins have been extensively used to investigate the nature of serotonergic innervation and associated receptor sites in rat brain tissue (Brunswick et al, 1992; Frankfurt et al, 1987; Hensler et al, 1994; O'Hearn et al, 1988). Although much of the serotonergic innervation throughout the brain is the result of overlapping projections from the dorsal and median raphe nuclei, these two serotonergic axon types can be characterised by their different sensitivities to neurotoxins. The finer dorsal raphe serotonergic processes are more susceptible to amphetamine derived neurotoxins such as methylendioxymethamphetamine (MDMA), methamphetamine (MDA) or parachloroamphetamine (PCA) than those from the median raphe (Mamounas and Molliver, 1988; Mamounas et al, 1991; Wilson et al, 1989). While site specific injections of PCA have no effect within the dorsal raphe, the destruction of serotonergic neurons using 5,7-dihydroxytryptamine (5,7-DHT) injected into the dorsal raphe causes a dramatic loss of serotonergic cell bodies in the area (Hensler et al, 1994) as well as extensive reductions in 5-HT and 5-HIAA concentrations throughout the forebrain (Wogar et al, 1993).

Gobbi and colleagues have assessed the effects of 5,7-DHT on ^{3}H-paroxetine binding in rat brain using quantitative autoradiography (Gobbi et al, 1990 and 1994), however the lesions were induced by injecting the 5,7-DHT i.c.v. and a very short wash out time was used (2 x 5 minutes) after incubation with ^{3}H-paroxetine. Therefore in the present study, the differential pattern of innervation contribution from the dorsal and median raphe on serotonin re-uptake sites was determined by selectively injecting 5,7-DHT into the dorsal raphe, as well as providing a detailed map of the effects of 5,7-DHT induced lesions on ^{3}H-paroxetine binding sites throughout the brain. In this case a longer wash time was used to reduce non-specific binding.

Another neurotoxin, tetanus toxin, has been demonstrated to interfere with 5-HT pathways. Aguilera et al (1987) and Aguilera and Gonzalez-Sastre (1988) demonstrated that an increase in CNS tissue levels of 5-HT could be induced by intraperotoneal injection of tetanus toxin. Further work by Aguilera (1991) demonstrated that 24 hours following intraventricular injection of tetanus toxin, an increase in 5-HT levels in the hypothalamus, hippocampus and spinal cord could be seen. Furthermore, a significant increase in 5-HT levels was observed following direct stereotaxic injection of tetanus toxin into the hypothalamus and hippocampus, whereas injection of the same dose of toxin into the dorsal or magnus raphe did not effect 5-HT levels in the brain.

The mechanism of tetanus toxin is unclear, however it is known to act at the pre-synaptic level to reduce calcium dependent, potassium stimulated neurotransmitter release (Osborne and Bradford, 1973; Mellanby, 1984). Bagetta et al (1990a) showed that tetanus toxin caused neuronal loss and reduction of $GABA_A$ binding sites 7 days after intrahippocampal injection. This effect was thought to be caused by unopposed excitation due to impairment of inhibitory transmission in the hippocampus (Mellanby and Thompson, 1977). The loss of binding sites could also be attributed to the direct result of toxin-induced degeneration of nerve terminals in the region. Therefore in the present study, it was determined whether tetanus toxin could be used as a locally applied neurotoxin on the serotonergic re-uptake sites, and whether it could be applied to a specific area without affecting adjacent or contralateral areas.

RESULTS

3.1. CHARACTERISTICS OF ^{3}H-PAROXETINE BINDING TO RAT BRAIN SECTIONS.

In this study, incubation and wash time experiments were performed to obtain optimum binding parameters. Data were obtained by scintillation spectometry for these experiments and the saturation and inhibition studies.

3.1.1. Effect of incubation and wash times.

Optimum wash and incubation conditions were investigated for the binding of 0.25nM ^{3}H-paroxetine to slide-mounted sections of rat brain. Wash times were determined over a range of 2 x 10 to 2 x 90 minute washes, following a 2 hour incubation (Fig. 3.1A). The total and non-specific binding decreased to approximately half its original value within 60 minutes (2 x 30 minute washes) and then stabilised at 120 minutes (2 x 60 minutes). The specific binding increased to reach a peak of 64% of total binding at an optimum wash-out time of 120 minutes.

Using a wash time of 2 x 60 minutes, the incubation time was varied from 20 minutes to 180 minutes (Fig. 3.1B). The total binding increased rapidly to 60 minutes and then remained at a plateau for a further 120 minutes. The specific binding peaked at 120 minutes. For all subsequent studies, sections were incubated for 120 minutes and rinsed for 2 x 60 minutes respectively.

3.1.2. Saturation analysis.

A saturation isotherm of specific ^{3}H-paroxetine binding to slide-mounted rat brain sections, over a concentration range of 0.02 to 3nM, was generated (Fig. 3.2).

Using the equation:

$$B = (B_{max} \times L)/(K_d + L)$$

to fit the curve, a dissociation constant (K_d) of 0.329 ± 0.091nM and the maximum binding capacity (B_{max}) of 216.84 ± 23.46 fmol/mg were calculated, where B is the amount of ^{3}H-paroxetine specifically bound at concentration L. Curve analysis using LIGAND indicated that binding was to a single site and was shown to be saturable and of high affinity. The protein concentration of each section per slide was measured as 0.107 ± 0.022mg/section using the Bradford method (Bradford, 1972).

3.1.3. Inhibition by selective serotonin re-uptake inhibitors.

The inhibition of ^{3}H-paroxetine specific binding by paroxetine, citalopram, sertraline and fluoxetine was analyzed by LIGAND and IC_{50} values were determined (Table 3.1). A single site model fitted in all cases, although citalopram appeared to expose an element of low affinity non-specific binding (Fig. 3.3). Despite an IC_{50} of 5.49 ± 0.67nM, which indicates a high affinity for the binding site, at 10μM, citalopram had not completely inhibited the^{3}H-paroxetine binding as had the other inhibitors at this concentration.

Inhibitor	IC_{50} (nM)	Rank order
Paroxetine	0.75 ± 0.16	1
Fluoxetine	3.87 ± 0.58	2
Sertraline	4.62 ± 0.78	3
Citalopram	5.49 ± 0.67	4

Table 3.1. Inhibition by SSRI's of specific ^{3}H-paroxetine binding to slide-mounted, parasagittal rat brain sections. A range of inhibitor concentrations (0.01nM to 10μM) were added to a 0.25nM ^{3}H-paroxetine incubation medium for 120 minutes. 4μM citalopram was included to define non-specific binding.

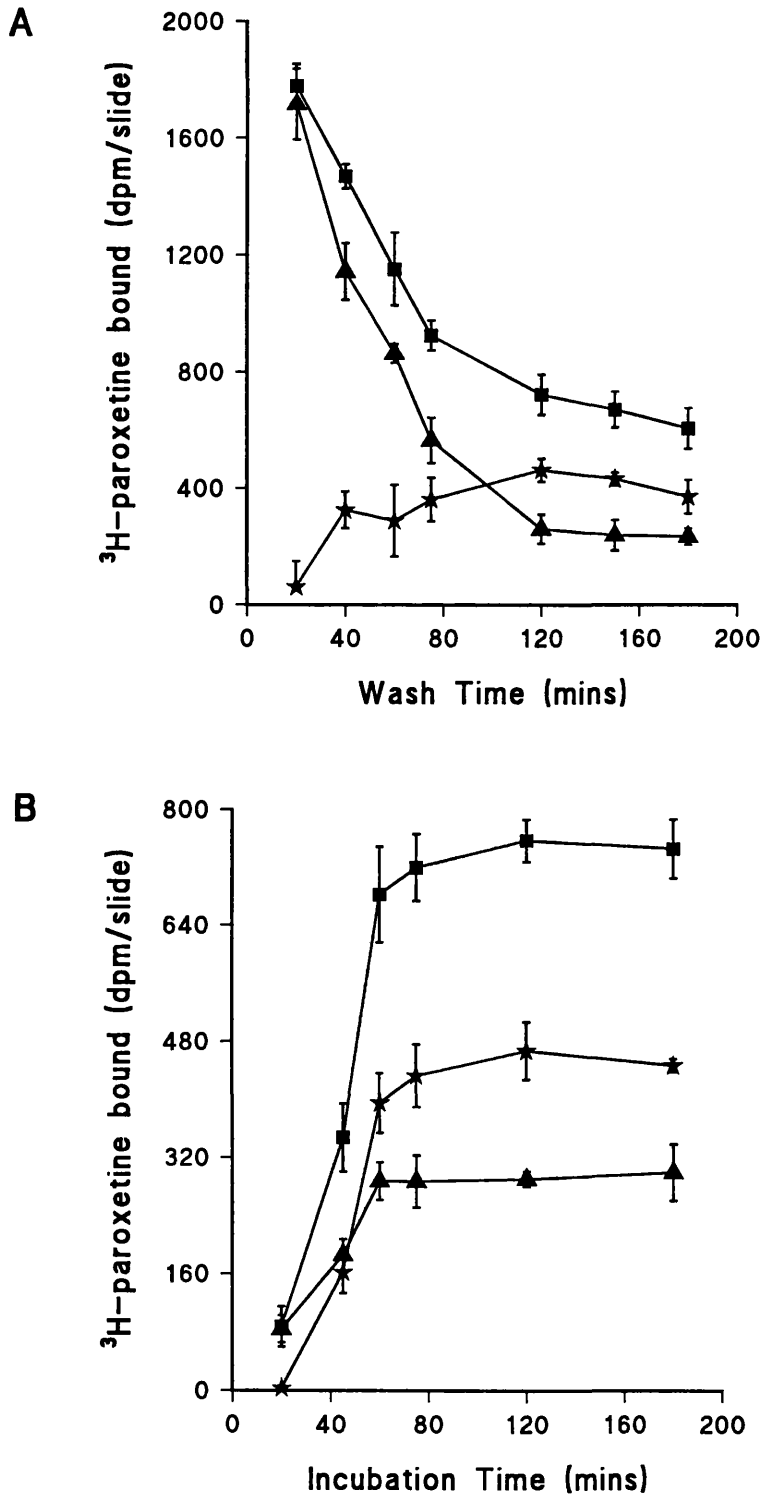


Fig. 3.1. Time course of dissociation (A) and association (B) of ^{3}H-paroxetine binding to slide mounted rat brain parasagittal sections cut at 1.4mm from the midline. Specific binding (★) was calculated as the difference between the binding of 0.25nM ^{3}H-paroxetine in the absence (■) and presence (▲) of 4μM citalopram. Data shown are the mean ± s.e.m of dpm per section of 3 experiments, n=4.

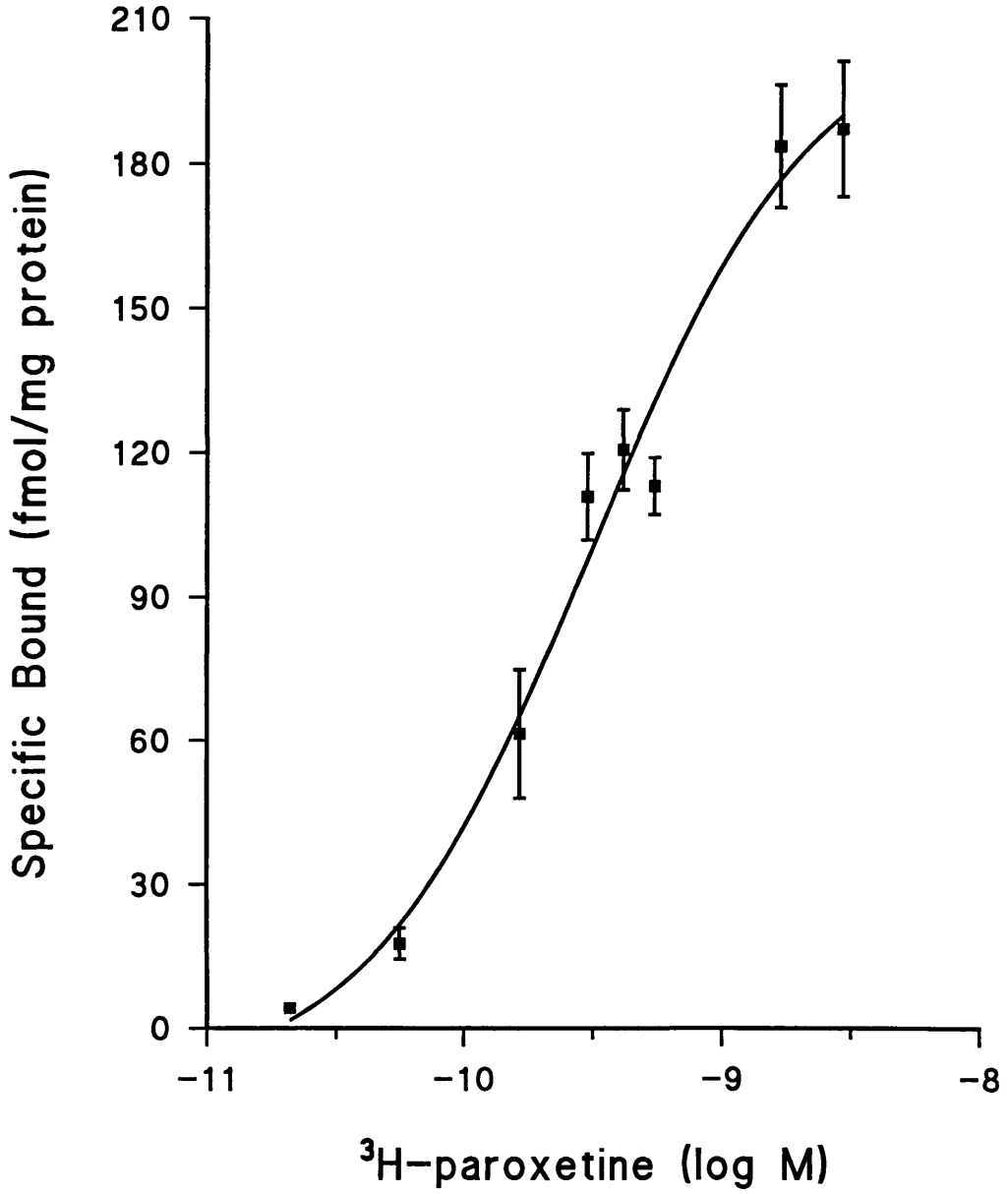


Fig. 3.2. Saturation isotherm of ^{3}H-paroxetine binding to sagittal rat brain sections (10μm) cut between 1.9 and 0.9mm from the midline. Sections were incubated for 120 minutes with increasing concentrations (0.02 to 3nM) of ^{3}H-paroxetine. 4μM citalopram was included to determine non-specific binding. Data points are the mean ± s.e.m of 3 experiments, n=4.

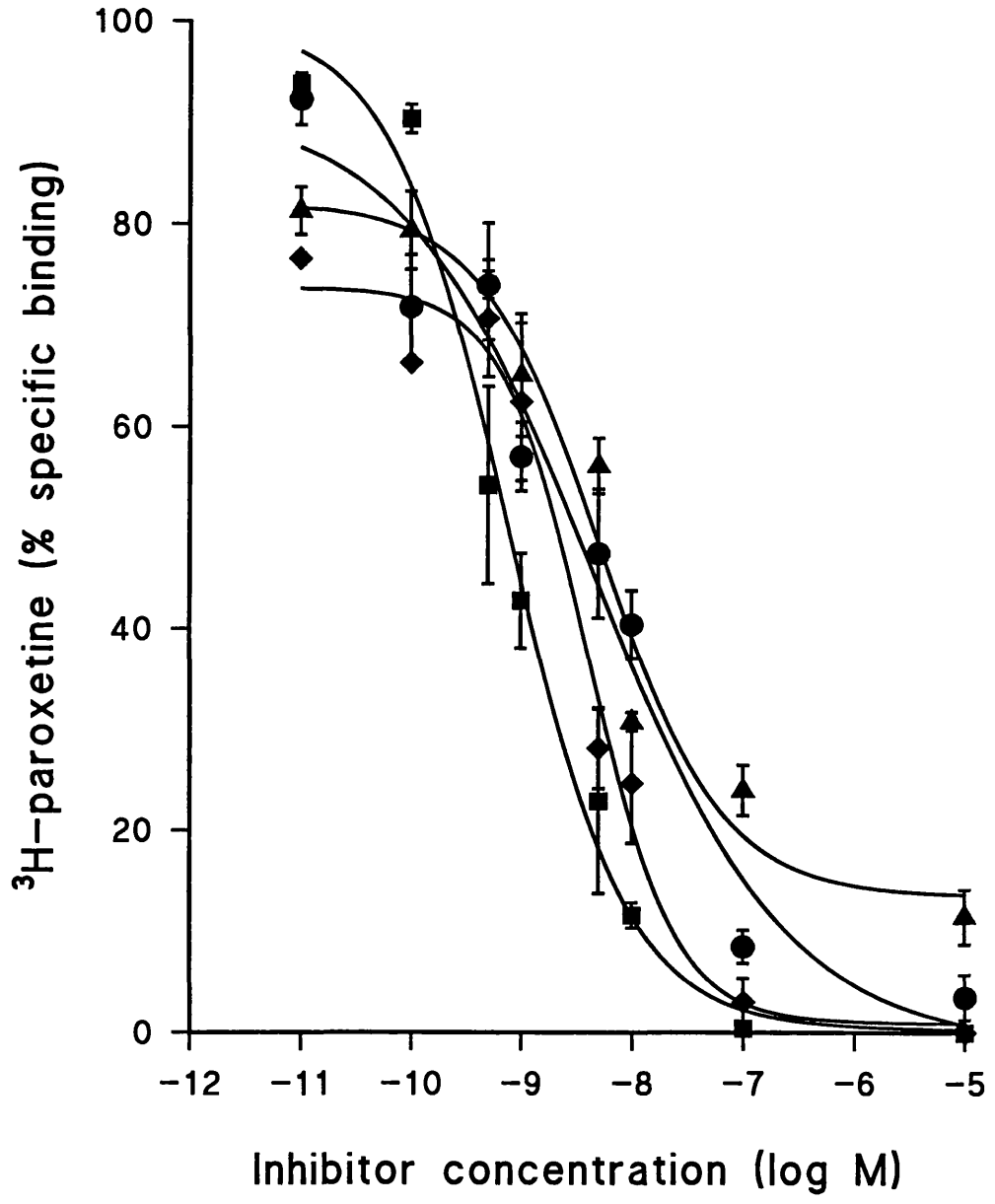


Fig. 3.3. Inhibition curves of specific ^{3}H-paroxetine binding to sagittal rat brain sections cut between 1.9 and 0.9mm from the midline. Sections were incubated for 120 minutes with 0.25nM ^{3}H-paroxetine in the presence of increasing concentrations (0.01nM to 10μM) of paroxetine (■), citalopram (▲), fluoxetine (●) and sertraline (◆). 4μM citalopram was included to determine non-specific binding. Data points are the mean ± s.e.m of 3 experiments, n=4 per experiment.

3.2. AUTORADIOGRAPHIC ANALYSIS OF ^{3}H-PAROXETINE BINDING IN RAT BRAIN SECTIONS.

3.2.1. Distribution of ^{3}H-paroxetine binding sites.

The autoradiographical distribution of ^{3}H-paroxetine binding sites at a concentration of 0.25nM was heterogeneous within the brain and selective for the grey matter with very little binding seen in the white matter (Table 3.2).

A high density of binding sites was found in the brainstem in the B groups of cell bodies and neurons containing 5-HT (Dahlström and Fuxe, 1964), with the highest density (340 ± 91 fmol/mg) observed in the dorsal raphe (B7). Other raphe nuclei such as the raphe pontis, median raphe and raphe magnus all contained a high density of binding sites (Fig. 3.5). Very high binding was seen in the facial nuclei, an area not measured in previous studies (Fig. 3.4).

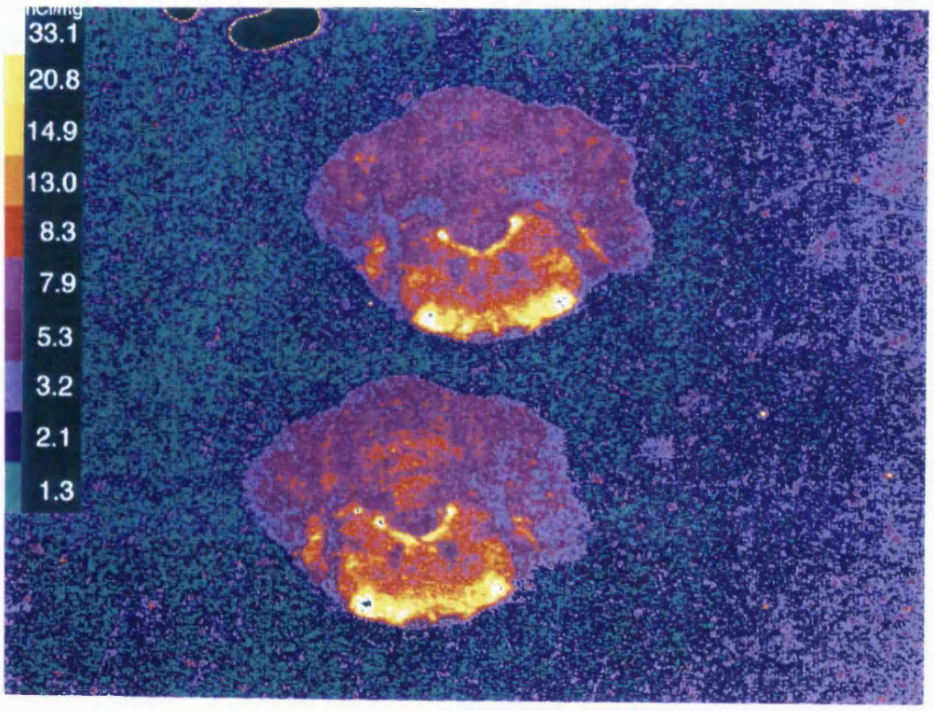
The inferior colliculus and superficial layer of the superior colliculus displayed moderate to high binding (62 -64 fmol/mg) in comparison to the lower levels (21 -39 fmol/mg) seen in the other layers of the superior colliculus (Fig. 3.5). The superficial layers of the superior colliculus are connected via the superior quadrigeminal brachium to the lateral geniculate which showed moderately high binding in comparison to the lower density of binding sites found in the medial geniculate.

Within the interpeduncular nuclei a heterogeneity of binding sites was revealed in the moderately high density of the rostral, anterior and intermediate nuclei and very low density in the caudal nuclei. In the substantia nigra there was little difference in the binding in the pars reticularis and pars compacta (Fig. 3.6).

Despite rich 5-HT innervation to the hippocampus, ^{3}H-paroxetine binding levels varied. Binding in the CA1, CA2 and CA3 fields of Ammon's horn ranged from 36 to 40 fmol/mg while the dentate gyrus had the lowest binding (6 ± 1

fmol/mg). Equally so, the hypothalamus showed low levels of binding (14 - 25 fmol/mg) in regions which are densely innervated with 5-HT fibres, the exception being the medial forebrain bundle which had higher levels of binding (38 ± 11 fmol/mg).

In general, binding site densities decreased in a rostral direction away from the raphe nuclei into the forebrain. In the thalamus, only the midline nuclei (paraventricular, intermedial, rhomboid and reuniens nuclei) showed a moderately high level of binding (Fig. 3.7). In the forebrain which is expected to contain many 5-HT terminals, the frontal cortex (43 ± 8 fmol/mg) and basal ganglia (18 - 27 fmol/mg) showed moderate to low levels of binding. The entorhinal cortex was the only area in the cortex to exhibit a high binding site density(80 ± 12 fmol/mg).



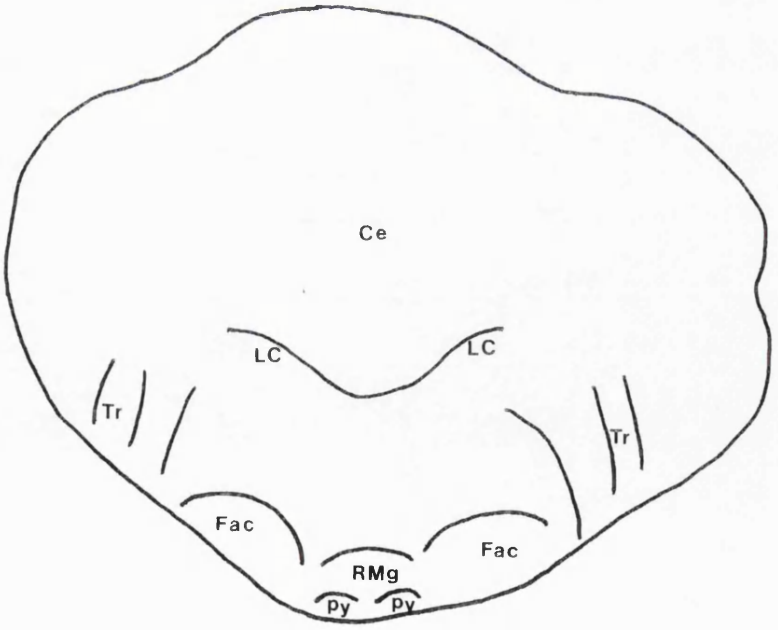
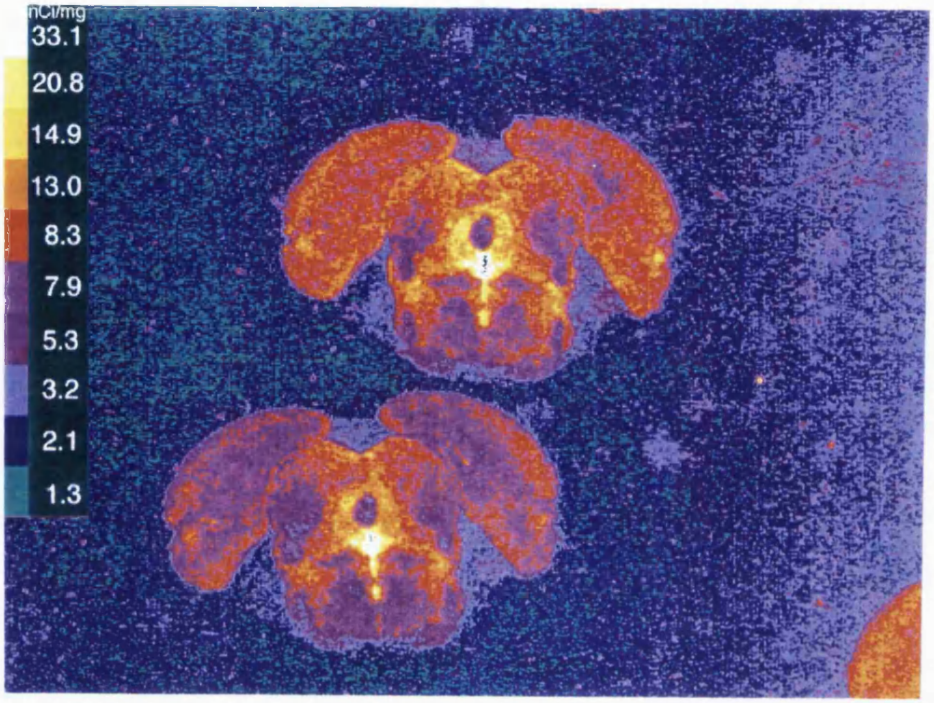


Fig. 3.4. Pseudocolour images of ^{3}H-paroxetine binding in the facial nuclei and locus coeruleus in the brainstem in coronal sections of rat brain. Binding site densities range from very high (white) to high (yellow), moderate (orange/red) and low (mauve). For abbreviation see Glossary.



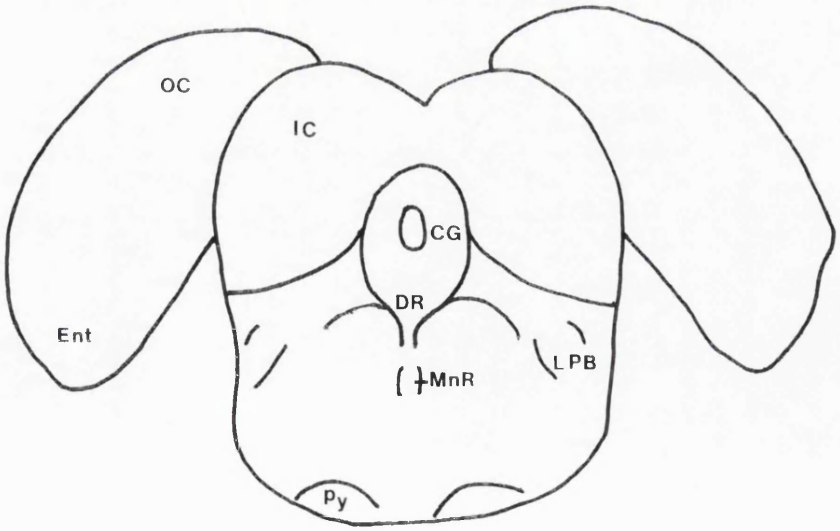
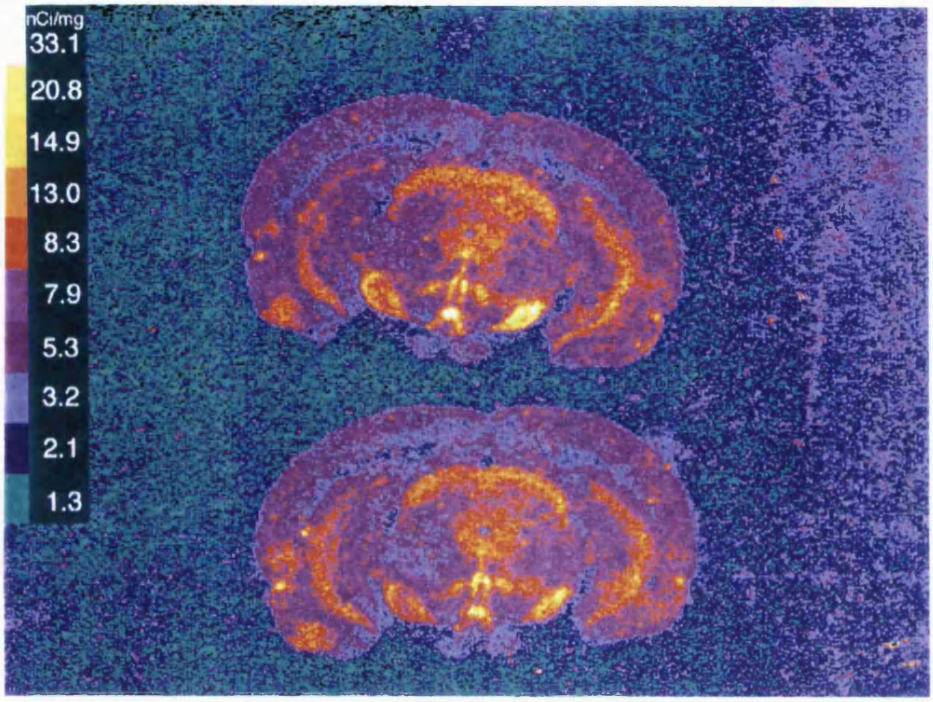


Fig. 3.5. Pseudocolour images of ^{3}H-paroxetine binding in the dorsal and median raphe in coronal sections of rat brain. Binding site densities range from very high (white) to high (yellow), moderate (orange/red) and low (mauve). For abbreviations see Glossary



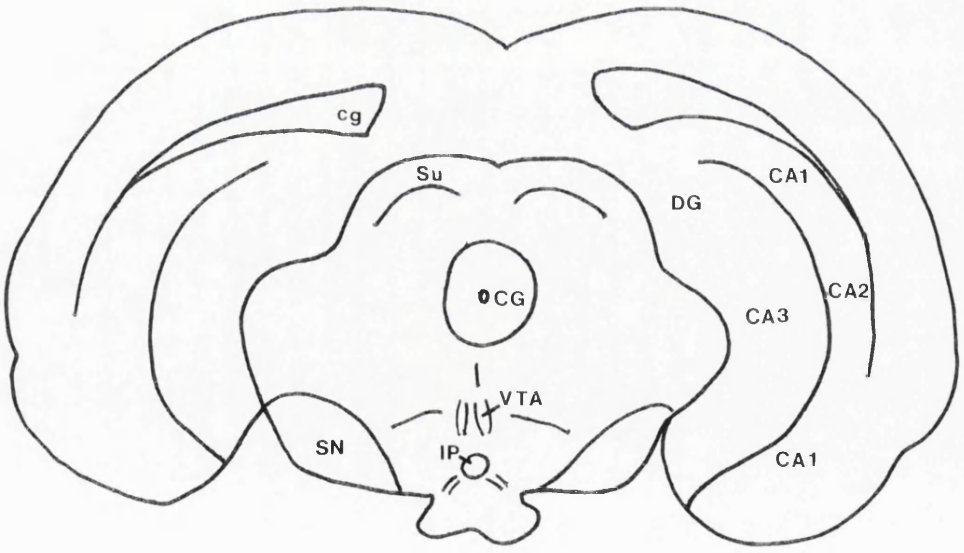
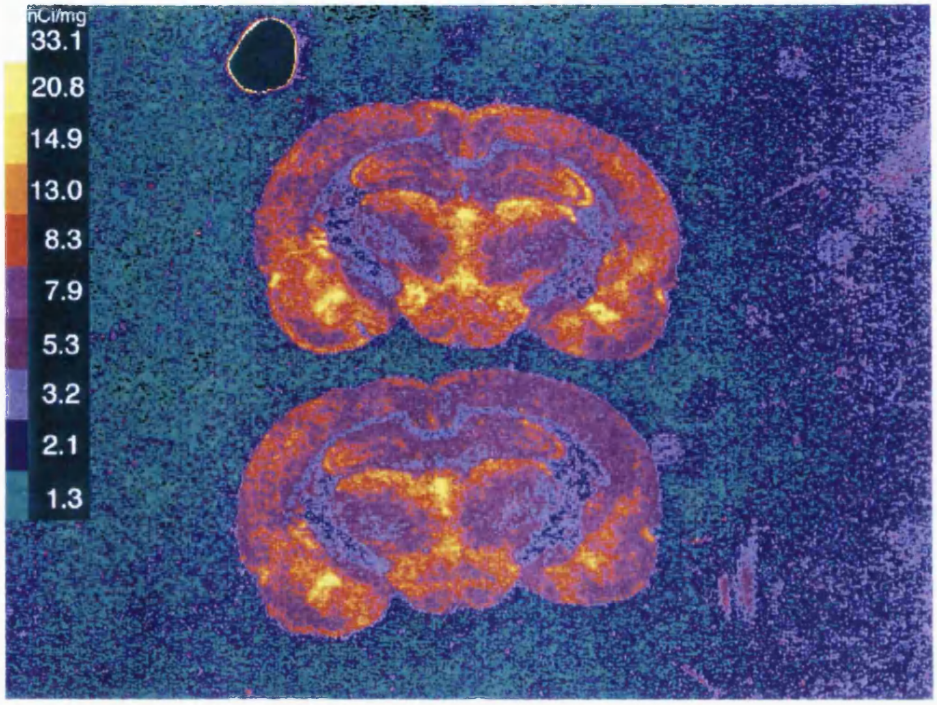


Fig. 3.6. Pseudocolour images of ^{3}H-paroxetine binding in the hippocampus, substantia nigra and superior colliculus in coronal sections of rat brain. Binding site densities range from very high (white) to high (yellow), moderate (orange/red) and low (mauve). For abbreviations see Glossary.



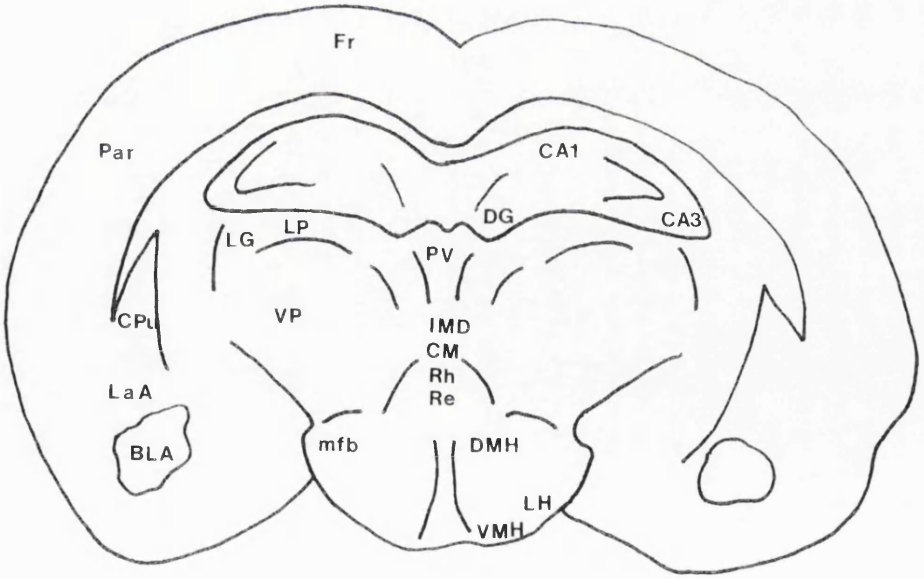


Fig. 3.7. Pseudocolour images of ^{3}H-paroxetine binding in the thalamus, hypothalamus and amygdala in coronal sections of rat brain. Binding site densities range from very high (white) to high (yellow), moderate (orange/red) and low (mauve). For abbreviations see Glossary.

Table 3.2. Quantitative autoradiographic analysis of the distribution of ^{3}H-paroxetine specific binding in rat brain. nu - nucleus. Data are the means ± s.e.m. of measurements taken from 3 sections per brain region. Experiments were carried out in triplicate. n = 3.

Brain Region	Abbreviation	^{3}H-paroxetine Bound (fmol/mg tissue)
Locus coeruleus	LC	290 ± 63
Pyramid	py	11 ± 3
Raphe magnus nu	RMg	97 ± 18
Trigeminal nu	Tr	62 ± 10
Facial nu	Fac	200 ± 44
Lateral parabrachial nu	LP	84 ± 5
Raphe pontis nu	RPn	170 ± 54
Cerebellum	Ce	14 ± 6
Dorsal raphe nu	DR	340 ± 91
Median raphe nu	MnR	83 ± 13
Central gray	CG	130 ± 32
Inferior colliculus	IC	64 ± 15
Rhabdoid nu	Rbd	97 ± 12
Reticulotegmental nu	RTg	34 ± 4
Superior colliculus	SC	
Superficial gray layer	Su G	62 ± 20
Optic nerve layer	Op	39 ± 12
Intermediate layer	In	27 ± 15
Deep gray layer	Dp	21 ± 10
Interpeduncular nuclei		
Rostral/apical subnu	IPR/IPA	75 ± 9
Intermediate subnu	IPI	69 ± 16
Caudal subnu	IPC	18 ± 8
Hippocampus		
Presubiculum	PrS	19 ± 9
Parasubiculum	PaS	19 ± 8
Subiculum	S	22 ±6
Dentate gyrus	DG	6 ± 1
CA1 field of Ammon's horn	CA1	36 ± 2
CA2 field of Ammon's horn	CA2	38 ± 9
CA3 field of Ammon's horn	CA3	40 ± 19
Fimbria	fi	0.37± 0.13
Substantia Nigra	SN	
Pars reticularis	SNR	42 ± 16
Pars compacta	SNC	55 ± 14
Ventral tegmental area	VTA	67 ± 10
Red nucleus	Red	7 ± 3
Medial geniculate nu	MG	11 ± 7
Lateral geniculate nu	LG	61 ± 17

Table 3.2. Cont.

Brain Region	Abbreviation	^{3}H-paroxetine bound (fmol/mg tissue)
Media mamilliary nu	MP	54 ± 11
Medial habenula	MHb	35 ± 5
Lateral habenula	LHb	12 ± 3
Thalamic nuclei		
Paraventricular, posterior	PVP	75 ± 18
Paraventricular, anterior	PVA	38 ± 2
Lateroposterior	LP	42 ± 12
Laterodorsal, ventrolateral	LDVL	33 ± 17
Laterodorsal, dorsomedial	LDDM	26 ± 7
Mediodorsal	MD	12 ± 4
Intermediodorsal	IMD	49 ± 11
Centromedial	CM	32 ± 21
Ventromedial	VM	11 ± 6
Ventroposterior	VP	4 ± 2
Ventrolateral	VL	7 ± 3
Rhomboid	Rh	44 ± 5
Reuniens	Re	39 ± 10
Anterodorsal	AD	8 ± 1
Anteroventral	AV	24 ± 2
Anteromedial	AM	21 ± 1
Reticular	Rt	15 ± 2
Paratenial	PT	33 ± 5
Amygdala		
Lateral	La A	42 ± 17
Basolateral	BL A	78 ± 18
Medial	Me A	17 ± 7
Central	Ce A	6 ± 1
Hypothalamus		
Posterior	PH	25 ± 6
Ventromedial	VMH	14 ± 8
Dorsomedial	DM	20 ± 5
Lateral	LH	19 ± 5
Anterior	AH	19 ± 2
Arcuate	Arc	21 ± 11
Suprachiasmatic	Sch	27 ± 1
Medial forebrain bundle	mfb	38 ± 11
Septal area		
Medial	MS	23 ± 4
Lateroseptal nu, dorsal	LSD	56 ± 5
Lateroseptal nu, intermediate	LSI	49 ± 3
Accumbens nu	Acb	20 ± 3
Septohippocampal nu	SHi	12 ± 3

Table 3.2. Cont:

Brain Region	Abbreviation	^{3}H-paroxetine bound (fmol/mg tissue)
Globus Pallidus	GP	18 ± 1
Caudate Putamen	CP	26 ± 3
Ventral Pallidum	VP	27 ± 10
Claustrum	CL	19 ± 7
Cortex		
Entorhinal	Ent	80 ± 21
Occipital	Ocp	46 ± 17
Frontal	Fr	43 ± 8
Parietal	Par	23 ± 7
Temporal	Te	24 ± 13
Piriform	Pir	7 ± 2
Perirhinal	PRh	17 ± 3
Cingulum	cg	19 ± 2
Olfactory tubercle	tu	59 ± 13
Islands of Calleja	ICj	74 ± 15

3.2.2. Effects of 5,7-dihydroxytryptamine injection in the dorsal raphe.

The effects of 5,7-DHT-induced lesions by direct stereotaxic injection into the dorsal raphe were assessed using quantitative autoradiography. Table 3.3. shows the mean ± s.e.m. (fmol/mg) specific binding of ^{3}H-paroxetine to 5-HT re-uptake sites in 5 control and 4 treated rats sacrificed 14 days after injection.

Binding in the area of the injection site, which was localized within the caudal subnuclei of the dorsal raphe, showed a 56.07 ± 10.65% reduction as compared to the rostral subnuclei which did not exhibit any reduction in binding (Fig. 3.8). The site of injection was confirmed in animals using cresyl violet staining.

The entorhinal and occipital cortex were the most affected with a decrease in binding of 65.69 ± 8.06 and 74.79 ± 5.37 % respectively. Specific binding was reduced by more than 50% in the central grey, CA1 field of Ammon's horn, medial septal area and the medial forebrain bundle (Fig. 3.9). Significant reductions in binding were also observed in the lateral parabrachial nuclei (49.47 ± 9.19%), inferior colliculus (30.94 ± 6.06%), superior colliculus (49.03 ± 11.45%), lateral hypothalamus (42.76 ± 12.60%) and caudate putamen (48.68 ± 10.86%). The substantia nigra and amygdala did not appear to be affected by the 5,7-DHT induced lesions. Whilst binding was inhibited in the frontal cortex by 59.07 ± 18.88%, statistical analysis did not confirm that this was significant.

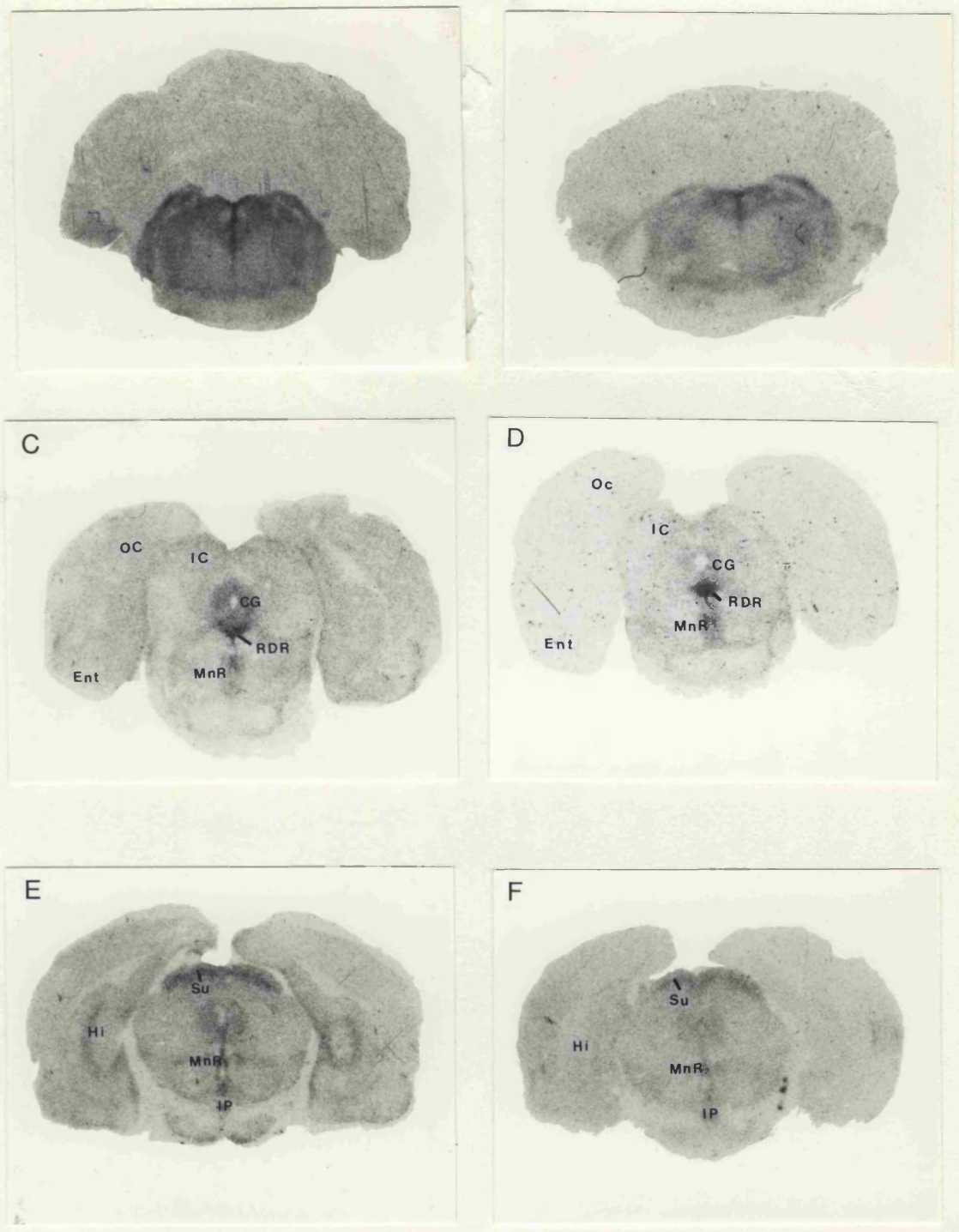


Fig.3.8. Photographic visualization of ^{3}H-paroxetine binding to coronal sections of rat brain following 5,7-DHT injection. Sections A,C, and E represent ^{3}H-paroxetine binding in the brainstem and hindbrain of sham-lesioned rats. Brain structures were identified by using the atlas of Paxinos and Watson (1986). Sections B,D, and F represent ^{3}H-paroxetine binding in similar brain regions of rats treated with 5,7-DHT injected directly into the dorsal raphe. Photographs were printed directly from autoradiograms. For abbreviations see Glossary.

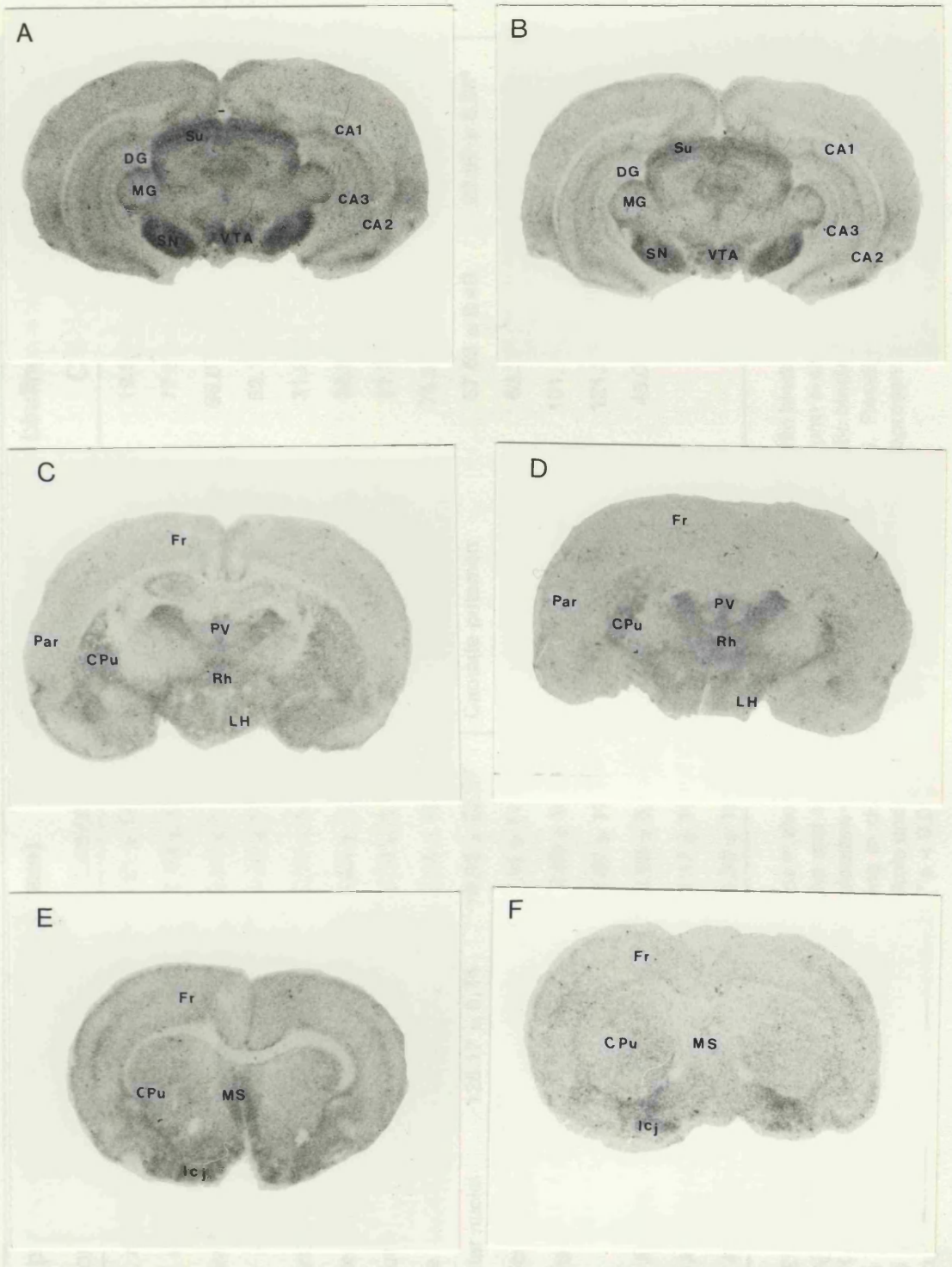


Fig. 3.9. Photographic visualization of ^{3}H-paroxetine binding to coronal sections of rat brain following 5,7-DHT injection. Sections A,C, and E represent ^{3}H-paroxetine binding in the midbrain and forebrain of sham-lesioned rats. Brain structures were identified by using the atlas of Paxinos and Watson (1986). Sections B,D, and F represent ^{3}H-paroxetine binding in similar brain regions of rats treated with 5,7-DHT injected directly into the dorsal raphe. Photographs were printed directly from the autoradiograms. For abbreviations see Glossary.

^{3}H-paroxetine binding (fmol/mg tissue)			^{3}H-paroxetine binding (fmol/mg tissue)		
Brain Region	Control	Lesion	Brain region	Control	Lesion
Dorsal raphe, caudal	159.83 ± 11.60	70.21 ± 17.01[a]	Dentate gyrus	19.96 ± 1.73	15.72 ± 3.85
Dorsal raphe, rostral	168.03 ± 12.73	151.68 ± 15.94	Paraventricular thalamus	77.59 ± 8.18	54.94 ± 11.76
Lateral parabrachial	84.16 ± 5.07	42.52 ± 7.73[a]	Rhomboid/reuniens nuclei	86.08 ± 11.93	63.39 ± 4.17
Central gray	77.17 ± 7.97	34.35 ± 7.32[a]	Lateral hypothalamus	53.16 ± 6.45	30.42 ± 6.69[b]
Entorhinal cortex	51.24 ± 9.85	17.58 ± 4.13[b]	Frontal cortex	31.26 ± 5.72	12.79 ± 5.9
Occipital cortex	25.35 ± 2.59	6.39 ± 1.36[a]	Parietal cortex	32.82 ± 4.21	21.82 ± 7.90
Inferior colliculus	39.55 ± 6.34	12.24 ± 2.40[a]	Lateral septal area	21.31 ± 4.02	9.43 ± 2.87
Median raphe nuclei	117.92 ± 7.37	93.28 ± 12.10	Medial septal area	75.52 ± 12.47	29.35 ± 9.20[b]
Interpeduncular nuclei	125.17 ± 6.74	79.05 ± 22.37	Caudate putamen	57.68 ± 8.49	29.60 ± 6.26[b]
Superior colliculus	96.63 ± 12.29	49.25 ± 11.07[b]	Ventral pallidum	63.31 ± 8.11	57.68 ± 8.49
Substantia nigra	105.26 ± 5.87	92.06 ± 9.40	Medial forebrain bundle	101.16 ± 28.45	52.81 ± 14.00[b]
Amygdala	93.01 ± 10.84	78.07 ± 17.15	Islands of Calleja	121.28 ± 22.25	72.25 ± 18.95
CA1, field of Ammon	64.73 ± 6.25	34.96 ± 5.63[b]	Accumbens	45.09 ± 10.27	23.07 ± 1.89
CA2, field of Ammon	54.64 ± 6.97	30.17 ± 10.64			
CA3, field of Ammon	50.72 ± 5.87	54.38 ± 15.22			

Table 3.3. Effect of 5,7-dihydroxytryptamine lesions in the dorsal raphe on ^{3}H-paroxetine specific binding in rat brain. Rats were injected with a single injection of 5,7 DHT into the dorsal raphe and sacrificed after 14 days. Coronal (10μm) brain sections were cut and incubated with 0.25nM ^{3}H-paroxetine for 120 minutes. 4μM citalopram was included to determine non-specific binding. Autoradiograms were analysed and the mean ± s.e.m. fmol/mg of specific binding in different brain regions was calculated. Results were determined from triplicate measurements of 3 sections from each region. 5 controls and 4 treated rats were used. Statistics represent the results of a two-tailed students t-test comparing binding in control and treated rats. [a] $p < 0.01$, [b] $p < 0.05$.

3.2.3. Effect of tetanus toxin injection in the ventral hippocampus.

Initial experiments were performed to determine the effect of intrahippocampal injection of phosphate buffer (vehicle) or tetanus toxin on binding of ^{3}H-paroxetine 7 days following treatment (Table 3.4). Quantitative analysis was performed on autoradiograms generated from brains injected unilaterally into the ventral hippocampus. ^{3}H-paroxetine binding in the contralateral side of sham-treated brains was compared with binding in the contralateral side of tetanus toxin-treated brains to see if tetanus toxin had any effect on binding in the adjacent hemisphere. Concurrent experiments were performed to determine whether levels of 5-HT and the metabolite 5-HIAA were affected (collaboration with P. Britton, School of Pharmacy). If tetanus toxin destroys just the 5-HT re-uptake binding sites then the B_{max} would be reduced and an increase in extracellular 5-HT and 5-HIAA would be expected. If the toxin destroys the whole nerve terminal, then the extracellular levels of 5-HT and 5-HIAA would be expected to decrease.

Measurements taken from the hemisphere of the injection site of phosphate buffer showed no significant difference in binding to the untreated hemisphere (Fig. 3.10). Unilateral injection of tetanus toxin into the ventral hippocampus resulted in a significant reduction in ^{3}H-paroxetine specific binding to 5-HT re-uptake sites in ventral CA1, CA3 pyramidal cell layers and also in the occipital cortex (Fig. 3.11). Although there appeared to be a reduction in the ventral dentate gyrus this was not significant. A comparison of contralateral binding in sham- and tetanus toxin-treated brains showed that tetanus toxin had no effect on ^{3}H-paroxetine binding in the adjacent hemisphere (Fig. 3.12).

Table 3.4. Effect of tetanus toxin injected into the ventral hippocampus on ^{3}H-paroxetine specific binding sites in rat brain. Values are expressed as mean ± s.e.m of 6 sections per rat, n = 3 rats per treatment. Contralat. - contralateral to injection site; Ipsilat - ipsilateral to injection site. * $p < 0.05$ compared to sham treated rats injected with phosphate buffer. Statistics represent the results of a Mann-whitney U-test.

	Specific ^{3}H-paroxetine binding sites (fmol/mg)			
Area	Vehicle injected		Tetanus toxin treated	
	Contralat.	Ipsilat.	Contralat.	Ipsilat.
CA1, ventral	14.2 ± 4.3	13.5 ± 5.4	13.4 ± 2.0	7.2 ± 0.9*
CA3, ventral	14.4 ± 5	12.0 ± 4.5	13.6 ± 1.3	7.4 ± 0.8*
Dentate gyrus, ventral	11.2 ± 2.5	14.3 ± 4.0	9.9 ± 2.0	7.5 ± 0.4
Dentate gyrus, dorsal	4.5 ± 1.3	4.6 ± 1.1	5.1 ± 1.3	3.9 ± 0.4
Occipital cortex	8.5 ± 0.8	7.1 ± 1.3	9.5 ± 1.5	4.6 ± 0.1*
Central gray	19.5 ± 2.4	20.5 ± 3.1	17.7 ± 1.5	17.6 ± 1.5
Ventral tegmental area	20.3 ± 1.2	25.7 ± 4.1	26.8 ± 3.3	28.2 ± 2.5
Substantia Nigra	20.6 ± 6.5	20.9 ± 6.5	22.5 ± 2.1	22.4 ± 3.6

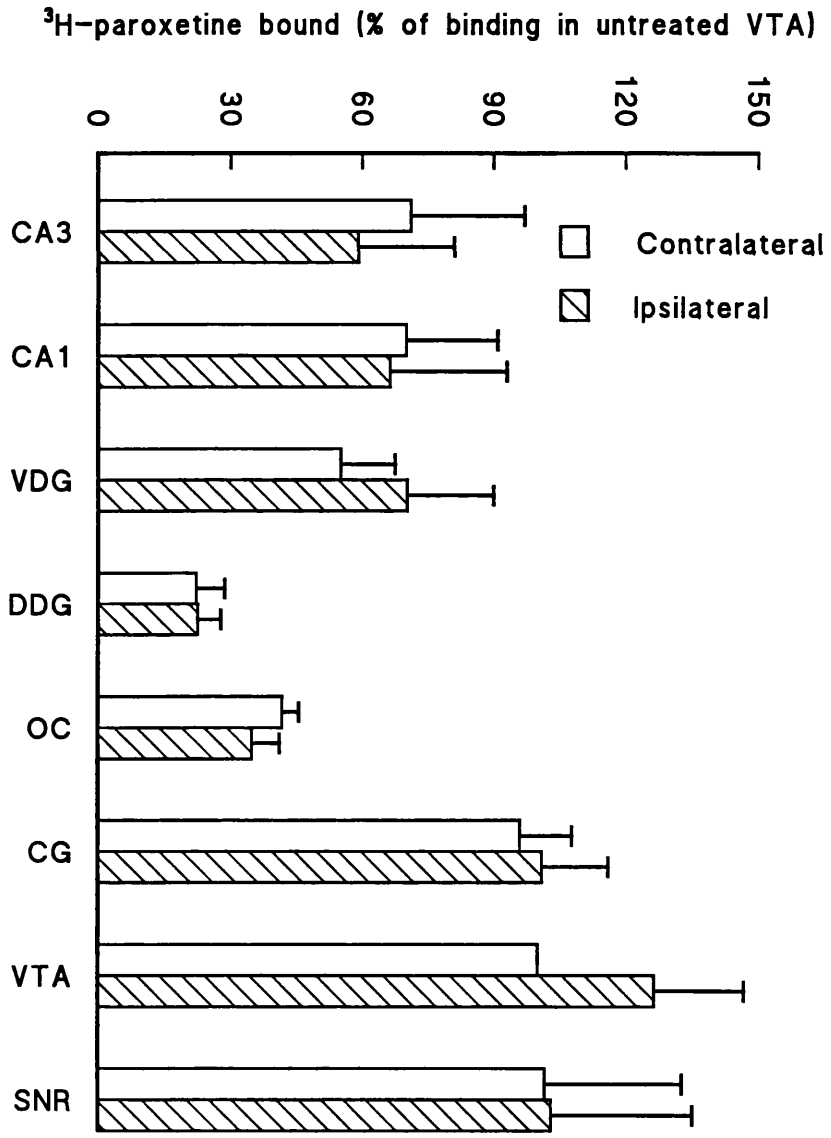


Fig 3.10. Effect of unilateral intrahippocampal injection of phosphate buffer (vehicle) on ^{3}H-paroxetine binding sites. Rats were sacrificed after 7 days and 10μm coronal sections cut for autoradiography. Results were expressed as a mean ± s.e.m. percent of specific binding in untreated VTA from 6 sections per rat, n=3 rats per treatment. CA3, CA1 - ventral pyramidal cell layers of fields of Ammon's horn; VDG, DDG - ventral and dorsal dentate gyrus granular layers; OC - occipital cortex; CG - central gray; VTA - ventral tegmental area; SNR - substantia nigra pars reticularis.

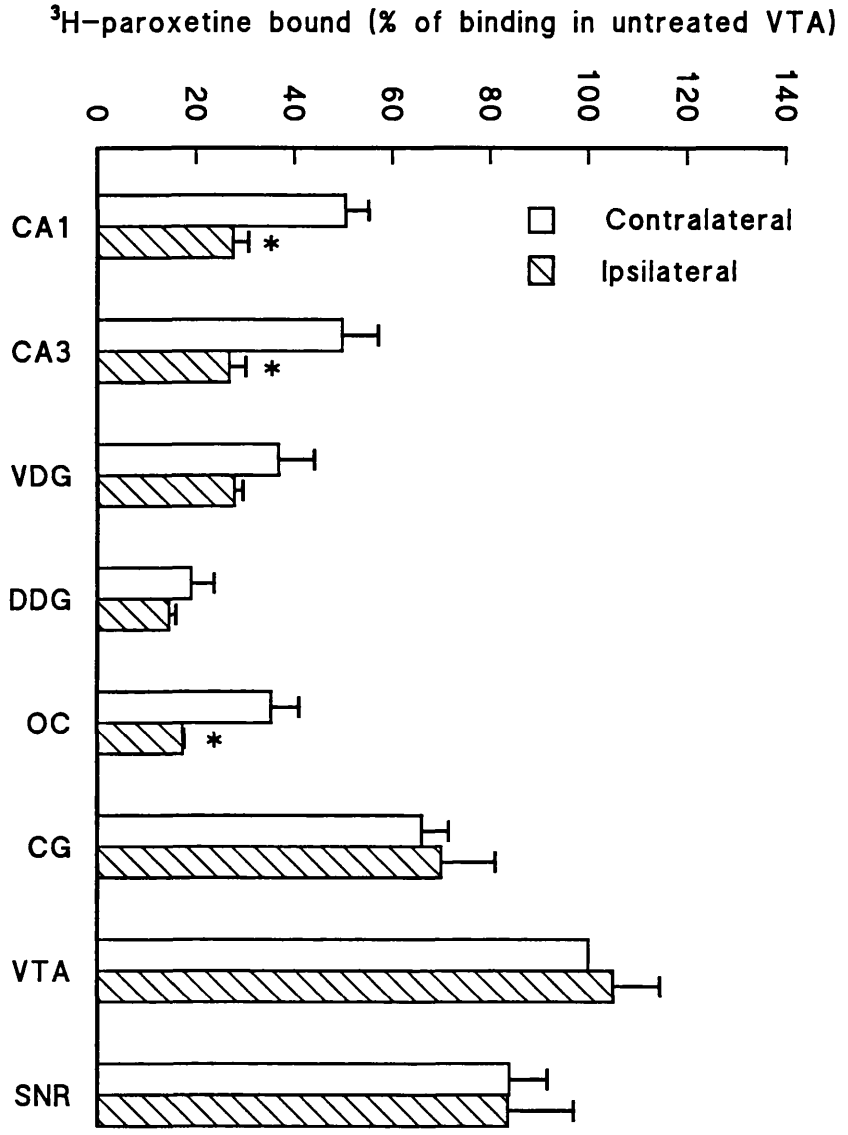


Fig. 3.11. Effect of unilateral intrahippocampal injection of tetanus toxin on ^{3}H-paroxetine binding sites. Rats were sacrificed after 7 days and 10μm coronal sections cut for autoradiography. Results were expressed as a mean ± s.e.m. percent of specific binding in untreated VTA from 6 sections per rat, n=3 rats per treatment. Statistics represent the results of a Mann-Whitney U-test comparing treated vs untreated hemispheres, * $p < 0.05$. CA3, CA1 - ventral pyramidal cell layers of fields of Ammon's horn; VDG, DDG - ventral and dorsal dentate gyrus granular layers; OC - occipital cortex; CG -central gray; VTA - ventral tegmental area; SNR - substantia nigra pars reticularis.

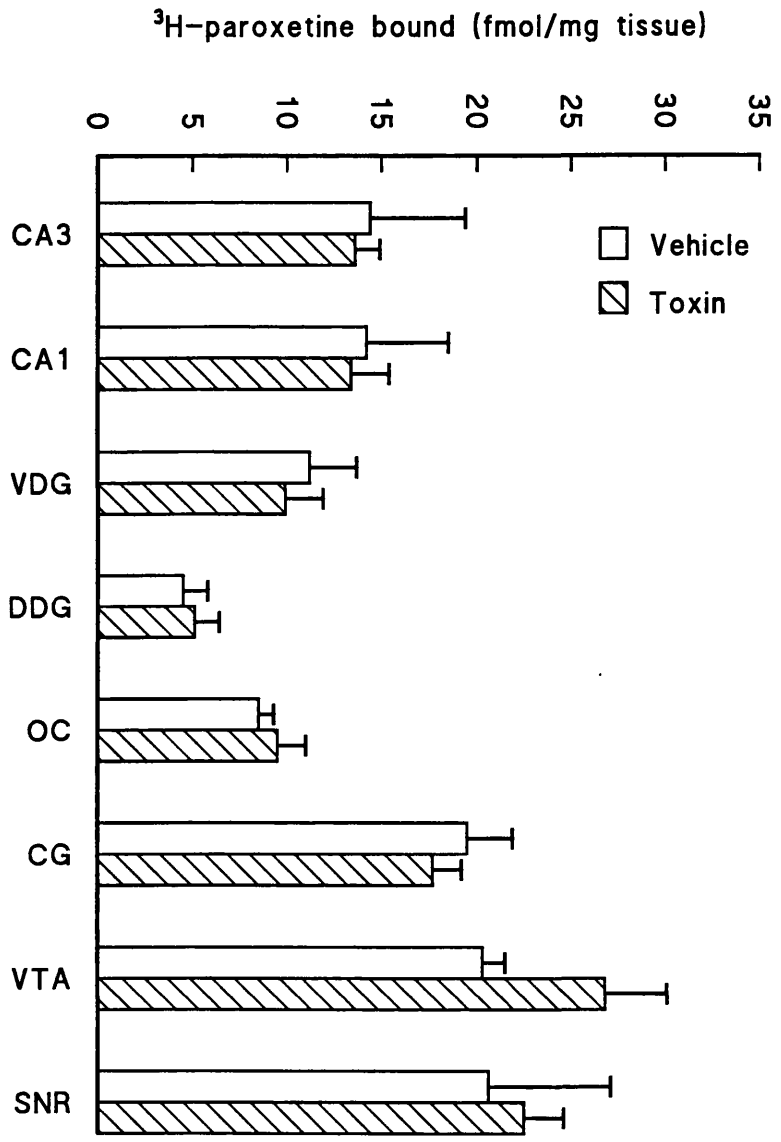


Fig 3.12. Comparison of ^{3}H-paroxetine binding in the contralateral hemisphere of tetanus toxin and vehicle treated rats sacrificed after 7 days. 10μm coronal sections were cut for autoradiography with ^{3}H-paroxetine. Results were expressed as a mean ± s.e.m. fmol/mg tissue of 6 sections per rat, n=3 rats per treatment. CA3, CA1 - ventral pyramidal cell layers of fields of Ammon's horn; VDG, DDG - ventral and dorsal dentate gyrus granular layers; OC - occipital cortex; CG -central gray; VTA - ventral tegmental area; SNR - substantia nigra pars reticularis.

DISCUSSION

The optimum binding parameters of 120 minutes incubation and two 60 minute washes chosen for this work are the same as those used by De Souza and Kuyatt (1987). These authors used 1μM citalopram to block the non-specific binding sites, whereas in the present study 4μM citalopram was used. Hrdina et al (1990) incubated for 60 minutes and had a wash time of 2 x 20 minutes at 37°C, however it was found that washing at 37°C reduced the total ^{3}H-paroxetine binding but not the non-specific binding. Since the specific ^{3}H-paroxetine binding in the present study reached an optimum level at 120 minutes and remained at this level for a further 60 minutes, it did not seem necessary to use the longer wash time of 2 four hour periods employed by Chen et al (1992)

In the optimum wash and incubation time experiments, the specific binding was found to be 64% at 120 minutes for the incubation and 2 x 60 minutes for washing. Although the specific binding of ^{3}H-paroxetine is known to reach 90%, these sections were cut on a sagittal plane 1.4mm from the midline which did not include the very high binding density areas of the raphe nuclei, and as a result the specific binding was not as high as expected. In autoradiographical analysis of sections taken in this plane, certain areas such as the substantia nigra and ventral tegmental area were found to have a specific binding percentage of up to 80% of total binding (Section 3.2.1).

K_d values obtained from saturation studies of ^{3}H-paroxetine binding in slide-mounted rat brain sections have varied from 0.001nM (De Souza and Kuyatt, 1987) to 0.36nM (Hrdina et al, 1990) indicating a high affinity of ^{3}H-paroxetine for its binding site. The K_d of 0.329nM measured in the present study is in agreement with the above results and in all cases the binding was saturable, reaching equilibrium within 60 minutes. Although the B_{max} value of 216 fmol/mg obtained in this study was lower than the B_{max} values measured in other autoradiographical studies of 276 fmol/mg (Hrdina et al, 1990) and 268 fmol/mg

(Chen et al, 1992), sagittal sections, as opposed to coronal sections, were measured here and therefore different regions in the brain were assessed.

Characterization of ^{3}H-paroxetine binding in rat brain homogenates revealed similar K_d values of 0.1 - 0.15nM (Habert et al, 1985; Mellerup and Plenge, 1986), although Marcusson et al (1988) found at lower tissue concentrations (0.75mg/ml) that the K_d was higher (0.03nM). While the K_d values were lower than those measured in autoradiographical studies, the B_{max} was found to be higher in the homogenate binding experiments (398 - 690 fmol/mg). This is because the binding values were expressed as fmol/mg protein whereas the autoradiographical values were expressed as fmol/mg tissue.

Initial saturation experiments performed in the present study revealed that the K_d was lower than 0.329nM. The results are not shown because insufficient data points were used to generate an accurate saturation curve, however at that time a concentration of 0.25nM was chosen for determining ^{3}H-paroxetine binding sites with the impression that this concentration was sufficiently high enough to bind at B_{max} values. At the correct K_d value of 0.329nM, a 0.25nM concentration of ^{3}H-paroxetine binds to less than 50% of the potential binding sites. Since the correct saturation experiments were performed once all the distribution and lesion experiments had been carried out, a decision was made to report the findings of experiments using 0.25nM ^{3}H-paroxetine. Under these circumstances, it would be assumed that in the case of lesion experiments, a decrease in binding sites is due to a reduced B_{max}, and in the case of reduced binding in the presence of the inhibitors used in this study, the apparent affinity of ^{3}H-paroxetine is lowered. In order to confirm whether a change in ^{3}H-paroxetine binding sites is due to a change in B_{max} or K_d or both, a saturation curve would have to be performed in the presence and absence of procedure-induced circumstances.

Potent inhibition (low nanomolar IC_{50} values) of ^{3}H-paroxetine binding to rat brain sections by SSRI's such as citalopram, sertraline and fluoxetine confirmed

that ^{3}H-paroxetine specific binding was associated with the serotonin transporter. Inhibition of ^{3}H-paroxetine binding in homogenates revealed a ranking order of paroxetine > sertraline > citalopram > fluoxetine (Cheetham et al, 1993; Mellerup and Plenge, 1986). The ranking order of the present investigation showed that while fluoxetine was more potent than sertraline or citalopram at inhibiting ^{3}H-paroxetine binding to rat brain sections, the IC_{50}'s measured were very similar (3.87 - 5.49nM) and could not be significantly distinguished from each other.

The distribution of ^{3}H-paroxetine binding sites in this study was in good agreement with autoradiographical studies using ^{3}H-paroxetine (De Souza and Kuyatt, 1987; Hrdina et al, 1990) as well as results obtained from studies using other serotonin re-uptake inhibitors such as ^{3}H-citalopram (D'Amato et al, 1987), ^{3}H-indalpine (Savaki et al, 1985) and ^{3}H-cyanoimipramine (Kovachich et al, 1988). A recent autoradiographic study of ^{3}H-paroxetine and ^{3}H-milnaciprin (a novel serotonin and noradrenaline re-uptake inhibitor) showed much higher ^{3}H-paroxetine binding densities in a number of areas which are generally accepted to have moderate to low binding (frontal cortex, caudate putamen, hypothalamus, hippocampus, ventral pallidum and globus pallidus). However, only two 5 minute washes were used which could account for the high binding values (Barone et al, 1994).

In general, the distribution of ^{3}H-paroxetine binding sites in slide-mounted brain sections correlated well with the distribution of serotonergic innervation in the rat brain (Kosofsky and Molliver, 1987; Lidov et al, 1980; Parent et al, 1981; Steinbusch, 1981). The high density of ^{3}H-paroxetine binding sites in the facial nuclei, and less so in the raphe magnus, correspond with the B1 and B3 groups of serotonin containing cell bodies. These represent the bulk of the descending serotonin system since most of the 5-HT containing fibres emerging from this region enter the spinal cord. At the levels of B7, B8 and B9, which contain the dorsal raphe, median raphe and raphe pontis, the binding of ^{3}H-paroxetine was also high, with the highest density of binding sites found in the dorsal raphe.

The serotonin content of the brain stem nuclei as measured by Palkovits et al (1974) correlated well with the binding of ^{3}H-paroxetine found in the brainstem with the highest concentrations seen in the dorsal raphe, median raphe and facial nuclei. High concentrations of 5-HT were also observed in the substantia nigra, central grey and locus coeruleus, areas which exhibit high levels of ^{3}H-paroxetine binding.

Parent et al (1981) observed that the majority of the collaterals which emerged from the periventricular system to innervate the colliculi, terminate in the superficial layer of the superior colliculus. This was reflected in the high levels of ^{3}H-paroxetine binding sites found in this area. A high density of ^{3}H-paroxetine binding sites was also seen in the ventral tegmental area and substantia nigra where the periventricular and transtegmental serotonin systems merge, indicating the presence of 5-HT re-uptake sites along these fibre systems. In the hypothalamus, which has a very dense 5-HT innervation, only low levels of ^{3}H-paroxetine binding could be detected, except in the medial forebrain bundle through which most of the ascending fibres pass. Equally so in the caudate putamen, globus pallidus and frontal cortex where many 5-HT fibres terminate, moderate to low levels of ^{3}H-paroxetine were seen.

In the limbic system the serotonin content was high in the olfactory tubercle and basolateral amygdala (Saavedra et al, 1974), areas which show high levels of ^{3}H-paroxetine binding. A two-fold difference in serotonin content was noted in the medial and the lateral habenula which was reflected in the levels of ^{3}H-paroxetine binding in these regions. Saavedra et al (1974) also noted that the lowest serotonin content in the limbic system was found in the hippocampus (8-fold less that in the median forebrain bundle), whereas in this study the levels of ^{3}H-paroxetine binding were similar in both regions. Since the hippocampus is richly innervated with 5-HT terminals (Oleskavich and Descarries, 1990) this suggests that higher levels of both serotonin content and ^{3}H-paroxetine binding should be expected.

The predominant source of afferents to the hippocampus originate in the median raphe (Kohler and Steinbusch, 1982) and projections from both the dorsal and median raphe innervate the entorhinal cortex which exerts a strong influence on the hippocampus. The function of serotonin in the entorhinal area and hippocampus is unclear, however the fact that many 5-HT fibres enter the hippocampus via the entorhinal cortex from the raphe nuclei suggest that modulation of raphe 5-HT activity will affect both structures (Steward, 1976).

The effects of stress are thought to influence 5-HT receptor binding in the hippocampus. An increase in radioligand binding to 5-HT_{1A} receptors was observed in the initial stages of restraint stress (Mendelson and McEwen, 1991), however binding of ^{3}H-paroxetine to 5-HT re-uptake sites in the hippocampus was unchanged by the effects of restraint stress (Watanabe et al, 1993). Mendelson et al (1993) found that chronic administration of estradiol benzoate reduced ^{3}H-paroxetine binding in the hippocampus, and that lower levels of ^{3}H-paroxetine binding were found in female rats treated with estradiol benzoate than male rats. Demographic studies have shown that females are more susceptible to depression than men, suggesting that a larger number of 5-HT re-uptake sites are present in females than males (Weissman and Klerman, 1985). Mendelson et al (1993) therefore suggested that, in contrast to increased levels of estradiol reducing ^{3}H-paroxetine binding, decreased estradiol levels could increase ^{3}H-paroxetine binding by up-regulating 5-HT re-uptake sites. Estradiol levels are reduced during menstruation, postpartum period and menopause, which could explain the increased susceptibility of females to depression during these periods in their lives. What is clear is that there is a steroidal modulatory role on serotonergic activity in the hippocampus.

The mis-match between the number of 5-HT terminals and 5-HT re-uptake sites labelled with ^{3}H-paroxetine in the hippocampus suggest that either the number of 5-HT re-uptake sites in the hippocampus are sufficient to play such a role or there are a greater number of 5-HT re-uptake sites present, only some of which are labelled by ^{3}H-paroxetine. Since the effect of 5,7-DHT lesions in the

hippocampus completely abolished ^{3}H-citalopram binding (Lawrence et al,1993) but not ^{3}H-paroxetine (Scheffel and Ricaurte, 1990) the latter case may be true. Scheffel and Ricaurte (1990) found that the reduction in levels of 5-HT (70-90%) in the frontal cortex, hippocampus and striatum was greater than the reduction of ^{3}H-paroxetine binding (30-40%). This could also mean that paroxetine binds to a site which is not sensitive to 5,7-DHT, a theory supported by Gobbi et al (1990) who showed that there was no correlation in the changes in ^{3}H-paroxetine binding induced by serotonergic lesions with changes in ^{3}H-5-HT uptake.

Other dorsal raphe selective neurotoxins such as MDMA and MDA significantly reduced ^{3}H-paroxetine binding (50-60%) in the hippocampus (Battaglia et al, 1987) while PCA treatment virtually abolished ^{3}H-paroxetine hippocampal binding (19%); (Hrdina et al, 1990). A more detailed investigation by Sharkey et al (1991) into the effect of chronic administration of MDMA on hippocampal function revealed an inconsistent loss of ^{3}H-paroxetine binding in the hippocampus, indicating that the dorsal CA2 and CA3 fields appear to be more vulnerable to the neurotoxin than their ventral counterparts or CA1 of both regions. In the case of other serotonergic re-uptake inhibitors a further disparity was noted. PCA treatment in the hippocampus reduced ^{3}H-cyanoimipramine (^{3}H-CN-IMI) binding in the CA2 and CA3 region by 39% and 38% respectively with the greatest loss seen in CA1 (51%); (Hensler et al, 1992).In contrasr, pre-treatment with MDA reduced ^{3}H-CN-IMI binding in the hippocampus by up to 50% and the greatest reduction was found in CA3, with the least seen in CA1 (Brunswick et al, 1992). 5,7-DHT lesioning in rat brain virtually abolished ^{3}H-citalopram binding sites in the hippocampus, with CA3 apparently more susceptible than CA1 (Lawrence et al, 1993a). This together with O'Hearn's finding (1988) that there is a subpopulation of 5-HT fibres which are apparently resistant to MDMA supports the theory that 5-HT re-uptake binding sites in the hippocampus are selectively susceptible to different neurotoxins.

In the present investigation, 5,7-DHT was injected directly into the caudal portion of the dorsal raphe nuclei. This produced a 56% reduction in ^{3}H-paroxetine binding 14 days after injection. No change in binding was detected in the median raphe which is consistent with evidence that 5,7-DHT is selective for the dorsal raphe nuclei (Hensler et al, 1994; Cudennec et al, 1988). A reduction in the levels of binding was found throughout the brain with about 50% decreases in most regions. The greatest reduction occurred in the entorhinal and occipital cortices (66% and 75% respectively). The only region in the hippocampus that was significantly affected was CA1. In no regions of the brain was the binding completely abolished.

Habert et al (1985) found a 90% reduction in ^{3}H-paroxetine binding to rat cortical membranes 15 days after 5,7-DHT injection. This was supported by Gobbi et al (1990) who found, by using autoradiography, that 5,7-DHT-induced lesions completely abolished ^{3}H-paroxetine binding in the majority of brain areas 7 days following injection. In both cases, however, the 5,7-DHT was administered i.c.v. and not directly into the dorsal raphe. Staining with cresyl violet to confirm the injection site in the present study showed that the injections were mainly in the caudal portion of the dorsal raphe (CDR). ^{3}H-paroxetine binding in the rostral dorsal raphe (RDR) was not affected and therefore the lack of complete abolition of binding sites could be due to innervation from the remaining intact rostral portion of the dorsal raphe.

Vertes (1991) analyzed the distribution of caudal and rostral specific projections from the dorsal raphe using immunohistochemistry. He found that there was a significant overlap in most regions in the brain, however dense labelling of efferents from the CDR could be seen in the entorhinal and occipital cortex which may explain the significant reduction in ^{3}H-paroxetine binding sites in those areas. Fluorescent retrograde tracing of serotonin innervation from the entorhinal cortex and hippocampus identified the dorsal raphe, in particular the caudal nuclei, as the major source of afferents to these areas (Kohler and Steinbusch, 1982). Vertes (1991) did find projections from both CDR and RDR

to the central gray, amygdala, lateral hypothalamus, caudate putamen, medial forebrain bundle all of which showed a reduction in ^{3}H-paroxetine binding sites.

A recent investigation by Gobbi et al (1994) showed a time dependent response of the dorsal and ventral portions of the dorsal raphe to 5,7-DHT-induced lesions following i.c.v. administration. ^{3}H-paroxetine binding decreased in the dorsal portion of the dorsal raphe 5 and 14 days after injection, whereas binding was found to increase in the ventral portion, 2 and 5 days post-lesion. These authors suggested that this could be a reflection of an up-regulation of the 5-HT re-uptake sites as the result of increased activity in serotonergic neurons resistant to 5,7-DHT.

The fact that 5,7-DHT injections into the dorsal raphe reduced, but did not completely abolish, ^{3}H-paroxetine binding in this study confirms that there is a significant overlap of innervation from the caudal and rostral dorsal raphe as well as from the median raphe. Since the selective lesioning of the CDR was unintentional in the current investigation, it is not possible to determine whether the lack of complete reduction in ^{3}H-paroxetine binding is due to binding to 5,7-DHT-insensitive sites or additional innervation from the rostral dorsal and median raphe. Multiple, serial injections of 5,7-DHT into the dorsal raphe would be needed to complete destruction within the whole dorsal raphe.

The effect of intrahippocampal tetanus toxin treatment on serotonergic re-uptake sites labelled with ^{3}H-paroxetine was investigated. This work was carried out in collaboration with P. Britton (School of Pharmacy, London) who measured the change in 5-HT and 5-HIAA brain tissue levels following intrahippocampal treatment with tetanus toxin. This was done by using microdialysis to collect daily samples from probes implanted into the ventral hippocampus. The monoamine content of the samples was measured using high performance liquid chromatography (HPLC);(P. Britton, Thesis 1994). Since the brains from animals used in the microdialysis experiments could not be used for autoradiography, the single dose tetanus toxin treatment for ^{3}H-

paroxetine binding and microdialysis experiments were carried out in parallel.

7 days following a single, intrahippocampal dose (1000 MLD's) of tetanus toxin, ^{3}H-paroxetine binding was found to be reduced in CA1 and CA3 of the ventral hippocampus and in the occipital cortex. Binding in other areas in the hippocampus (dorsal and ventral dentate gyrus) and surrounding regions (central gray, substantia nigra, ventral tegmental area) was unaffected. Intrahippocampal injection of ^{125}I-tetanus toxin in rats was found to be localised in the ventral hippocampus, with some residue along the injection tract (Mellanby, 1977), however no indication was given of the extent of diffusion of tetanus toxin within the ventral hippocampus. Bagetta et al (1990a) demonstrated that neurodegeneration caused by tetanus toxin could extend only 400μm either side of the injection site (granular layer of the dentate gyrus). Since CA1 and CA3 are, respectively, approximately 1200μm and 800μm in distance from the site of injection, it is unclear whether the reduction in ^{3}H-paroxetine binding sites is the direct result of diffusion of tetanus toxin into these areas. The reduction in binding seen in the occipital cortex is very likely due to the effect of toxin contamination along the needle tract.

Following tetanus toxin treatment, extracellular 5-HT levels decreased to approximately 35% of controls by day 3 and by day 7, 5-HT levels were returning to basal concentrations. Extracellular levels of 5-HIAA decreased to approximately 78% of controls and by day 7 had resumed normal basal concentrations (P. Britton, Thesis 1994). This is in contrast to the results obtained by Aguilera and colleagues (1987, 1988) who found an increase in 5-HT levels following i.p. injections of tetanus toxin. The reduction in 5-HT and 5-HIAA levels indicates that destruction of the serotonin nerve terminals has occurred since neurodegeneration will result in a loss of 5-HT release as well as a loss of re-uptake sites. It is also possible that the neuronal damage is the result of tetanus toxin-induced impairment of inhibitory neurotransmission creating unopposed neurotoxic excitation as suggested by Bagetta et al (1990b). However, it is doubtful that the reduction in ^{3}H-paroxetine binding sites

observed in CA1 and CA3, but not in the dentate gyrus (site of injection) is produced by this effect.

Therefore, the most likely explanations for the reduction in ^{3}H-paroxetine binding sites observed in CA1 and CA3 is that 1) tetanus toxin is able to diffuse within the ventral hippocampus to CA1 and CA3 from the injection site, or 2) the tetanus toxin-induced reduction in neuronal 5-HT release caused a down-regulation of 5-HT re-uptake sites. Both cases are speculative, however, and immunohistochemical procedures would have to be carried out to visualize the effects of tetanus toxin on the neurons in order to confirm that tetanus toxin does cause direct neurodegeneration. Measurements of extracellular concentrations of MDMA would have to be taken to determine if the damage is caused by unopposed excitation.

Although it is unclear as to how tetanus toxin produces a reduction in ^{3}H-paroxetine binding sites, it appears that tetanus toxin does exert its effect on 5-HT re-uptake sites without exhibiting any direct neurotoxic effect on the contralateral region. However, a unilateral injection of 5,7-DHT into the hippocampus was found to produce a reduction in tryptophan hydroxylase in both the ipsilateral and contralateral hippocampus indicating that damage was caused to both sides of the hippocampus (Clewans and Azmitia, 1984). In addition, a unilateral injection of 5,7-DHT was found to reduce ^{3}H-5HT uptake in the ipsilateral hypothalamus with partial reduction observed in the contralateral hypothalamus (Frankfurt and Azmitia, 1983). Therefore, it is possible that compensation by the contralateral region to increase 5-HT release, or up-regulation of the remaining 5-HT sites, could mask any neurotoxic effects in the contralateral region and this should be taken into account.

CHAPTER 4

DEVELOPMENT OF A NOVEL METHOD OF HUMAN WHOLE BRAIN AUTORADIOGRAPHY USING ^{3}H-PAROXETINE.

INTRODUCTION

Ullberg first developed the method of large section, whole body autoradiography in the 1950's while researching the distribution of radiolabelled benzylpenicillin in mice for his Ph.D. thesis. He found that by pre-injecting animals with a radiolabelled substance before sacrificing and then freezing them, he could maintain the localized distribution of the radioligand without risking leakage of water soluble substances (Ullberg, 1954). Whole body sections were prepared by pressing adhesive tape over the frozen, embedded animal in order to hold the section as it was being cut. The sections were freeze-dried and then apposed to a photographic emulsion prior to developing. For a comprehensive review of the techniques used to prepare and process large specimen sections for autoradiography see Ullberg (1977).

The method of cutting whole body, frozen sections has developed somewhat since those days of standing in fur coats in a refrigerated room at -15°C, holding a hand-driven sledge microtome. It is now possible to cut large specimen sections from 2µm to 200µm in an automated macrocut cryostat, however the majority of work generated from this still involves whole body imaging following *in vivo* pre-injection with the radiolabeled substance. The problem with cutting large sections for *in vitro* autoradiography lies in preparing sections which can then be subjected to incubation and washing procedures.

Gillberg et al (1986) were the first to develop a method which involved cutting a whole hemisphere section with tape and then transferring it to glass plates. The tissue was embedded in a block of frozen carboxymethylcellulose (CMC), a piece of tissue paper was placed over the block and a strip of tape pressed onto the paper. In this way they could collect 40 - 80µm sections on the paper/tape which were transferred to gelatin coated glass plates for the duration of incubating and washing.

The following year Quirion and colleagues (1987) described another method of preparing whole hemisphere coronal sections for *in vitro* autoradiography. This

involved floating the section onto a piece of card as it was being cut and subsequently transferring it onto a glass plate for incubation and washing.

In the studies reported above, a single hemisphere was used in each case. Therefore, the intention in the present study was to develop a method of cutting whole brain sections (both hemispheres) for use in autoradiography in order to map the distribution of ^{3}H-paroxetine binding sites in healthy human whole brains. Once this had been achieved it would then be possible to determine if there is a difference in receptor density or distribution in healthy and diseased human brains, as well as evaluate the viability of animal models of disease states in predicting receptor changes in diseased human brains.

In the results and discussion of this chapter, "background binding" is taken to mean radioligand binding to any material other than the tissue section.

RESULTS

4.1. CUTTING WHOLE BRAIN CORONAL SECTIONS.

A number of requirements were necessary in order to carry out successful experiments using human whole brains, the first of which was to obtain whole brains postmortem. When a brain is removed during autopsy, under normal conditions, one hemisphere is fixed in formaldehyde for neuropathological assessment of the patient and the other can be used for experimental procedures. The availibility of whole intact brains is scarce, especially in light of increasing concern over the risk from working with human tissue infected with Hepatitis B, HIV, slow viruses (Creutzfeldt-Jakob disease) or other pathogenic materials. Brains from patients with neurological disorders are more readily available than brains from patients with no apparent neuropathology or drug history, and therefore the control brains required for preliminary experiments are more difficult to aquire. Altogether 4 whole brains were obtained from various sources plus 2 cortical slabs each from 2 other controls.

For demographic details see Methods, Table 2.1.
To perfect the technique of preparing sections, pig brain (about the size of a human fist) obtained from the slaughterhouse was used, prior to using human tissue.

Initially the method described by Quirion et al (1987) was tried, whereby as the section is being cut it is floated onto a piece of card kept in the cryostat so the section does not melt as it touches the card. Even on small sections of pig brain, the tissue cracked or curled up before it could float onto the card. As the section appeared above the knife blade it began to curl back onto itself. Various attempts were made to design a roll bar to fit above the blade, but the piece of metal holding the blade in place prevented a workable solution from being reached. Attempts were made to guide the section onto the card by gently holding another piece of card above the section to prevent it from rolling up. In this manner it was possible to produce a whole section, however as the section began to slide onto the card it started to break up. Occasionally it was possible to get a whole section onto the card but in the process of transferring it to a glass plate it either broke up or became distorted. The same problem was encountered when trying to cut sections directly onto glass plates.

Another method was tried as described by Gillberg et al (1986) using tissue paper and tape. It was found that the brain tissue rarely adhered to the tissue paper long enough to cut a whole section and in the process of transferring the section to a glass plate, the section either fell off the tissue paper or broke up on reaching the glass. As quoted by Gillberg et al (1986); "these last steps of the procedure require special training for good results". However, after many attempts to perfect the technique, both with pig brain and human whole brain, it became apparent that the above methods were not feasible for human whole brain work and an alternative method had to be developed.

4.2. USING TAPE AS A BACKING MATERIAL.

Since whole body autoradiography is performed using tape to cut large sections, the obvious place to start was to try and cut sections with an adhesive tape which could withstand low temperatures. For this purpose a 5" wide, transparent adhesive tape (Tesa Tape, Leica) was used. Inital experiments were performed on pig brain sections to conserve human tissue while perfecting the cutting technique.

In all the experiments performed on sections adhered to tape, the following method was used, unless otherwise specified: 20µm whole brain sections were cut onto tape at -16°C and briefly brought up to room temperature to thaw-mount before the tape was secured to perspex frames (20 x 20 cm) and stored at -20°C overnight. The following morning the sections were brought up to room temperature for 1 hour. The frames and tape were placed in specially designed perspex wash baths with 3 litres of Tris-HCl buffer (50mM, pH 7.4) containing 120mM NaCl and 5mM KCl for a 15 minute pre-incubation. When the sections were dry, the frames were placed on glass plates so that the tape rested flat on the glass with the section facing upwards. A 2 hour incubation was carried out by covering the section with 1nM ^{3}H-paroxetine in Tris-HCl buffer in a drop-by-drop manner to ensure the entire section was covered. 4µM citalopram was included to define non-specific binding. Approximately 15ml radioligand in buffer was required for a human whole brain coronal section.

When the sections were incubating, they were covered with a perspex lid to minimise evaporation of the ligand. The incubation medium was then aspirated off and the sections dipped in Tris-HCl buffer for 1-2 seconds to wash off the excess ligand. Washing was carried out in the wash baths containing Tris-HCl buffer for 2 x 60 minutes. The sections were rinsed in ice-cold, distilled water for 3 seconds and dried under a stream of cold air for 30 minutes. When the sections were partially dry they were placed in a freeze dryer for 1 hour to ensure complete dryness and prevent diffusion of the radioligand during development.

In order to prevent the tape from sticking to the radiographic film, a fine cover of talc powder was carefully applied around the sections on the tape using a 1/8" paintbrush. If powder gets onto the section it can block the β particles of the radioligand from reaching the film as well as creating a "static" artifact during development. Once the tape was sufficiently powdered, the sections were stuck to a piece of card and apposed to Hyperfilm-^{3}H for 4 weeks. The films were developed as described in Methods, section 2.5.

4.2.1. Pre-incubation with bovine serum albumin (BSA)

It was relatively easy to produce 20μm sections of good quality by cutting sections onto tape. Preliminary experiments were performed by incubating pig brain sections with ^{3}H-paroxetine to determine the extent to which the ligand bound to the tape and section. As it was apparent that the tritiated ligand bound to the tape to produce substantial background binding, attempts were made to reduce this binding by pre-incubating the tape and sections for 3 - 5 hours with increasing concentrations (1, 3 and 5%) of bovine serum albumin (BSA).

The BSA concentrations were used following a discussion with Mary-Anne Weaver at Brunel University. She found that pre-incubating with BSA substantially reduced the background in her experiments on radioligand binding in trout. However, despite lengthy pre-incubations with BSA in the current investigation, no discernable difference was made to the background binding.

At this point another tape became available which was a 6" wide, opaque adhesive tape from 3M (810). There did not appear to be any difference in the quality of sections gained from cutting onto either type of tape and therefore the effect of ^{3}H-paroxetine background binding was investigated in the presence and absence of pre-incubation with BSA on the new tape. This time, 1.5 x 1.5cm (2.25cm^{2}) pieces of tape were used without any brain tissue on them to reduce the amount of buffer, BSA and radioligand used. There appeared to be no substantial difference in 1nM ^{3}H-paroxetine binding to either tape. The amount of binding reached a minimum in 3M tape pre-incubated with 3% BSA,

however the average dpm count for a single 2.25cm^2 piece of tape was still more than 25 000dpm's (Fig. 4.1).

Tests revealed that after 2 hours of immersion in buffer, the 3M tape began to distort and the adhesive separated from the tape. The Tesa tape maintained its shape and adhesive qualities even after lengthy exposure to liquid. As a result, the use of 3M tape for this type of autoradiography would not be feasible.

In order to determine whether the radioligand was binding to the adhesive on the tape or the backing material, 100µl ^{3}H-paroxetine was pipetted onto 2.25cm^2 pieces of tape on the adhesive side or backing side. Care was taken not to let the incubation medium come into contact with the reverse side of the tape. Fig. 4.2. shows extensive ^{3}H-paroxetine binding on the adhesive side of the tape, with very little binding observed on the backing side. Since the backing side has no adhesive qualities to hold the sections, another method of preventing the radioligand from coming into contact with the adhesive would have to be found.

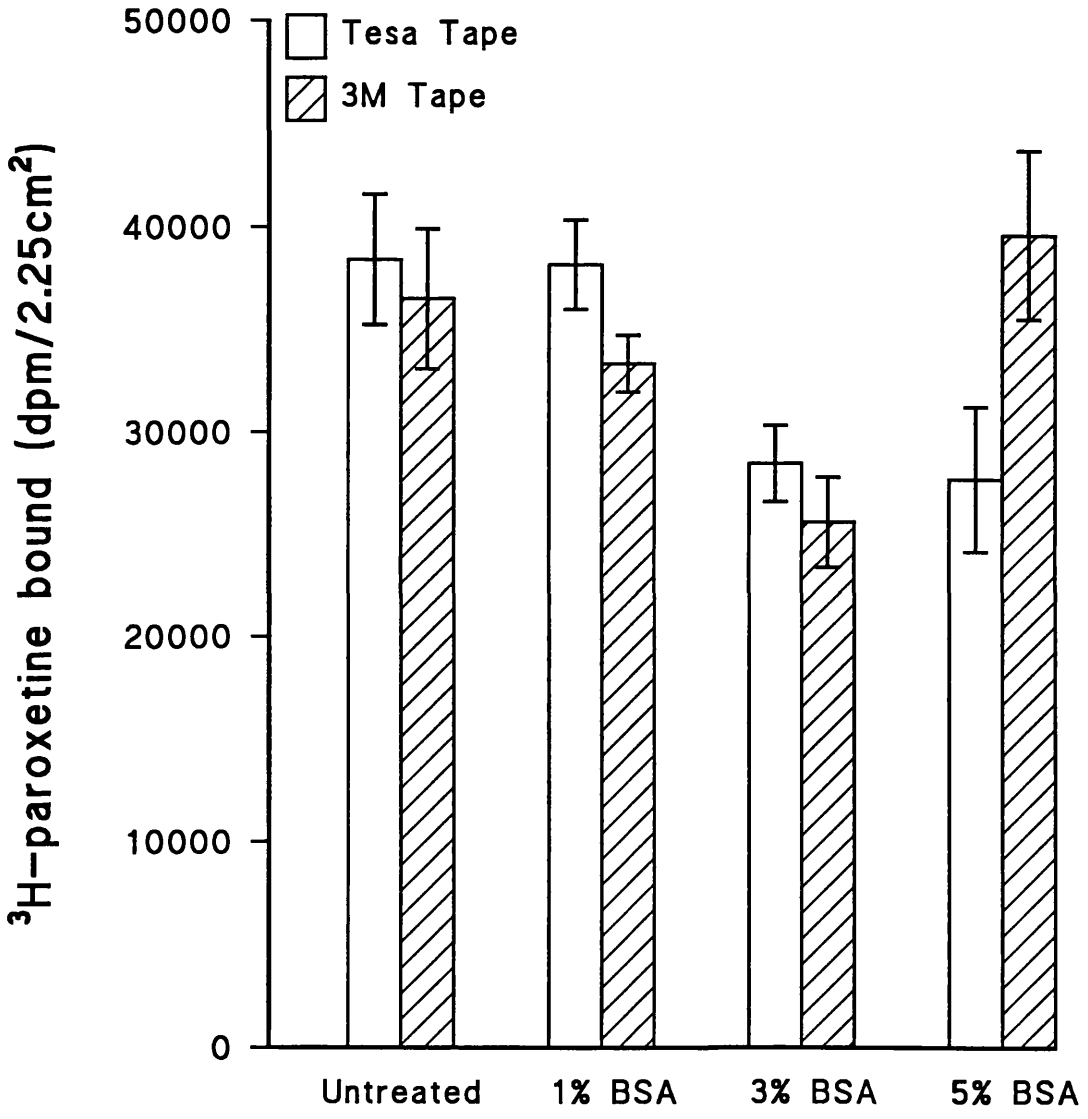


Fig. 4.1. ^{3}H-paroxetine binding to different types of tape pre-incubated with increasing concentrations of BSA. 2.25cm^2 pieces of tape (Tesa and 3M) were incubated with 1nM ^{3}H-paroxetine. The radioactivity of each piece of tape was measured using scintillation spectometry. Results are the mean ± sd. dpm of 3 pieces of tape per condition. Experiments were carried out in duplicate.

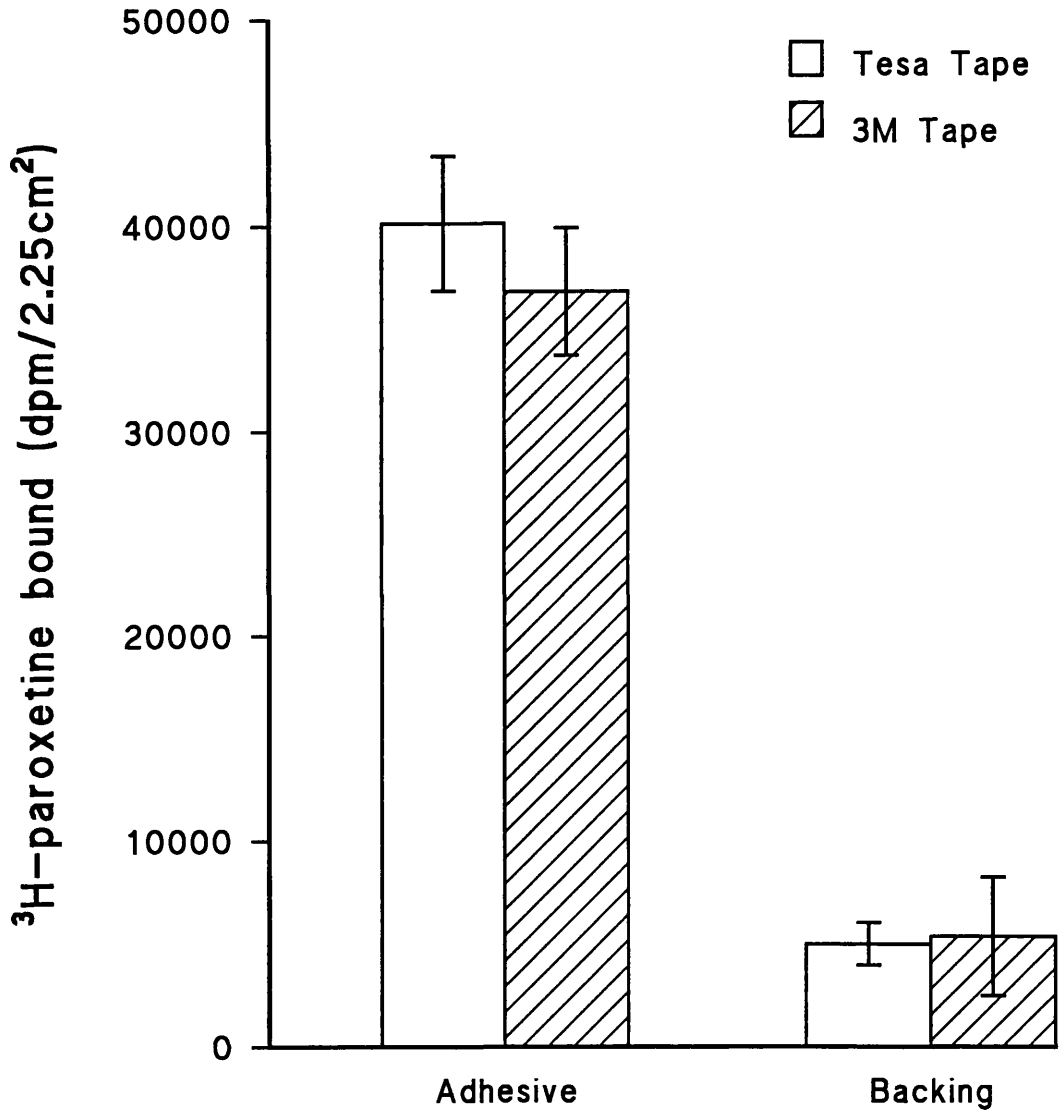


Fig. 4.2. ^{3}H-paroxetine binding to adhesive or backing material of tape. The adhesive or backing side of 2.25cm^2 pieces of tape were incubated with 100µl 1nM ^{3}H-paroxetine and then measured in the β counter. Results are the mean dpm ± s.d. dpm of 3 pieces of tape per condition. Experiments were carried out in duplicate.

4.2.2. Silicone treatment of tape.

Silicone has been used to alleviate non-specific binding of radioligands by creating a hydrophobic covering over glassware, and therefore it was decided to try coating the tape with liquid silicone prior to cutting sections. Strips of tape were immersed in either Repelcote (BDH Chemicals, U.K. Ltd) or Sigmacote (Sigma Chemicals, U.K. Ltd) for 5 seconds and allowed to dry. While the silicone treatment reduced the adhesive quality of the tape, it was still possible to cut adequate 20µm whole brain sections. The results in Fig. 4.3. show that treating the tape with silicone did not reduce the background ^{3}H-paroxetine binding.

4.2.3. Pre-treatment with polyethylenimine.

In membrane binding studies, pre-soaking filters with polyethylenimine (P.I.) has been used to reduce the amount of radioligand binding to the filters, therefore the use of pre-treatment with 0.1, 0.5 and 1% P.I. was investigated to see if that would reduce the background ^{3}H-paroxetine binding to tape. A non-significant reduction in the amount of ^{3}H-paroxetine binding was seen in tape pre-treated with 0.5% and 1% P.I. (Fig. 4.4). No difference was observed in the tape pre-treated with 0.1%. The radioactivity on the Tesa tape pre-treated with 0.5% and 1% P.I. was reduced by 21% and 18% respectively.

4.2.4. Effect of extended rinse times.

Since the background binding of ^{3}H-paroxetine was not affected by treating the tape, a longer rinse time (up to 8 hours) was investigated to see if that would allow more of the radioligand to dissociate from the tape. A short wash time of 2 x 5 minutes was included to see if there was any difference from the longer wash times. Fig. 4.5. indicates a small decrease in background binding as the wash time increased.

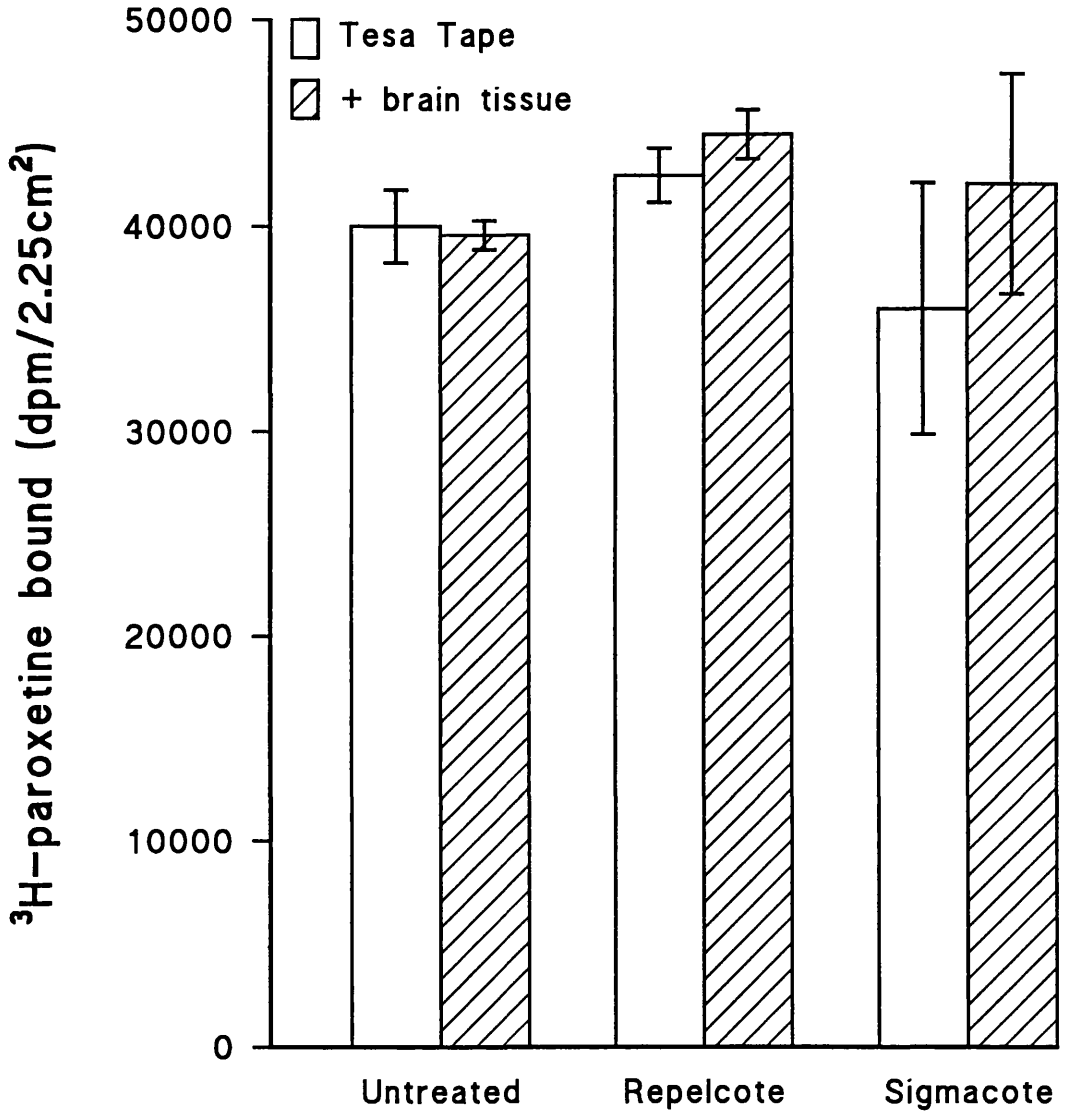


Fig 4.3. The effect of silicone treatment on background ^{3}H-paroxetine binding to tape. 20µm human whole brain coronal sections were cut onto Tesa tape which had been pre-treated with 2 different types of liquid silicone. The sections were then subjected to autoradiographic procedures as described in Section 4.1. 2.25cm^2 strips of tape covered with brain tissue were cut out and their dpm content measured in the β counter. Results are the mean ± sd. dpm of 3 pieces of tape per condition. Experiments were carried out in duplicate.

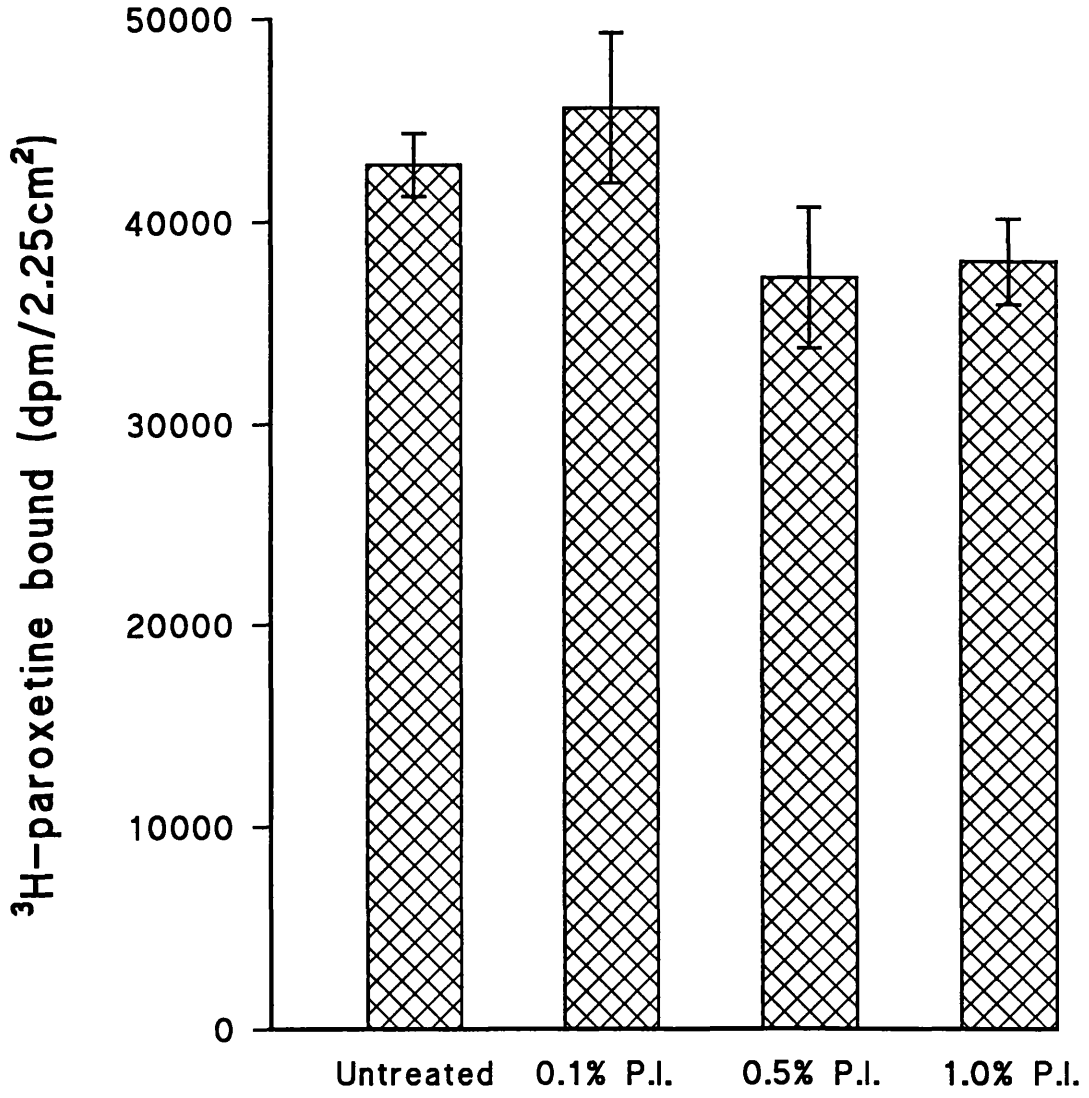


Fig 4.4. The effect of pre-treatment with polyethylenimmine on background ^{3}H-paroxetine binding to tape. 2.25cm^2 pieces of Tesa tape were pre-incubated with 0.1, 0.5 and 1% P.I. overnight and then subjected to autoradiographic procedures as described in Section 4.1. Results are the mean ± sd. dpm of 3 pieces of tape per condition. Experiments were carried out in duplicate.

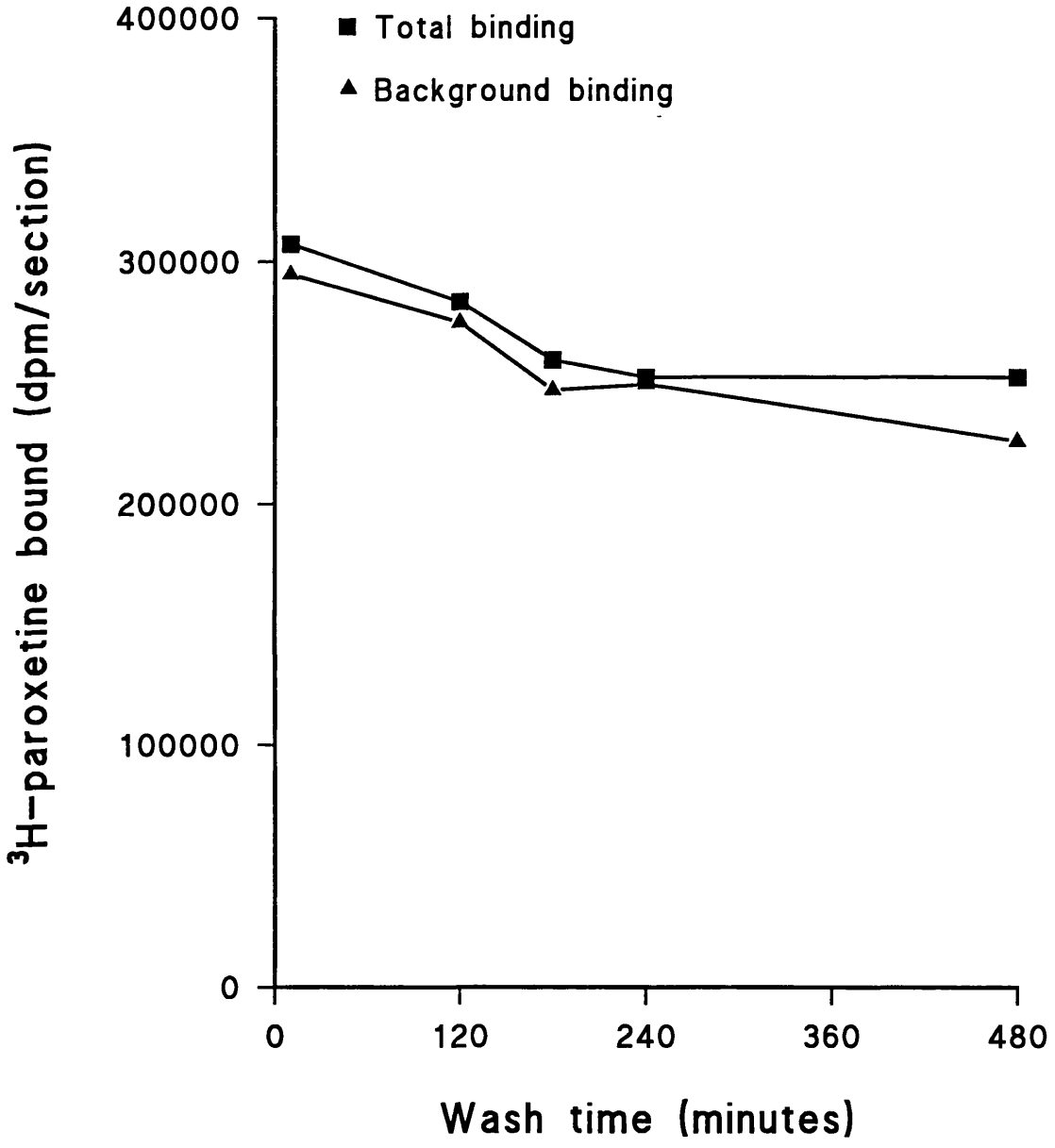


Fig. 4.5. The effect of extended wash times on background ^{3}H-paroxetine binding to tape. 20µm whole brain coronal sections were cut onto Tesa tape and incubated with 1nM ^{3}H-paroxetine for 120 minutes. 4µM citalopram was included to determine non-specific binding. The wash times were varied from 2 x 5 minutes to 2 x 240 minutes. The sections were then cut into 4 and each piece counted separately in the β counter. The results are the mean dpm of the sum of each section. Experiments were carried out in duplicate.

4.2.5. Effect of different radioligands binding to tape.

Since all previous experiments on tape had been carried out with ^{3}H-paroxetine, the possibility that it might be only ^{3}H-paroxetine binding to the tape was investigated. Therefore, two types of tape, Tesa tape and 3M tape, were incubated with a 1nM concentration of 3 different ligands; ^{3}H-paroxetine, ^{3}H-fluoxetine and ^{3}H-5HT to determine their ability to bind to the adhesive.

The amount of radioligand bound to tape was substantially less (Fig. 4.6) in the case of ^{3}H-fluoxetine and ^{3}H-5-HT despite having a higher specific activity (42.6 and 53.5 Ci/mmol respectively) than paroxetine (16.6 Ci/mmol). A difference was also seen in the background binding in the two tapes. ^{3}H-fluoxetine binding was the same in both tapes whereas ^{3}H-5HT binding was less in the Tesa tape than the 3M tape. Despite the lower levels of background binding found with ^{3}H-fluoxetine and ^{3}H-5-HT, the total radioactivity of each piece of tape was in excess of 20 000dpm's. Although ^{3}H-paroxetine appeared to bind less to the 3M tape, the standard error was too large to determine if there was any real difference. In Fig. 4.1. where the two tapes are compared, there was no difference in ^{3}H-paroxetine binding.

4.2.6. Use of grease to prevent background ^{3}H-paroxetine binding.

Attempts were made to cut sections with a variety of other backing materials such as lens tissue, nescofilm, clingfilm, freezer wrap and a selection of plastics of varying thickness. Since the tissue sections did not adhere to these materials, another means of holding the sections had to be devised. High-pressure vacuum grease can withstand temperatures of -40^{0}C and therefore attempts were made to cut sections onto the various backing materials which were coated with vacuum grease. Unfortunately the clingfilm and nescofilm distorted or cracked when left at -16^{0}C for any length of time. The freezer wrap, some of the plastics and the lens tissue allowed the cutting of relatively good sections. These were incubated with ^{3}H-paroxetine to determine their ability to prevent background binding (Fig 4.7).

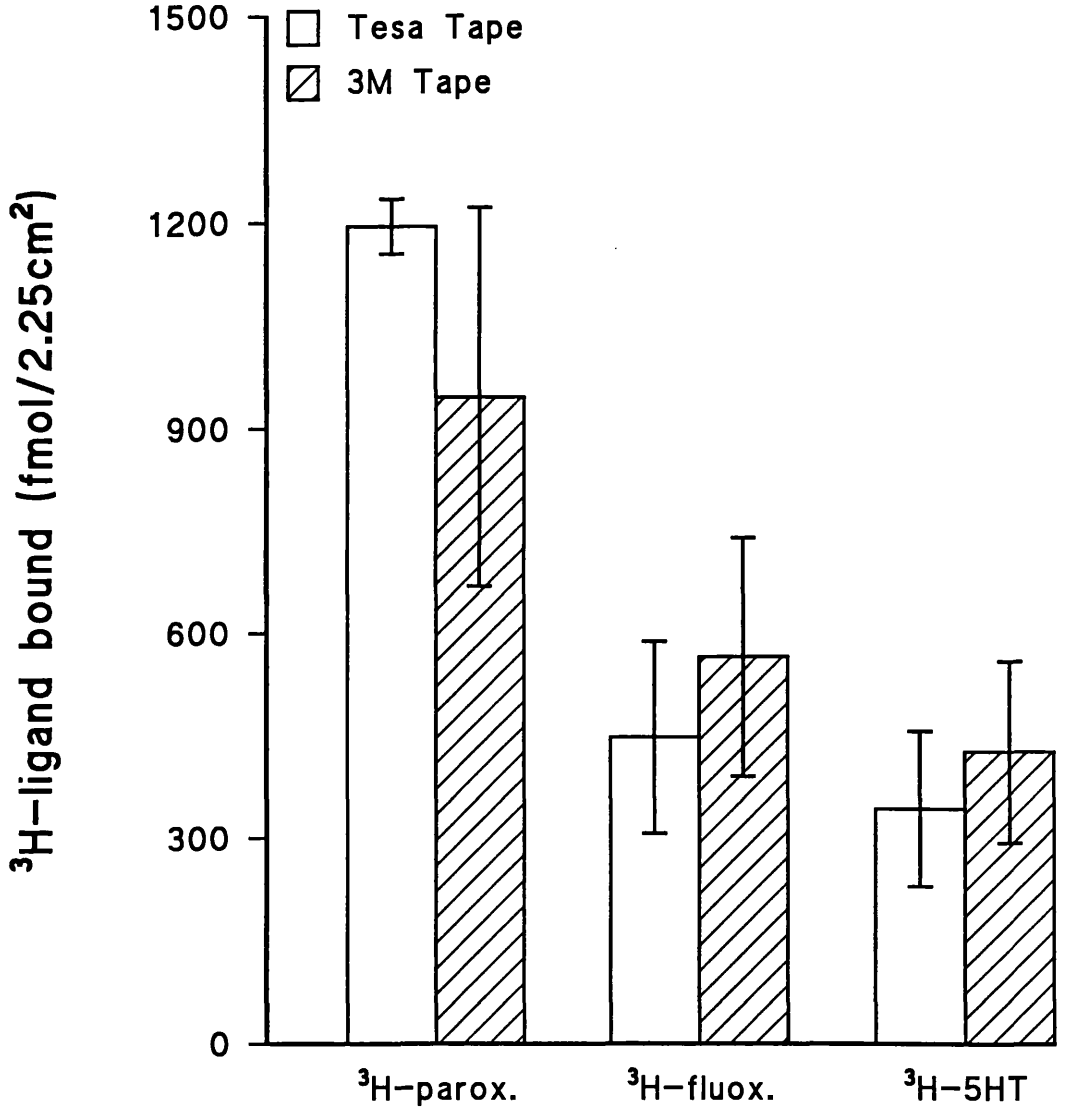


Fig 4.6. Different radioligand binding to two types of tape. 2.25cm^2 of either Tesa tape or 3M tape were incubated with 1nM ^{3}H-paroxetine, ^{3}H-fluoxetine or ^{3}H-5HT. Results are the mean ± sd. fmol of 3 pieces of tape per condition. Experiments were carried out in duplicate. parox. - paroxetine; fluox. - fluoxetine.

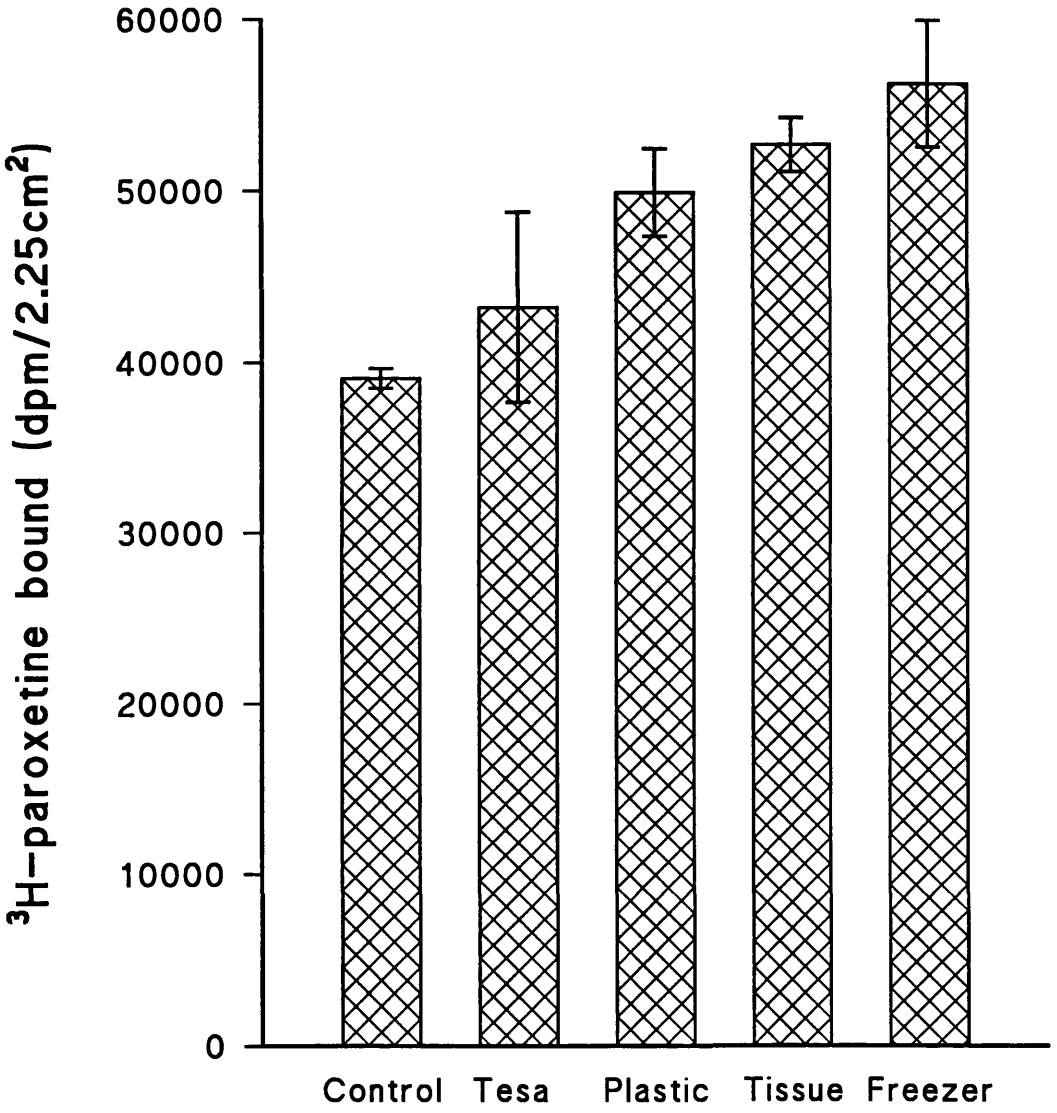


Fig 4.7. The use of backing materials coated with high-pressure vacuum grease. 2.25cm^2 pieces of Tesa tape, plastic, lens tissue and freezer wrap were coated with high-pressure vacuum grease prior to incubation with 1nM ^{3}H-paroxetine. Results are the mean ± sd. dpm of 3 pieces of material per condition. Experiments were carried out in duplicate.

Fig. 4.7. shows that the high-pressure vacuum grease increased ^{3}H-paroxetine binding in all cases. The Tesa tape alone had the lowest levels of binding with the freezer wrap having the highest. The lens tissue which was expected to have a lower binding capacity because it was paper based and not plastic, had the second highest level of binding. It was not possible to use ordinary Kleenex tissue paper because it was too fragile.

4.3. USING MEMBRANES AS A BACKING MATERIAL

It was now apparent that the use of tape was not feasible as part of a method for ^{3}H-paroxetine binding to whole brain coronal sections in autoradiography, therefore attempts were made to find another suitable backing material. From the above experiments it was determined that the new material had to meet a number of criteria; it had to:

a) be strong enough to withstand the sectioning procedure
b) withstand temperatures as low as -20^{0}C
c) hold a large tissue section sufficiently to prevent the section from breaking.
d) withstand lengthy immersion in buffer (up to 5 hours)
e) maintain its integrity and shape throughout the cutting, autoradiographic and drying procedures.
f) keep the background binding of ^{3}H-paroxetine to a minimum.

Since the sections needed to be held to the backing material by means other than adhesives, it was suggested that the sections could be held by some sort of absorbtion process. Professor P. Cruz Sanchez in Barcelona had some success with cutting sections onto nitrocellulose membrane filters for *in situ* hybridization procedures and therefore the use of filters as a backing material was investigated. Samples of membrane filters were obtained from various manufacturers and tested for their ability in section cutting and autoradiographical procedures (Table 4.1).

MEMBRANE FILTER	TYPE	PORE SIZE	BACKGROUND (dpm)	SECTIONING ABILITY
NITROCELLULOSE (CN)	Biotrace NT		39504.8	+
	Whatman	0.65µm	33166.53	++
	Whatman	0.20µm	50414.4	++
	Hybond C extra		35894.3	+++
REGENERATED CN	Cuprophan dialysis tubing	20µm thickness	302.7	-
	Cuprophan flat membrane	11.5µm thickness	420.9	-
PTFE	Mupor 1E Hydrophobic	4µm	235.2	-
	Mupor 1E Hydrophilic	4µm	2945.3	-
	Mupor 2X Hydrophobic	5-10µm	349.13	-
GLASS FIBRE	GF/F		336.2	-
	GF/B		512.9	-
PVDF	Durapore GVWP	0.22µm	5307.1	-
MIXED CELLULOSE ESTERS	HA	0.45µm	30509.4	+
	GSWP	0.22µm	47476.2	+
	VSWP	0.025µm	37798.4	+
NYLON	Hybond N+		9838.2	++

Table 4.1. Table showing the binding capacity and sectioning ability of membrane filter materials. 2.25cm^2 pieces of membrane filters were incubated with 1nM ^{3}H-paroxetine for 120 minutes and washed for 2 x 60 minutes in Tris buffer. Each membrane was investigated as a suitable backing material for holding 40µm whole brain coronal sections during sectioning: +++ - Good; ++ - moderate; + - Poor; - - unable to hold sections. Results are the mean dpm of three 2.25cm^2 pieces of membrane used in experiments performed in duplicate.

Bearing in mind that some practice may be required in order to cut decent sections, section cutting was tested with each membrane over 3 days to a week using varying section thickness (20 - 80μm), angle of blade (20 - 60^0) and cryostat temperature (-25 to -12^{0}C).

4.3.1. Nitrocellulose membrane filters.

Four different nitrocellulose membranes (Biotrace NT, Whatman 0.20μm, Whatman 0.65μm, Hybond C extra) and 2 regenerated nitrocellulose membranes (Cuprophan dialysis tubing, Cuprophan dialysis flat membrane) were tested (Fig. 4.8). The amount of ^{3}H-paroxetine binding in the nitrocellulose membranes was much the same as for the Tesa tape. Whatman filter "B" had higher background binding levels than Whatman filter "A", this may be attributed to the reduced pore size (0.20μm and 0.65μm respectively) which may hold the paroxetine more firmly. The lowest levels of background binding were seen in the regenerated nitrocellulose membranes, however they had no ability to hold the sections and completely altered shape when exposed to liquid.

4.3.2. Miscellaneous membrane filters.

A variety of other membrane filters were subsequently tested (Table 4.1). Hydrophobic and hydrophilic PTFE membranes (pore size 4μm) were tested and while they maintained a low level of background binding, they were unable to hold the tissue sections. The hydrophobic membranes had a lower level of background binding, presumably because they repelled the aqueous based incubation medium. The mixed cellulose esters had similar levels of background binding to Tesa tape. The glass fibre filters were expected to have low levels of background binding due to their successful use in homogenate binding experiments, however they had no capacity to hold the tissue sections during cutting.

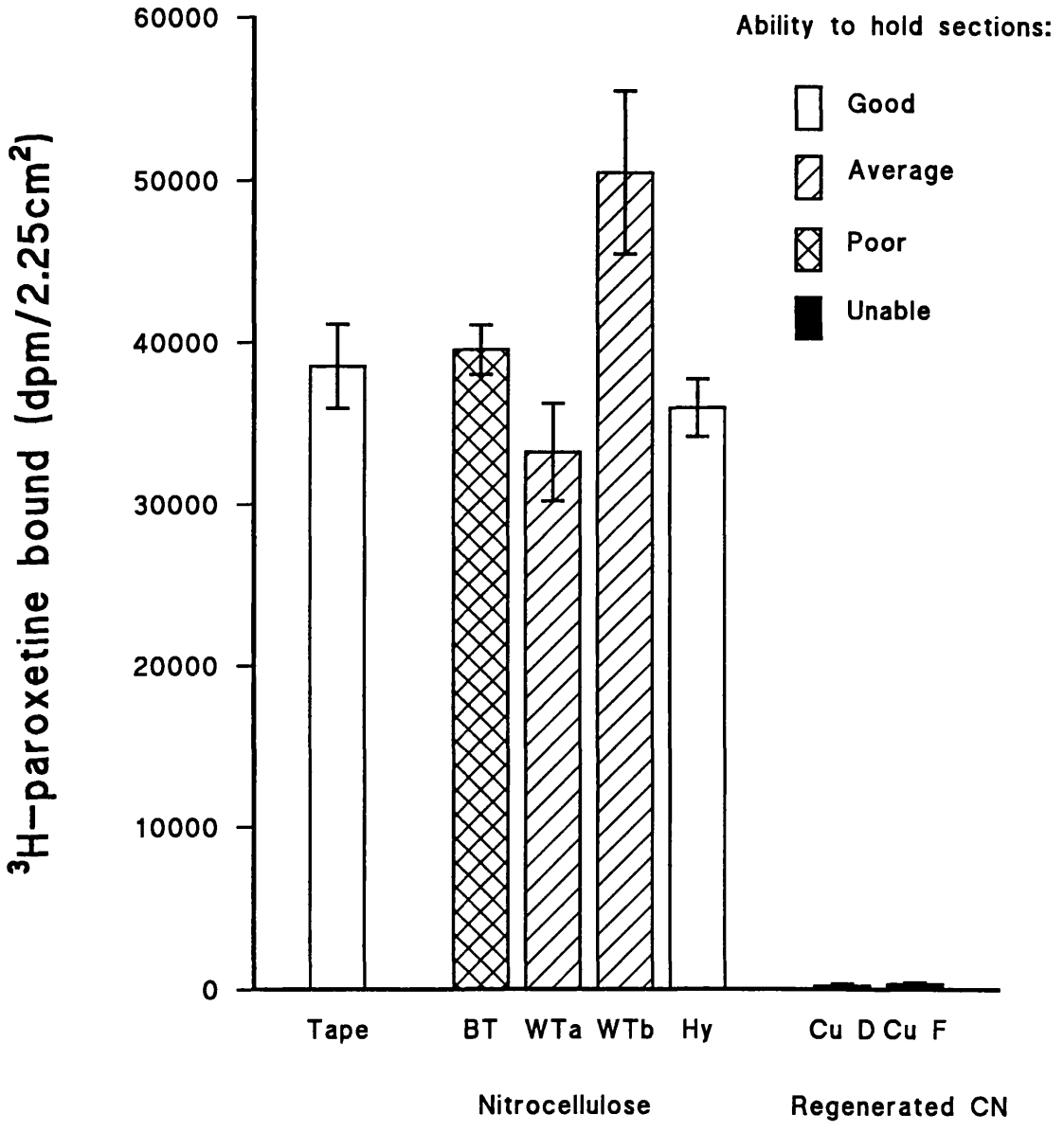


Fig 4.8. Nitrocellulose membranes as backing materials for cutting tissue sections. Four different nitrocellulose membranes and 2 regenerated nitrocellulose membranes were incubated with 1nM ^{3}H-paroxetine. Their ability to hold tissue sections during cutting was assessed as indicated by the shading in the bar graph. Results are the mean ± sd. dpm of 3 pieces of each material. Experiments were carried out in duplicate. BT - Biotrace; WTa - Whatman filter "A" (0.65µm pore size); WTb - Whatman filter "B" (0.20µm pore size); Hy - Hybond C Extra; Cu D - Cuprophan dialysis tubing; Cu F - Cuprophan flat dialysis membrane.

4.3.3. Nylon membrane filters.

Since Hybond C membranes (Amersham, U.K.) showed the best holding ability of the membranes investigated, different samples of Hybond material were tested, in particular the nylon based membranes, Hybond N and Hybond N+, where the + indicates that the membrane is positively charged (Fig 4.9). The Hybond N+ had the lowest levels of background binding and was relatively easy to use as a backing material. It held the tissue sections well and kept them intact throughout the incubation and washing procedures. On the basis of these results, it was decided to use the Hybond N+ as a backing material in a series of experiments using ^{3}H-paroxetine binding to human cortical sections (40μm). Two concentrations of ^{3}H-paroxetine were used (0.1 and 1nM) to determine the length of time required for the photographic film to be exposed to the sections. After 6 weeks, the 0.1nM concentration was not visible, while the 1nM film was still barely visible.

Therefore a 5nM concentration of ^{3}H-paroxetine with 20μM citalopram to define non-specific binding was used in sections apposed to film for 4 weeks. In these sections, tissue binding was visible without the background binding being a hindrance to the quantitative analysis process. The difference between total and non-specific binding was quite clear in areas where ^{3}H-paroxetine is known to bind with moderate to high density. In the cortex and other areas where ^{3}H-paroxetine levels are low, the percentage specific binding was lower, however specific binding was still present (25%). On the basis of these results, the decision was made to map the distribution of ^{3}H-paroxetine binding sites in human whole brain coronal sections using a concentration of 5nM ^{3}H-paroxetine with 20μM citalopram to determine the non-specific binding.

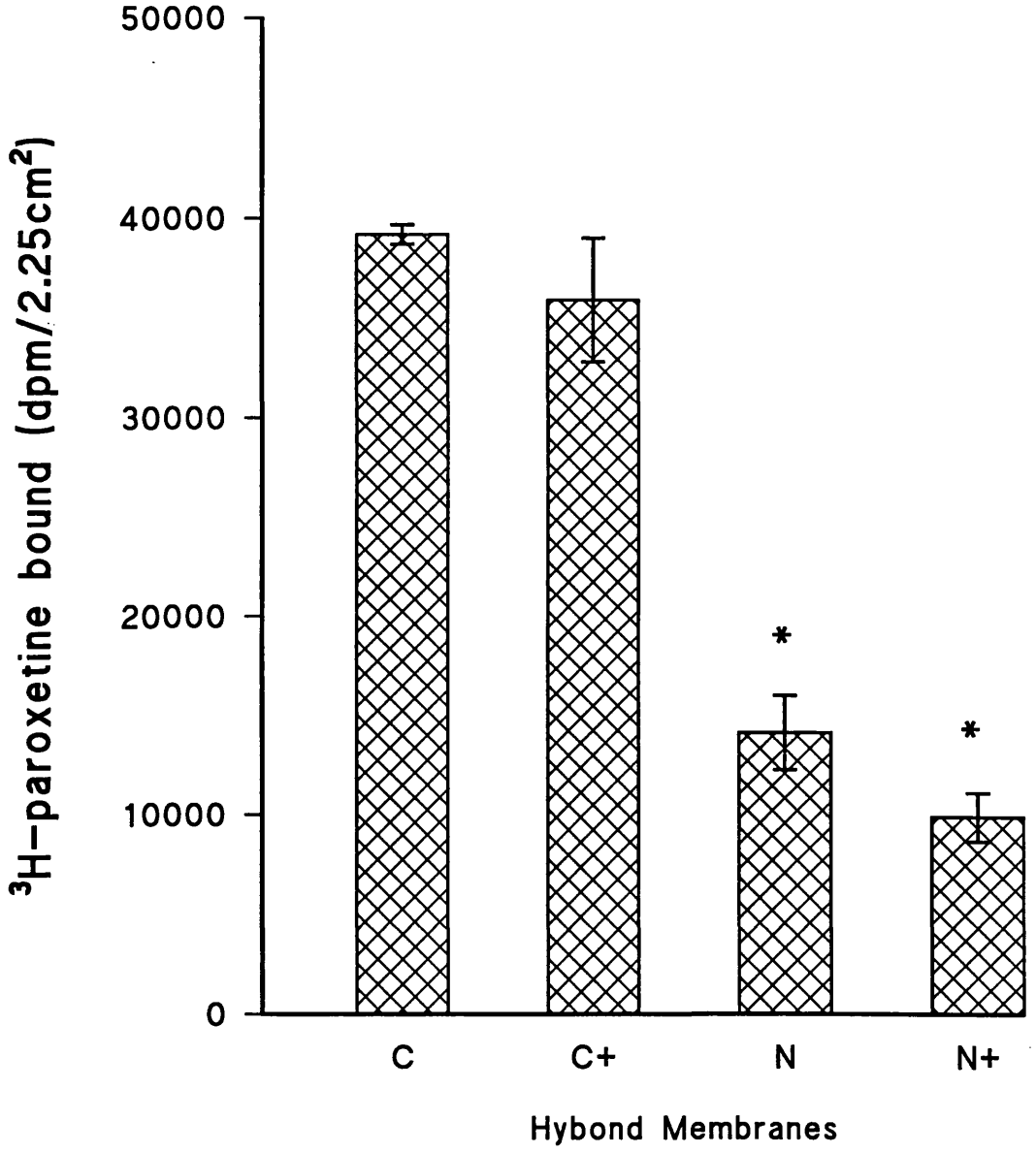
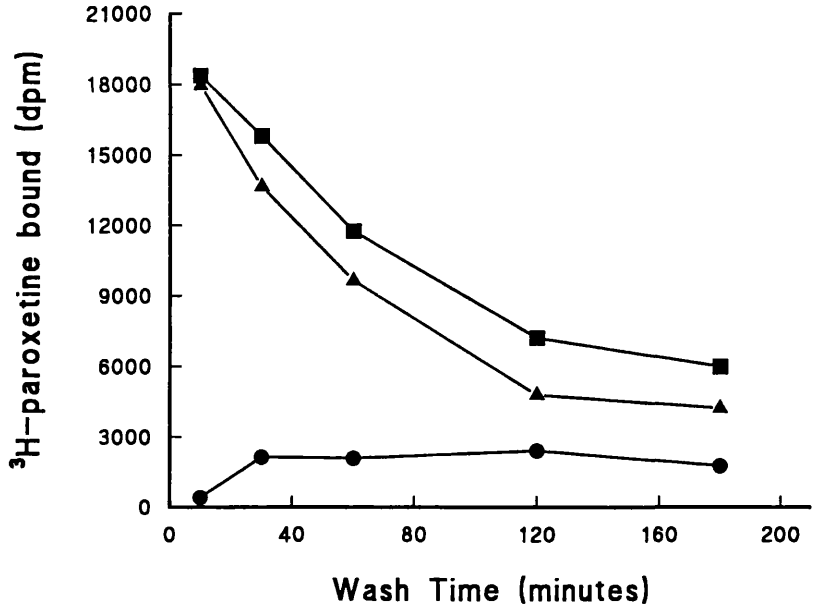


Fig. 4.9. Nylon membrane filters as a suitable backing material for tissue sections. Nitrocellulose or nylon Hybond membranes were incubated with 1nM ^{3}H-paroxetine and their radioactive content measured in the β counter. Results are the mean ± sd. dpm of 3 pieces of each material. * $p < 0.05$, statistics represent the results of a student t-test comparing ^{3}H-paroxetine binding in Hybond N membranes to binding in Hybond C membranes. Experiments were carried out in duplicate. C - nitrocellulose; C+ - positively charged nitrocellulose; N - nylon; N+ - positively charged nylon.

4.4. OPTIMUM WASH AND INCUBATION TIMES FOR ^{3}H-PAROXETINE BINDING IN AUTORADIOGRAPHY.

4cm^2 blocks of human cortex were used to determine the optimum binding parameters for ^{3}H-paroxetine (Fig. 4.10). 40µm sections were thaw-mounted onto gelatin coated microscope slides and incubated with 5nM ^{3}H-paroxetine for 120 minutes and then washed for different times (10, 30, 60, 120 and 180 minutes) in Tris-HCl buffer which was changed halfway through the wash. Both the total and non-specific binding decreased rapidly until 120 minutes (2 x 60) where they reached a plateau. The specific binding determined by 20µM citalopram reached a steady level at 30 minutes where it remained for a further 150 minutes.

Using a wash time of 2 x 60 minutes, the incubation time was varied from 10 to 240 minutes. The total binding reached equilibrium at 120 minutes, while the non-specific binding peaked at 60 minutes. The specific binding reached a maximum at 120 minutes. From these results the binding parameters were set at 120 minutes incubation and 2 x 60 minutes wash time respectively.



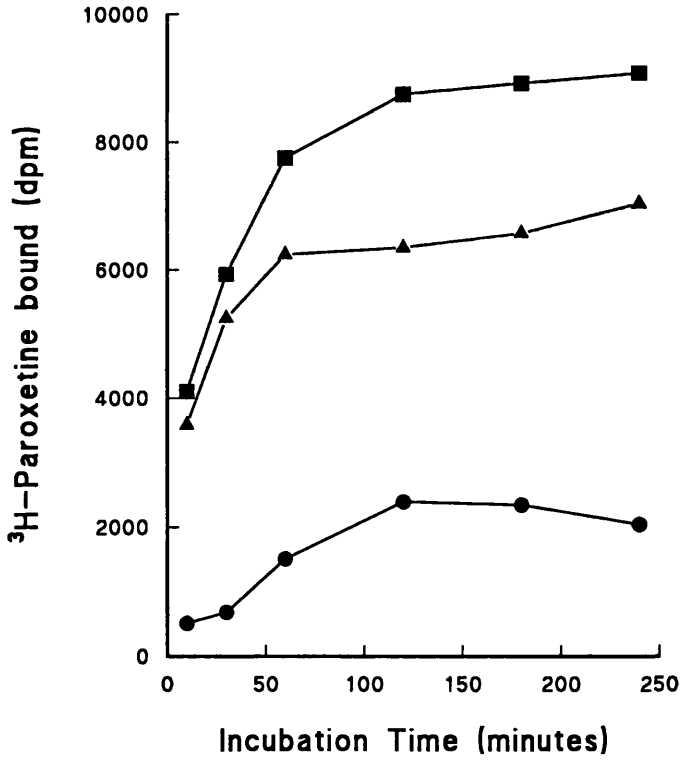


Fig 4.10. Optimum wash and incubation times for ^{3}H-paroxetine binding to small sections of human brain. 40µm sections were thaw mounted onto gelatin coated microscope slides and incubated with 5nM ^{3}H-paroxetine for 120 minutes and then washed for different times (10, 30, 60, 120 and 180 minutes) in Tris buffer. Using a wash time of 2 x 60 minutes, incubation was carried out over increasing time periods (10, 30, 60, 120, 180, 240 minutes). Non-specific binding was determined by the inclusion of 20µM citalopram. Results are the mean dpm of 3 measurements taken from each experiment performed in triplicate. ■ - total binding; ▲ - non-specific binding; ● - specific binding.

DISCUSSION.

The most obvious artifact found with human whole brain autoradiography is the formation of ice-crystals within the tissue. The larger the volume of tissue being frozen, the more slowly the heat dissipates out from the centre of the block. This results in ice-crystal formation which can distort the microstructure by physically breaking up the tissue. When the tissue section is brought up to room temperature, the ice-crystals melt and evaporate leaving cracks in the tissue.

Cracks in the tissue due to ice-crystal formation and mechanical breakage mean that the backing material is exposed to the radioligand during incubation and subsequently show up when the film is developed. Since the amount of exposure to the film through these cracks is not constant, any quantitative image analysis will include variable amounts of so-called "high density binding" in each measurement. If the background binding can be substantially reduced the variability is minimized and a more accurate measurement of binding is achieved.

Since one of the objectives of the experiments was to compare binding sites in regions from both hemispheres, it is important that the brain remains in its original shape during freezing. To this end, the whole brain is placed in a "brain shaped" polystyrene container for the duration of the freezing process. The main problem with freezing the brain whole is the formation of ice-crystals. Ideally the brain should remain at -80^{0}C during all handling procedures until it is brought up to -20^{0}C for sectioning, however this is not possible. If the brain is frozen whole, it has to be brought up to -20^{0}C to cut it into 1.5 - 2cm slabs which are then stored at -80^{0}C until required. When each slab is embedded, the outer surface of the tissue comes into contact with semi-liquid CMC which is chilled to 4^{0}C. This is then stored at -20^{0}C overnight before sectioning. During these procedures, it is impossible to avoid ice-crystal formation.

Quirion et al (1987) suggested that slicing the brain into 1cm slabs immediately after autopsy and rapidly freezing them in iso-pentane at -40^{0}C would reduce ice-crystal formation. Rosene et al (1986) carried out an extensive study on the effect of ice-crystal freezing artifacts and the use of cryoprotectants. They found that rapid freezing of large CNS tissue blocks in iso-pentane at -40^{0}C produced a cooling rate twice as fast as that obtained by freezing with dry ice. To produce optimum freezing conditions they submerged the brain in a cryoprotectant solution of 20% glycerol and 2% dimethylsulphoxide (DMSO) for a minimum of 4 days at -4^{0}C before freezing the tissue. 2% DMSO was employed to improve the infiltration rate of the glycerol. It is unclear how the glycerol reduces ice-crystal formation, however what is apparent is that the use of these solutions facilitates the cutting of sections by reducing the brittleness of the tissue. Therefore they not only improve the quality of the sections by reducing ice-crystal formation, but they also prevent tissue damage from brittle cracking (Rosene et al, 1986).

The use of cryoprotectants is dependent on the tissue being fixed postmortem, however, the use of fixative reduces the amount of radioactive binding during autoradiography. If a method could be found whereby tissue fixing had no effect on radioligand binding, the use of a cryoprotectant would be invaluable in preventing damaging freezing artifacts. Since the brains that were obtained for the present study had already been frozen whole immediately after autopsy, it was not possible to dictate the circumstances in which they were frozen or the condition in which they were received. Some coronal slices of brain tissue were obtained which were cut before freezing, however these were unsuitable for cutting whole brain sections because they had distorted so much that all symmetry was lost.

In order to prevent ice-crystal formation as much possible, all handling procedures were carried out as swiftly as possible. When slices of brain were embedded, chilled CMC was used to reduce melting at the contact surface. Attempts were made to use sections which had not been thaw-mounted onto

the membranes, however these could not withstand the incubation and washing times.

When Rosene et al (1986) divided the brain into 3 concentric zones, centre core, midzone and peripheral zone, these authors found that the distribution of ice-crystal formation was concentrated in the midzone with an outward progression into the peripheral zone. The central zone consists of the multi-nuclear structures such as the thalamus, hypothalamus, substantia nigra and brainstem nuclei. The mid zone comprises larger structures such as the putamen and globus pallidus, and the cortical regions form the bulk of the peripheral zone. Since the majority of the areas of interest in the present study were found in the central zone, these were less affected by freezing artifacts. Care was taken at all times to avoid measuring areas which showed obvious tissue damage.

The use of a sharp, machine-honed knife is essential for preventing undue tissue damage during the sectioning process. It was noticed that an increase in the presence of cracks in the tissue and the thickness of each section became more variable, as the knife became less sharp. Even with the advent of automated cryostats, it is difficult to cut large sections of uniform thickness. Ito and Brill (1991) investigated the variation in thickness of sections cut for whole body autoradiography using a macrotome cryostat. They found that in 30 and 50μm sections there was a mean thickness variation of 1.15 and 1.21μm respectively. The amount of variation within the slice was found to be 0.92μm in a 30μm section and 0.91μm in a 50μm section. This level of variation could be maintained provided the knife was kept sharp at all times and therefore knives were changed on a regular basis.

When the whole brain is being cut into 1-2cm slices, it is important to cut them as symmetrically as possible. This is necessary not only for analytical reasons but also because it is easier to identify structures from an atlas if they have a common plane. At present the whole brains are cut using a portable, motorised

meat slicer. The temperature of the tissue has to be brought up to -20°C, if it is any colder the blade cannot pass through the whole brain. Even at this temperature it is not always possible to cut slices that are absolutely symmetrical. The size and shape of the frozen tissue makes it difficult to maintain a firm grip on the brain as it is being cut, and it can only be held with one hand. Therefore a bandsaw is currently being purchased which will cut slices of brain at -80°C by using a frozen meat blade. The design of the bandsaw is such that it will also be possible to hold the brain with two hands in order to steady it as it is being cut.

A number of different tapes can be used to hold sections whilst they are being cut.There are three main types of adhesives used: synthetic adhesives, rubber-and-resin-type adhesives and silicone treated adhesives. Tesa tape and 3M (810) tape both contain synthetic adhesives. In the above experiments the tapes were coated with a silicone treatment in the laboratory. When it became apparent that ^{3}H-paroxetine was binding to the adhesive on the tape, queries were made to the companies which manufacture tape for whole body autoradiography in order to ascertain which component of the adhesive was binding with the radioligand. However, informations regarding the exact composition of the adhesives was not available and therefore it was assumed that ^{3}H-paroxetine would bind to any of the adhesives available.

The results of the experiments using various radioligands (^{3}H-paroxetine, ^{3}H-fluoxetine, ^{3}H-5HT) with tape suggest that different radioligands bind with different affinity to the tape. Certainly it is much easier to cut good quality sections onto tape than any of the other methods tried above, therefore while the use of tape as a backing material for autoradiography with ^{3}H-paroxetine is not feasible, it should not be ruled out as an option in experiments with other radioligands. A suggestion for the reason as to why paroxetine binds more to tape than other radioligands may lie in the fact that paroxetine is a lipophilic drug, which is necessary for passing through the blood brain barrier. It may be this component that binds to the adhesive, however this is purely speculative.

Since the binding parameters for ^{3}H-paroxetine binding in autoradiography require long incubation and wash times, the use of 3M tape is not possible due to its deterioration in liquid after lengthy immersions although it maybe feasible in experiments with ligands which require shorter incubation and wash times.

The reason for the success of certain membrane filter materials, in particular Hybond N+, in holding tissue sections efficiently and maintaining low background binding levels is unclear. The humidity in a cryostat is minimal and the constant input of cold, dry air creates a build up of static electricity which can make the handling of sections difficult. Since the Hybond N+ chosen for this method is positively charged, it is possible that a small but significant attraction is generated between the section and the membrane. This attraction, complemented with absorption of the surface layer of tissue of the section into the membrane structure, is sufficient to generate a firm hold by the membrane on the section. The amount of pressure placed on the membrane over the tissue block is crucial to the integrity of the section being cut. If the pressure is too great, a better section is cut but it tends to compact the tissue, resulting in a section far smaller than the original block size. If the pressure is too light, the section does not hold to the membrane and falls off as it is being handled. Presumably the filter characteristic of the nylon membrane allows the ^{3}H-paroxetine to wash through without being contained by the physical structure of the membrane, however these suggestions are purely speculative.

Under optimum binding conditions of 15 minute pre-incubation, 120 minute incubation and 2 x 60 minute wash time, the specific binding reached a maximum of approximately 25%, however these experiments were performed on pieces of cortex which are known to have low levels of ^{3}H-paroxetine binding. Small pieces of cortex were used to minimize the quantity of radioligand used. The size of the cortex blocks had to be small enough to be picked up by a glass slide. Technically it would have been too complex to carry out these experiments on sections adhered to membranes and therefore glass slides were used, based on the assumption that the conditions of ^{3}H-paroxetine

binding to human 5-HT re-uptake sites would be the same regardless of the backing material.

By using nylon membrane filters, it was possible to produce 40μm whole brain coronal sections which could be used for autoradiography without affecting the integrity of the tissue section and maintaining a low level of background binding. This type of nylon membrane filter is commonly used for protein blotting procedures and therefore would be ideal for adjusting to *in situ* hybridization methodologies. The size of the section would require large quantities of probes, however with increasing technology it will become easier and cheaper to produce such quantities. This particular method of cutting whole brain sections has advantages over current methods of cutting single hemisphere sections. Symmetrical measurements can be made from both hemispheres and the visual image obtained can be compared with images obtained from PET scanning. The effect of a unilateral lesion on receptors in the adjacent hemisphere can be determined, and the distribution of receptor sites affected by disease states can be mapped across the width of the brain to see if there is an assymetrical pattern of receptor modification or loss.

CHAPTER 5

AUTORADIOGRAPHIC DISTRIBUTION OF ^{3}H-PAROXETINE BINDING SITES IN HUMAN WHOLE BRAIN CORONAL SECTIONS.

INTRODUCTION

Given the success of the SSRI's in the treatment of depression and the extensive research carried out using ^{3}H-paroxetine as a marker of the 5-HT re-uptake site in rat brain, it was a natural progression to the use of ^{3}H-paroxetine in human postmortem brain tissue. ^{3}H-paroxetine binding to the 5-HT re-uptake site in the human brain has been utilized in a number of studies using neuronal membranes prepared from different brain regions taken from postmortem human brain tissue (Hrdina et al, 1993; Laruelle et al, 1988; Lawrence et al, 1990a; Plenge et al, 1990a). The characteristics of ^{3}H-paroxetine binding to the human serotonin transporter appear to be very similar to those found in rat brain (Arranz and Marcusson, 1994; Bäckström et al, 1989; Erreboe et al, 1995; Laruelle et al, 1988, Plenge et al, 1990a), however, inhibition and displacement studies have revealed species differences in the 5-HT re-uptake site (Bäckström et al, 1989; Erreboe et al, 1995; Plenge et al, 1991).

In 1988, Cortés et al carried out an autoradiographical study of the distribution of ^{3}H-paroxetine binding sites in human brain from blocks of tissue dissected at autopsy, however a very high concentration of ^{3}H-paroxetine (0.5μM) was used. This is seemingly the only study using autoradiography to determine the distribution of ^{3}H-paroxetine labeled 5-HT re-uptake sites. Therefore, in the present study, a detailed investigation of the distribution of ^{3}H-paroxetine binding sites in human postmortem brain tissue was carried out using a lower concentration of ^{3}H-paroxetine. The experiments that were undertaken in this investigation were the first to label 5-HT re-uptake sites with ^{3}H-paroxetine in large human brain sections, as well as the first time any autoradiography has been performed on human whole brain sections.

The symmetrical distribution of ^{3}H-paroxetine binding sites in human brains has been investigated in several brain regions (Lawrence et al, 1990b; Plenge et al, 1990a) as a result of the finding that ^{3}H-imipramine labeled antidepressant

binding sites in an asymmetrical manner, in the frontal cortex of controls and patients suffering from depression (Demeter et al, 1989). However, since one of the advantages of using whole brain sections is the opportunity to measure binding sites in the left and right hemisphere of the same brain section, it was decided to investigate the symmetrical distribution of ^{3}H-paroxetine binding sites in a wider selection of brain regions.

It is now accepted that ^{3}H-paroxetine binds in a more selective manner to the 5-HT re-uptake site than ^{3}H-imipramine which also labels a low affinity binding site (Hrdina, 1984; Marcusson and Ross, 1990; Moret and Briley, 1986). Therefore, the distribution of both ligands was mapped in human whole brain sections in order to determine unique and common binding sites. Initial attempts were made to compare the distribution of ^{3}H-paroxetine, ^{3}H-fluoxetine and ^{3}H-sertraline binding sites in human brain sections, however this was not possible due to the problems encountered with ^{3}H-fluoxetine and ^{3}H-sertraline. These are discussed in Chapter 6.

All the experiments described in this chapter were carried out using the new method as detailed in Chapter 4. Part of the intention of this study, was also to confirm that the results obtained from this method were comparable with those obtained using other methods, and therefore viable for use in further experiments.

RESULTS

5.1. SATURATION ANALYSIS.

Coronal frontal cortex sections were incubated with increasing concentrations of ^{3}H-paroxetine (0.005 to 6nM) in the presence or absence of 20μM citalopram to define non-specific binding. Specific binding was measured in nCi/mg tissue from the resulting autoradiograms using the Quantimet, and converted to fmol/mg tissue.

Attempts were made to fit the data from the saturation curve to three different equations:

$$B = B_{max} \times L / (K_d + L) \qquad (1)$$

$$B = B_{max1} \times L / (K_{d1} + L) + B_{max2} \times L / (K_{d2} + L) \qquad (2)$$

$$B = B_{max1} \times L / (K_{d1} + L) + B_{max2} \times L / (K_{d2} + L) + NSB \qquad (3)$$

where B is the amount of ligand bound (fmol/mg tissue) and L is the concentration of ligand used. The saturation curve generated by the programme "Fig. P" was best fitted to equation (1) to reveal a K_d of 0.371 ± 0.067nM and B_{max} of 18.78 ± 0.16 fmol/mg tissue (Fig. 5.1). B_{max} was taken as the point at which the saturation curve reached equilibrium and binding values remained static regardless of an increase in ^{3}H-paroxetine concentration.

Therefore, using the above equation, ^{3}H-paroxetine appeared to bind to a single site in frontal cortex sections within the range of concentrations used in this experiment.

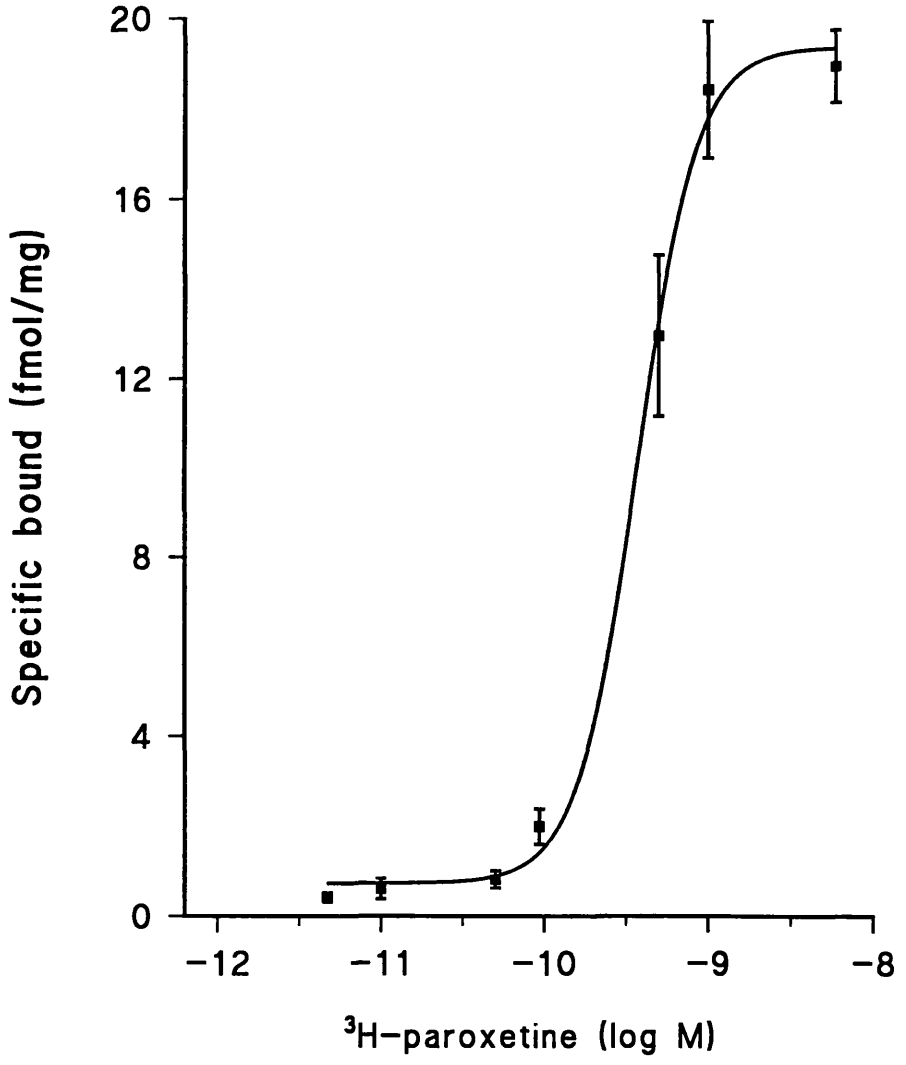


Fig. 5.1. Saturation curve of ^{3}H-paroxetine binding in human brain. Frontal cortex sections (40µm) were cut onto membrane backing material and incubated for 120 minutes with increasing concentrations of ^{3}H-paroxetine (0.005 - 6nM). Results are the mean ± s.e.m. fmol/mg tissue of measurements taken from the frontal cortex of 5 brains. Experiments were carried out in triplicate.

5.2. INHIBITION BY SELECTIVE SEROTONIN RE-UPTAKE INHIBITORS.

^{3}H-paroxetine binding to coronal frontal cortex sections was inhibited by increasing concentrations (1nM to 10μM) of paroxetine, citalopram, fluoxetine and sertraline. The inhibition of the specific binding of 5nM ^{3}H-paroxetine, as defined by the inclusion of 20μM citalopram, was measured in values of nCi/mg tissue taken from autoradiograms. A plot of ^{3}H-paroxetine binding in the presence of inhibitors revealed a set of inhibition curves (Fig. 5.2), where specific binding in the absence of inhibitors was taken as 100%. Inhibition of ^{3}H-paroxetine binding was exhibited by all the SSRI's at the lowest concentration point (1nM), where citalopram showed the greatest inhibition and paroxetine the least inhibition. Therefore the IC_{50} values determined from the curves generated by the programme "Fig P" are probably not true values (Table 5.1) as they do not cover the full inhibition range of each competitor. They do, however, reveal a ranking order of inhibition; paroxetine > citalopram > fluoxetine > sertraline. In order to gain a general idea of the inhibition constant (K_i), this was calculated according to the Cheng-Prussoff equation:

$$K_i = IC_{50} / (1 + L/K_d)$$

where L is the concentration of ^{3}H-paroxetine (5nM) and K_d is the dissociation constant (0.37nM).

Inhibitor	IC_{50} (nM)	K_i (nM)	Ranking Order
Paroxetine	0.87 ± 0.12	0.05	1
Citalopram	7.41 ± 1.89	0.51	2
Fluoxetine	22.38 ± 6.35	1.54	3
Sertraline	23.98 ± 4.02	1.65	4

Table 5.1. Inhibition of ^{3}H-paroxetine binding in frontal cortex sections.

Displacer concentration	^{3}H-paroxetine specific binding (mean ± s.e.m. %)			
	Citalopram	Paroxetine	Fluoxetine	Sertraline
1 nM	36.13 ± 7.12	57.14 ± 9.54	39.04 ± 7.20	49.59 ± 8.53
10 nM	20.67 ± 3.21	15.15 ± 1.80	30.23 ± 7.58	38.96 ± 7.83
100 nM	9.25 ± 2.77	8.54 ± 1.52	8.32 ± 3.40	18.19 ± 4.91
10 µM	7.64 ± 2.02	3.87 ± 0.84	3.96 ± 1.93	10.39 ± 1.93

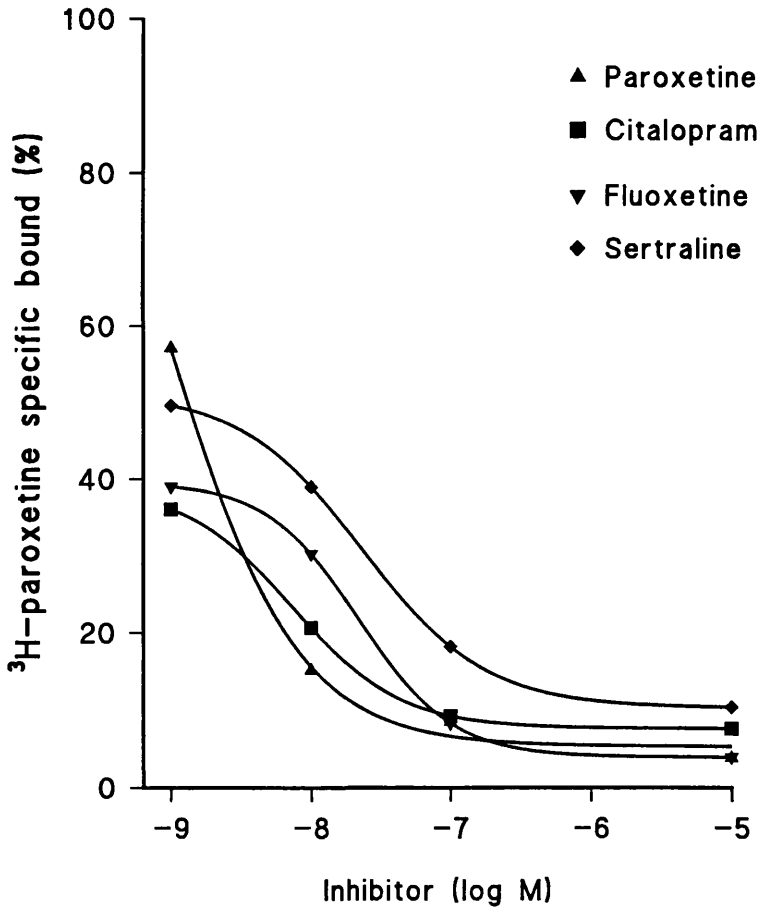


Fig. 5.2. Inhibition of specific ^{3}H-paroxetine binding in human brain. Frontal cortex sections (40µm) were incubated with 5nM ^{3}H-paroxetine in the absence and presence of increasing concentrations (1nM to 10µM) of paroxetine (▲), citalopram (■), fluoxetine (▼) and sertraline (◆). Results are the mean fmol/mg tissue of measurements taken from the frontal cortex of 5 brains. Experiments were carried out in triplicate.

5.3. DISTRIBUTION OF ^{3}H-PAROXETINE BINDING SITES IN CORONAL BRAIN SECTIONS.

In order to determine the binding site densities in the brain, binding levels were categorised into groups of: very high (250-500 fmol/mg), high (100-249 fmol/mg), moderate (50-99 fmol/mg), low (25-49 fmol/mg) and very low binding (0-24 fmol/mg). The actual binding site densities (fmol/mg tissue) are illustrated in Table 5.2. Since a concentration of 5nM ^{3}H-paroxetine was used in these experiments, complete saturation of the binding sites would be expected and therefore the binding values expressed are likely to be a reflection of the B_{max} values found in each region.

^{3}H-paroxetine binding sites were found throughout the brain, with the highest levels of binding exhibited in the brainstem region of the dorsal raphe. Binding in the superior central and linear raphe nuclei (described as the median raphe nuclei in rat brain) was also very high (Fig. 5.3).

The majority of moderate to high levels of binding were found in the grey matter set deep within the brain, with low to very low binding exhibited in the cerebral cortex. The exceptions to this were the entorhinal area and uncus which showed moderate levels of binding. Although binding in the inferior frontal gyrus appeared to be higher than the middle and superior gyri, this was not reflected in the binding values due to a high standard error of the mean.

In the thalamus, the distribution of binding sites was concentrated in the midline nuclei (high binding). These comprise the paraventricular, rhomboid and reuniens nuclei, however because they cover such a small area, they were measured as a single area. High levels of binding were also observed in the hypothalamus surrounding the fornix (Fig. 5.4). In the forebrain, the stria terminalis and diagonal band had high levels of binding with moderately high levels found in the septal nuclei.

Binding in the hippocampus was difficult to measure with no clear distinction between structures. In general the levels of binding were low. Where it was possible to discern Ammon's horn, the binding levels were moderate. A distinct triangular area was apparent in the pre-hippocampal gyrus which showed a high level of binding (Fig. 5.5). Since there is some confusion over exactly what this region is, it was named as "? area of high binding" in the hippocampal region. It is not directly within the hippocampus and appears to be part of the lacunosum.

5.3.1. Comparison of binding sites in left and right hemispheres.

In the 33 brain regions measured, there was no difference in binding site densities in the left and right hemispheres (Table 5.2). While the binding site densities generated by ^{3}H-paroxetine appeared to be higher in some regions in the left hemisphere (substantia nigra, ventromedial hypothalamus, subiculum) these differences were not significant. Due to the fact that not all the sections were absolutely symmetrical, not all left and right measurements were taken from the same section, and this could account for the variations in left and right binding.

Table 5.2. Distribution of ^{3}H-paroxetine binding sites in the left and right hemispheres of human whole brain coronal sections. Results are the mean ± s.e.m. of 3 measurements taken from each of 2 sections. Experiments were carried out in triplicate. n = 3.

Brain Region	^{3}H-paroxetine binding (fmol/mg tissue)		
	Total	Left	Right
Brainstem			
Dorsal Raphe nuclei	486.66 ± 87.68		
Superior Central nuclei (median raphe)	429.80 ± 78.84		
Locus Coerulus	381.71 ± 61.17		
Cerebral Aqueductal Gray	153.23 ± 31.75		
Central Tegmental Tract	265.91 ± 43.78		
Inferior Colliculus	67.45 ± 10.74	64.14 ± 5.37	69.69 ± 9.05
Superior Cerebellar Peduncle	15.74 ± 5.91	20.51 ± 9.07	14.23 ± 3.04
Corticospinal Tract	27.16 ± 9.85		
Basal Pons	39.86 ± 10.35		
Cerebellum	20.33 ± 2.78		
Red Nuclei	31.21 ± 6.19	33.79 ± 5.50	23.09 ± 1.96
Substantia Nigra	122.17 ± 17.45	145.81 ± 13.08	109.56 ± 16.32
Hippocampus	45.33 ± 10.11	54.36 ± 6.98	40.36 ± 9.26
Dentate Gyrus	52.02 ± 10.13	59.41 ± 10.33	34.62 ± 11.99
Ammon's Horn	86.98 ± 15.92	106.82 ± 7.23	95.15 ± 22.35
Subiculum	34.43 ± 10.05	56.55 ± 2.18	34.22 ± 5.46
? area of high binding	180.41 ± 12.82		
Amygdala	72.26 ± 18.81	78.65 ± 13.49	70.82 ± 23.74
Thalamic Nuclei			
Midline	151.67 ± 33.99	181.20 ± 34.87	192.78 ± 25.48
Centromedial	50.46 ± 15.25	66.26 ± 19.28	52.04 ± 18.11
Anterior	33.68 ± 8.59	36.72 ± 9.16	33.94 ± 4.33
Ventral	27.37 ± 4.67	27.31 ± 1.98	19.80 ± 4.26
Lateral	21.63 ± 7.59	24.53 ± 10.59	19.33 ± 2.34
Pulvinar	62.16 ± 12.02	55.36 ± 6.98	68.97 ± 14.88

Table 5.2 Continued.

Brain Region	^{3}H-paroxetine binding (fmol/mg tissue)		
	Total	Left	Right
Hypothalamus			
Lateral	107.87 ± 10.61	152.80 ± 4.99	130.27 ± 14.95
Ventromedial	100.46 ± 17.87	123.63 ± 10.88	99.78 ± 8.03
Fornix	6.13 ± 0.92		
Anterior Commissure	5.49 ± 2.65		
Caudate Nuclear Body	48.07 ± 18.38	64.52 ± 27.32	45.33 ± 19.49
Putamen	39.65 ± 13.22	34.42 ± 9.3	36.30 ± 9.71
Globus Pallidus			
External segment	32.88 ± 4.03	31.83 ± 2.52	25.87 ± 5.26
Internal segment	21.78 ± 7.72	32.53 ± 9.52	18.08 ± 7.51
Claustrum	39.11 ± 8.67		
Corpus Callosum	16.02 ± 5.52		
Septal Nuclei	88.18 ± 20.32	96.99 ± 25.73	82.55 ± 19.72
Stria Terminalis	128.40 ± 37.53		
Substantia Innomonata	39.79 ± 9.47	43.62 ± 12.43	35.59 ± 7.74
Accumbens Area	52.29 ± 12.30	55.86 ± 10.87	54.99 ± 3.58
Diagonal Band	133.60 ± 63.51		
Optic Tract	54.46 ± 14.83	66.01 ± 21.41	51.10 ± 9.69
Olfactory Tubercle	96.60 ± 26.46		
Cortex			
Cingulate gyrus	48.37 ± 5.63	48.80 ± 9.66	47.96 ± 1.21
Superior Frontal gyrus	19.89 ± 8.157	11.57 ± 2.24	11.08 ± 2.02
Middle Frontal gyrus	20.76 ± 4.90	19.16 ± 4.98	22.36 ± 5.77
Inferior Frontal gyrus	32.91 ± 14.91	8.01 ± 4.65	15.80 ± 7.72
Entorhinal Area	58.92 ± 15.36	63.28 ± 2.31	80.47 ± 16.79
Uncus	55.22 ± 12.72	52.27 ± 13.91	66.16 ± 8.58
Temporal Lobe	41.16 ± 13.27	40.73 ± 6.04	53.04 ± 17.46
Insula	25.38 ± 4.44	28.01 ± 8.24	22.36 ± 7.7
Paraolfactory gyrus	46.53 ± 12.84		

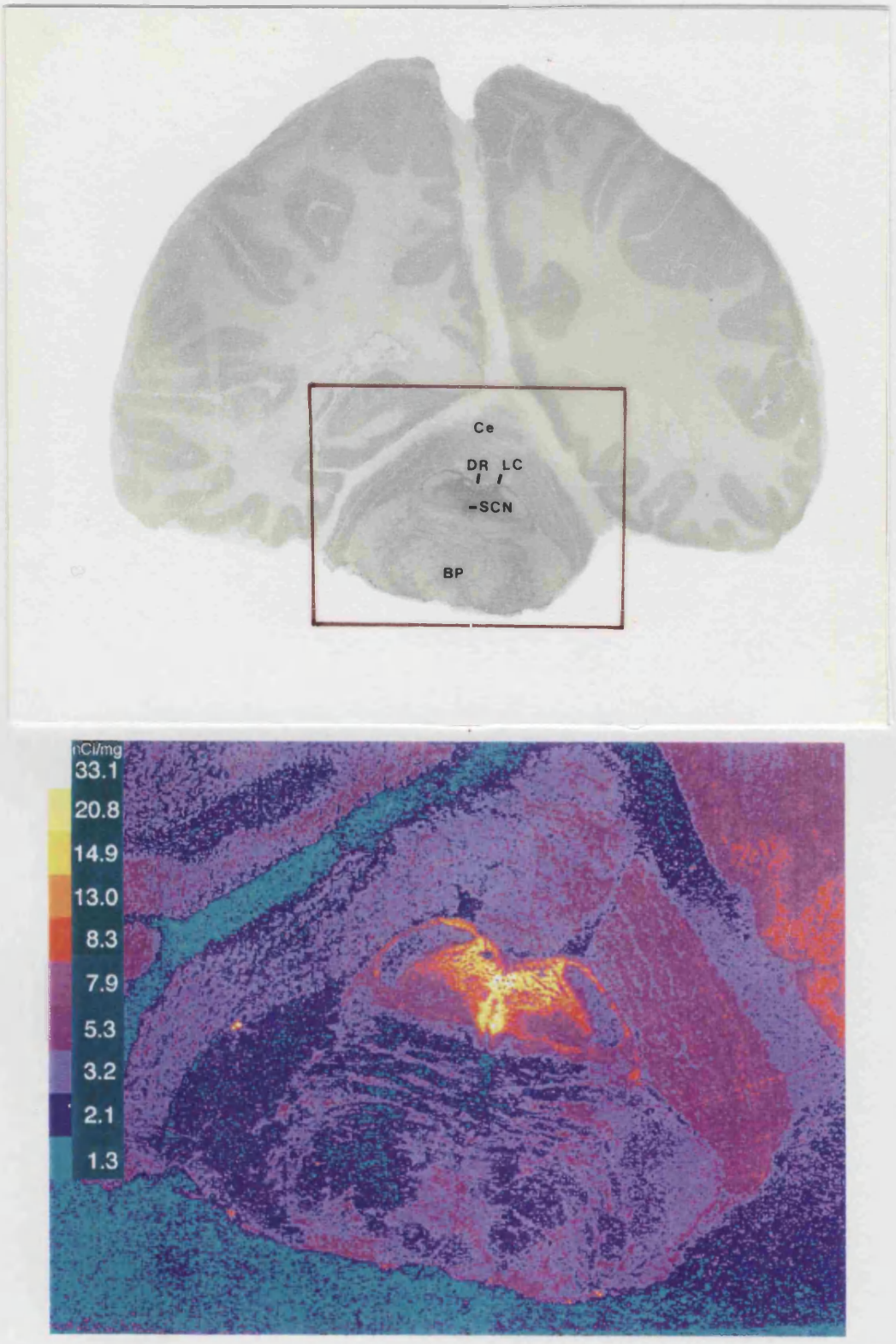
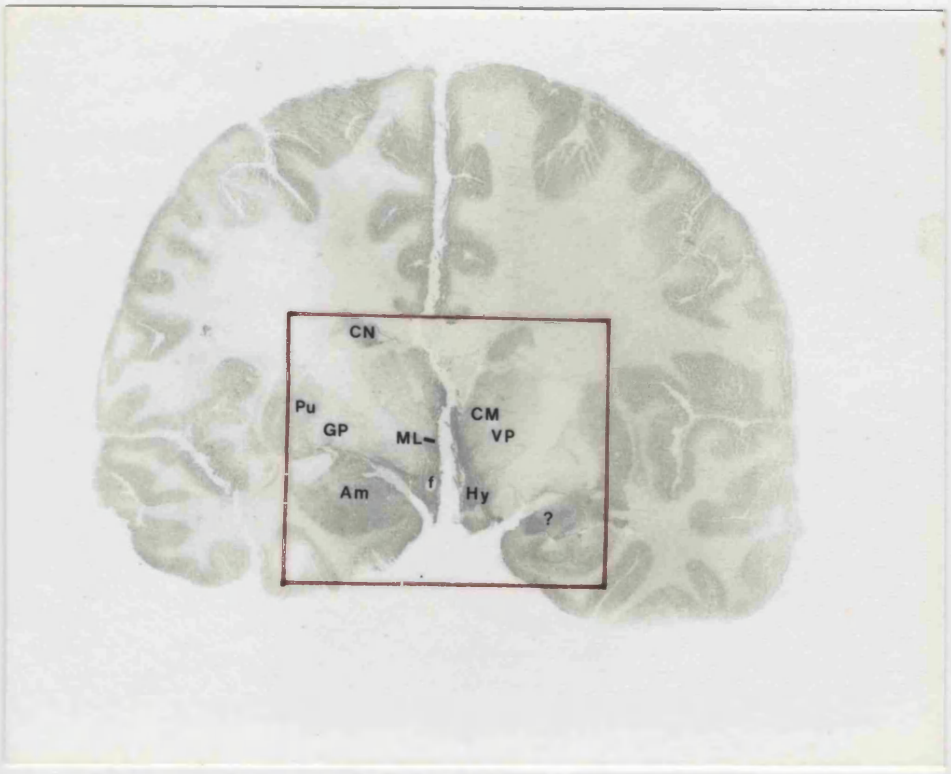


Fig. 5.3. Photographic visualization of ^{3}H-paroxetine binding in the brainstem of a human whole brain coronal section taken directly from autoradiograms. Pseudocolour images represent a higher magnification of the boxed area defined on the whole brain images. For abbreviations see Glossary.



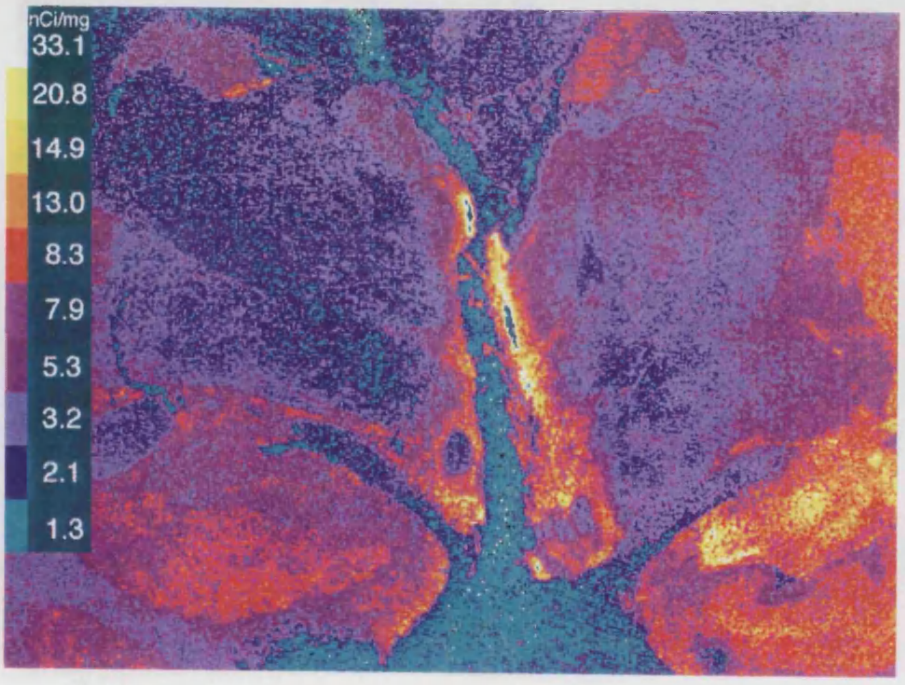
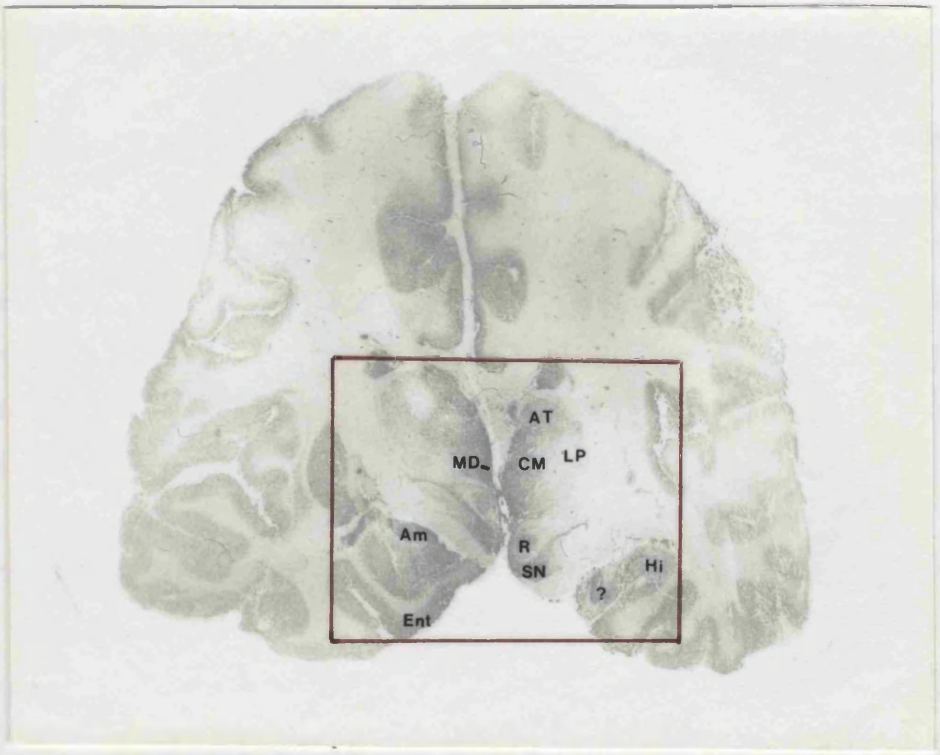


Fig. 5.4. Photographic visualization of ^{3}H-paroxetine in the thalamus and hypothalamus of a human whole brain coronal section taken directly from autoradiograms. Pseudocolour images represent a higher magnification of the boxed area defined on the whole brain images. For abbreviations see Glossary.



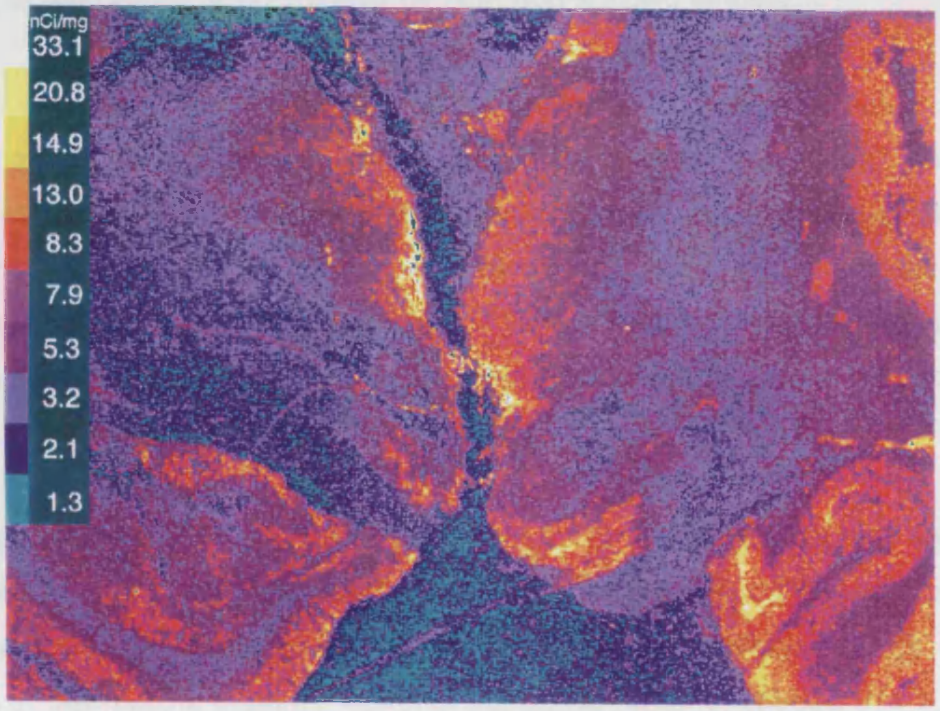


Fig. 5.5. Photographic visualization of ^{3}H-paroxetine in the substantia nigra of a human whole brain coronal section taken directly from autoradiograms. Pseudocolour images represent a higher magnification of the boxed area defined on the whole brain images. For abbreviations see Glossary.

5.3.2. Comparison of binding sites in human and rat brain.

The distribution of ^{3}H-paroxetine binding sites in rat and human brain was compared by normalizing the binding values to a percentage of the binding in the dorsal raphe as different concentrations of ^{3}H-paroxetine (0.25nM in rat; 5nM in human) were used for each experiment. At a concentration of 0.25nM, (less than K_d) ^{3}H-paroxetine binds to approximately 40% of binding sites in the rat and therefore the binding values were adjusted (fmol/mg tissue x 100/40) in order to gain a reflection of the true B_{max} values in rat brain (Table 5.3).

In general the pattern of distribution of binding sites was similar in rat and human brain, with the highest densities found in the brainstem in the dorsal raphe, central gray and locus coeruleus (Fig. 5.6A). In the human brain, however, binding in the superior central raphe nucleus was 5-fold higher than the median raphe in the rat. Equally low specific binding was observed in the cerebellum, with the very low levels in the human accompanied by high non-specific binding.

In the midbrain (Fig. 5.6B) the most obvious difference was noted in the hypothalamus, where low levels were found in the rat in contrast to the high levels in human lateral and ventromedial hypothalamus. Binding in the hippocampus was low in both species although the levels of binding in the dentate gyrus appeared to be higher in human. There is no area in the rat hippocampus which corresponds with the "? area of high binding" found in the human hippocampal gyrus.

Binding in the thalamus (Fig. 5.7A) was similar (moderate to low) with the exception of the midline thalamic nuclei. The binding levels in these nuclei in the rat brain are considered to be high in comparison with other thalamic nuclei, however the midline thalamic nuclei in the human brain exceeded this 3-fold.

In the basal ganglia and cortex (Fig. 5.7B) no extreme differences were observed. Binding levels in the frontal and entorhinal cortex were higher in rat, however once again the specific binding in human brain was obscured by high non-specific binding.

A comparison of the specific binding values (as a percent of total binding) in rat and human brain tissue was made (Fig. 5.8). A significant correlation was found ($r = 0.822$, $p < 0.0001$), confirming the similarities in ^{3}H-paroxetine binding sites in rat and human brain.

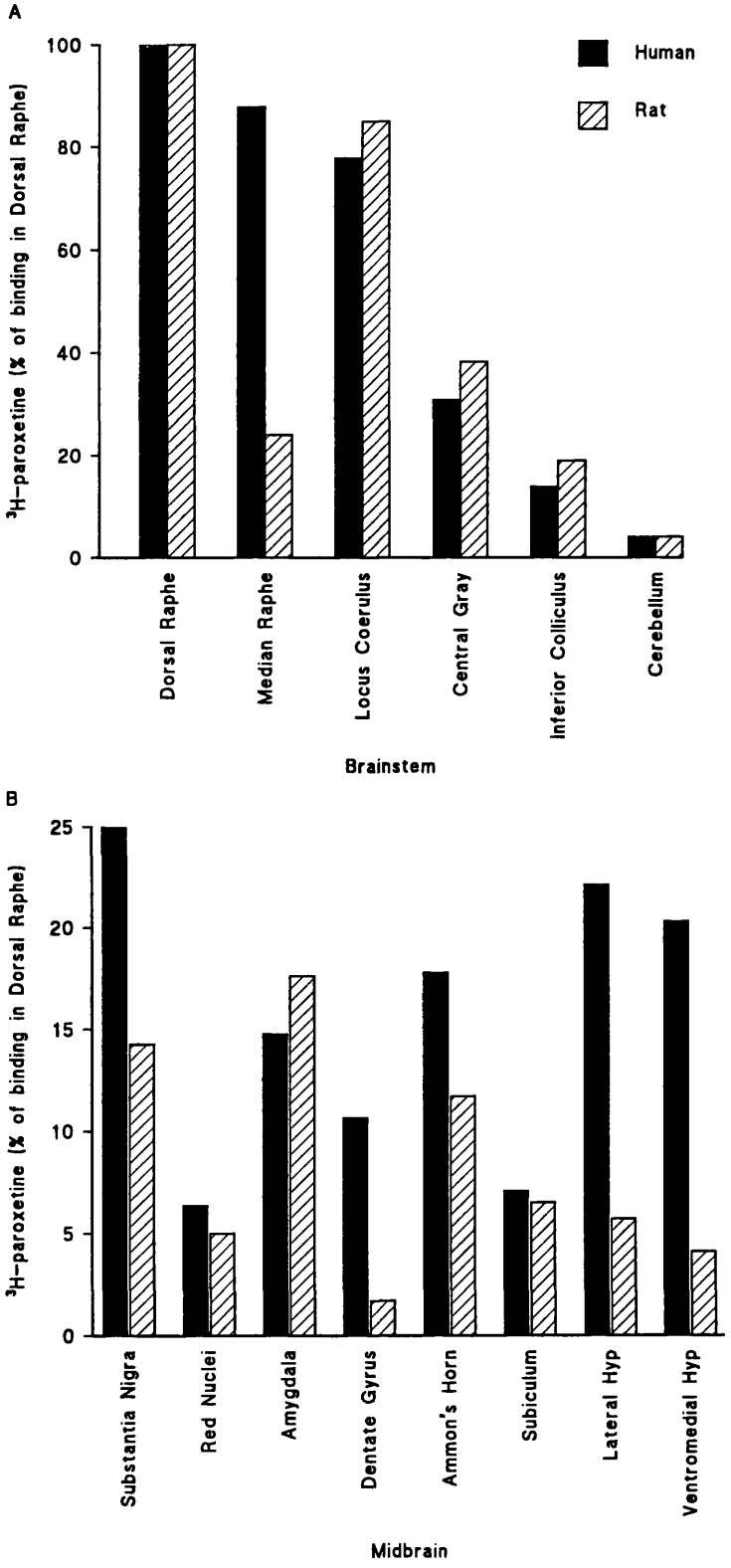


Fig. 5.6. A comparison of the levels of distribution of ^{3}H-paroxetine in rat and human brainstem (A) and midbrain (B). Results represent the means of data normalized to a percentage of the binding levels (fmmol/mg) found in the dorsal raphe of rat and human brain. n = 3, human; n= 3, rat.

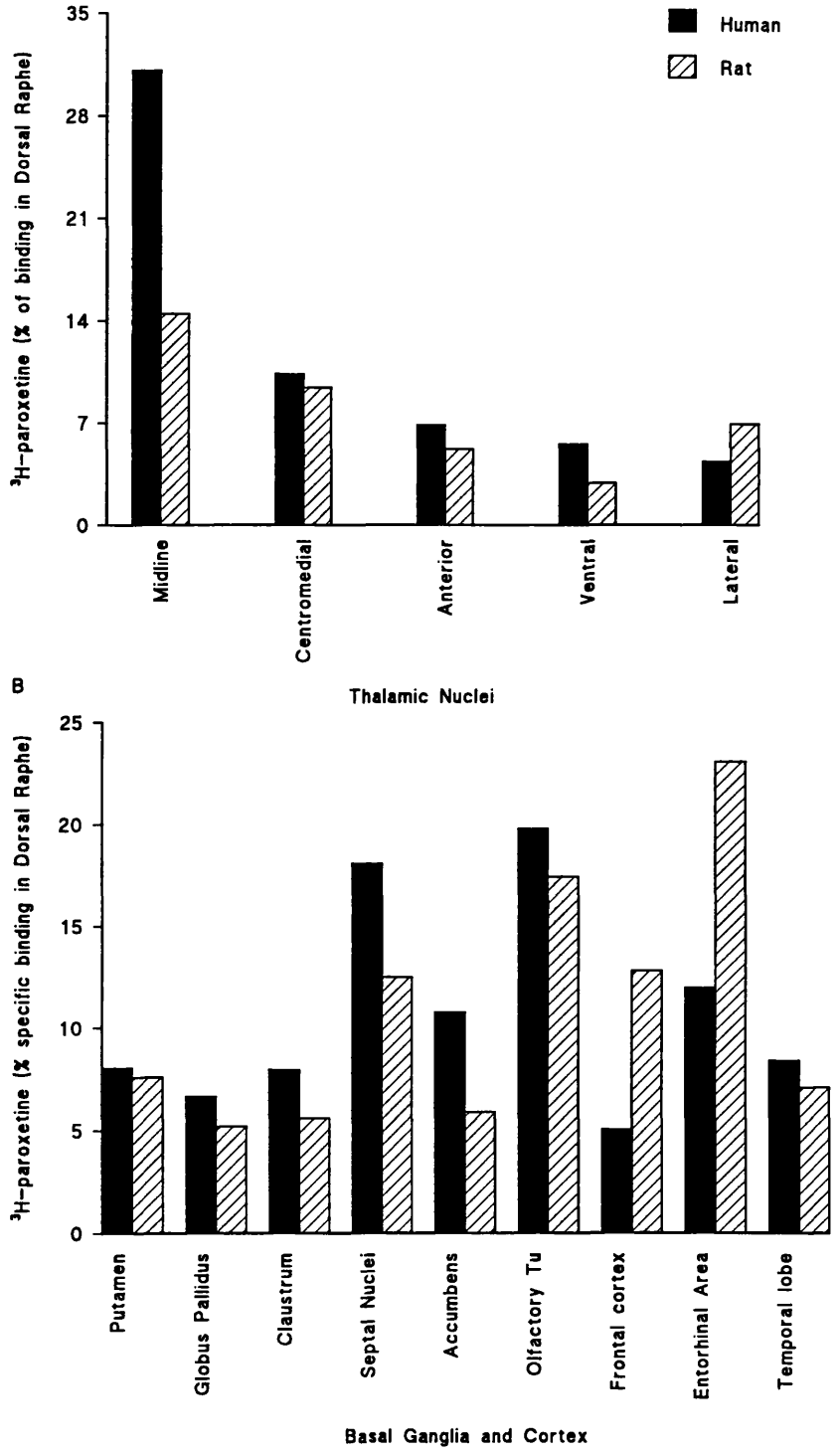


Fig. 5.7. A comparison of the levels of distribution of ^{3}H-paroxetine in rat and human thalamus (A) and basal ganglia and cortex (B). Results represent the means of data normalized to a percentage of the binding levels (fmmol/mg) found in the dorsal raphe of rat and human brain. n = 3, human; n= 3, rat.

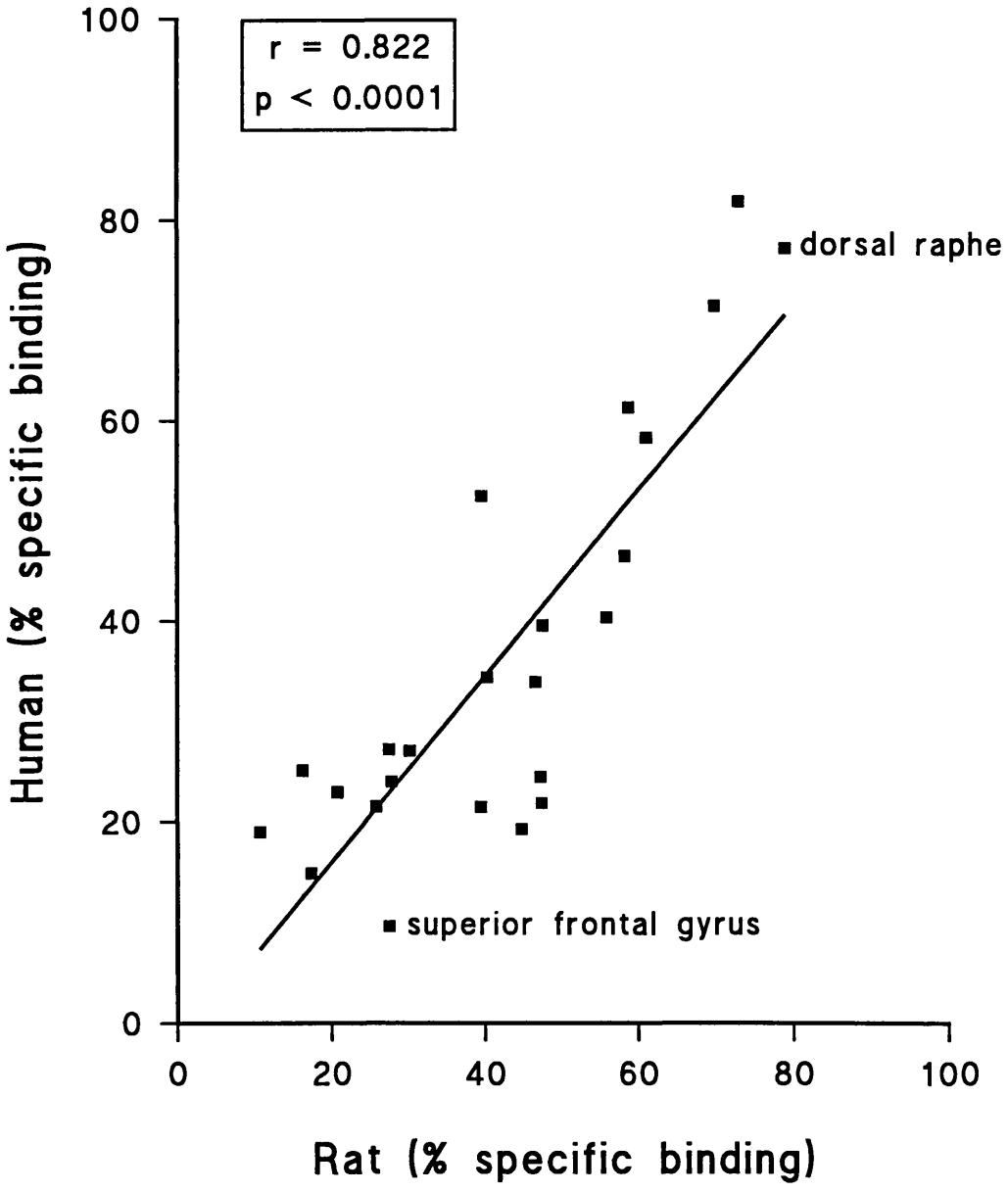


Fig. 5.8. A comparison of ^{3}H-paroxetine specific binding in rat and human brain. Measurements of specific binding (as a percentage of total binding) were analyzed on "Instat" for a correlation co-efficient (Pearsons) assuming a Gaussian distribution of binding sites.

Table 5.3. Comparison of ^{3}H-paroxetine binding sites in human and rat brain.

Region	B_{max} (fmol/mg tissue)	
	Human	Rat
Brainstem		
Dorsal raphe	487 ± 88	850 ± 228
Median raphe	430 ± 79	208 ± 8
Locus coeruleus	382 ± 61	725 ± 158
Central grey	153 ± 32	325 ± 80
Inferior colliculus	67 ± 11	160 ± 38
Cerebellum	20 ± 3	35 ± 15
Midbrain		
Substantia nigra	122 ± 17	121 ± 30
Red nucleus	31 ± 6	18 ± 8
Amygdala	72 ± 19	150 ± 45
Hippocampus		
Dentate gyrus	52 ± 10	15 ± 3
Ammon's horn	87 ± 16	95 ± 20
Subiculum	34 ± 10	55 ± 15
Hypothalamus		
Lateral	108 ± 11	48 ± 13
Ventrolateral	100 ± 18	35 ± 20
Thalamus		
Midline	152 ± 34	122 ± 63
Centromedial	50 ± 15	80 ± 52
Anterior	34 ± 9	44 ± 15
Ventral	27 ± 5	18 ± 8
Lateral	22 ± 8	60 ± 15
Basal ganglia		
Putamen	40 ± 13	65 ± 8
Globus pallidus	33 ± 4	45 ± 3
Claustrum	39 ± 9	47 ± 18
Septal nuclei	88 ± 20	107 ± 13
Accumbens	52 ± 12	50 ± 8
Olfactory area	97 ± 26	148 ± 33
Cortex		
Cingulate gyrus	48 ± 6	48 ± 5
Frontal	25 ± 6	109 ± 20
Entorhinal	59 ± 15	200 ± 53
Temporal	41 ± 13	60 ± 33

Results are expressed as mean ± s.e.m fmol/mg tissue. Data for rat brain are the result of original binding values adjusted (i.e. x 100/40) to reflect B_{max}. n = 3, human; n = 3, rat.

5.4. A COMPARISON OF THE DISTRIBUTION OF ^{3}H-PAROXETINE AND ^{3}H-IMIPRAMINE BINDING SITES.

Initial experiments to determine the minimal background binding levels of ^{3}H-imipramine to the backing material which holds the sections, showed that the two tapes tested (Tesa Tape and 3M tape) had the highest levels of binding. Of the two positively charged nylon membranes tested, the Merck membrane had lower levels than the Amersham membrane (Fig. 5.9A).
The effect of temperature on background binding by ^{3}H-imipramine to the membranes (Fig. 5.9B.) was assessed. Incubation with the ligand at 4^{0}C, as opposed to room temperature, further reduced the ^{3}H-imipramine binding, with the Merck membrane still the better of the two in maintaining minimal background binding levels. This was used in all subsequent experiments.

5.4.1. Distribution of ^{3}H-imipramine binding sites in whole brain coronal sections.

In the brain regions measured, the superior central raphe nucleus exhibited the highest density specific binding with 127 ± 6.16 fmol/mg. The lowest binding densities were recorded in the superior cerebellar peduncle and basal pons with 5.01 ± 1.8 and 4.97 ± 1.76 fmol/mg respectively. The cerebral aqueductal area, thalamus, hypothalamus and caudate nucleate body showed high levels of binding while the amygdala and basal ganglia showed moderate specific binding (Table 5.4). Non-specific binding was high (60 - 85 %) in most regions with the exception of the superior central nucleus which exhibited 20-35% non-specific binding.

5.4.2. Comparison of binding sites with ^{3}H-paroxetine.

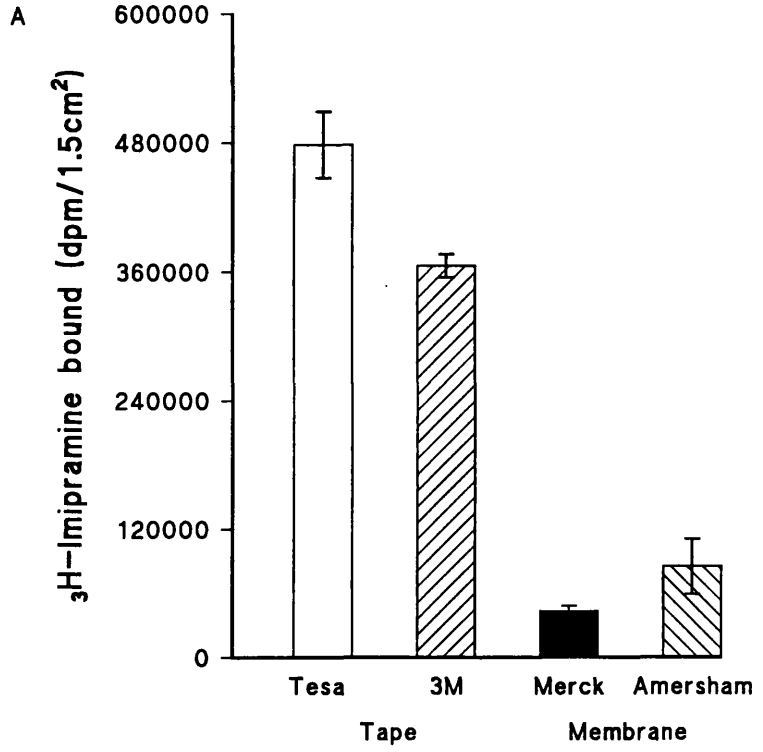
A comparison of ^{3}H-imipramine and ^{3}H-paroxetine binding in the human brain was made to determine the selectivity of these ligands to serotonin re-uptake sites. The total binding values (fmol/mg tissue) of ^{3}H-imipramine were higher

than those measured using ^{3}H-paroxetine, however due to the high levels of non-specific binding, the resulting specific binding values were lower for ^{3}H-imipramine in some regions (Table 5.4). In order to compare ^{3}H-imipramine and ^{3}H-paroxetine binding in similar sections taken from the same brain all values measured were normalised to a percentage of the specific binding in the superior central raphe nucleus in each experiment (Fig. 5.10).

^{3}H-imipramine binding was found to be relatively higher in many areas giving a more diffuse picture of receptor sites than ^{3}H-paroxetine which showed greater variation in binding to 5-HT reuptake sites. In particular, the thalamus is clearly defined by ^{3}H-paroxetine binding with the highest values in the midline nuclei (i.e. reuniens, rhomboid and periventricular nuclei) and decreasing substantially in the other thalamic nuclei (anterior, lateral and centromedial nuclei) while the ^{3}H-imipramine binding was similar in all the regions measured in the thalamus (Fig. 5.11). Binding in the midline nuclei was slightly higher (56.26 ± 9.59 fmol/mg) but not of the same magnitude as ^{3}H-paroxetine which indicated a 3-fold increase in comparison to values measured in other thalamic nuclei.

The basal ganglia also showed a similar pattern of ^{3}H-imipramine binding in the putamen and globus pallidus (31-35 fmol/mg tissue), whereas ^{3}H-paroxetine had a higher density of binding sites in the putamen than in the globus pallidus. The caudate nucleate body which had moderate to low levels of ^{3}H-paroxetine binding exhibited a high density of sites by ^{3}H-imipramine.

In the hippocampus, ^{3}H-paroxetine binding is moderate to low in the fields of Ammon's horn and the dentate gyrus. The most prominent feature is the discrete area of high density binding in the lacunosum (? area of high binding). Moderate ^{3}H-imipramine binding can be seen in the dentate gyrus and CA3,and while the binding in the lacunosum is higher it is less defined than that of ^{3}H-paroxetine.



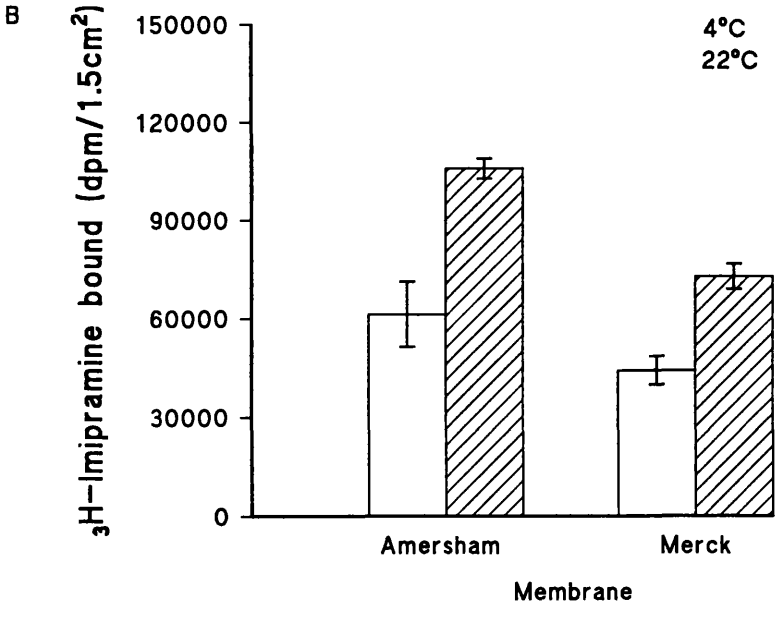


Fig. 5.9. The background radioactive binding levels of ^{3}H-imipramine; A: to Tesa Tape (open bars), 3M tape (/ hatched bars), Merck membrane (solid bars) and Amersham membranes (\ hatched bars), B: to positively charged nylon membranes incubated at 4^{0}C (open bars) or room temperature (hatched bars). Disintegrations per minute (mean ± s.d.) were measured from six 1.5cm^2 pieces of each material incubated with 10nM ^{3}H-imipramine.

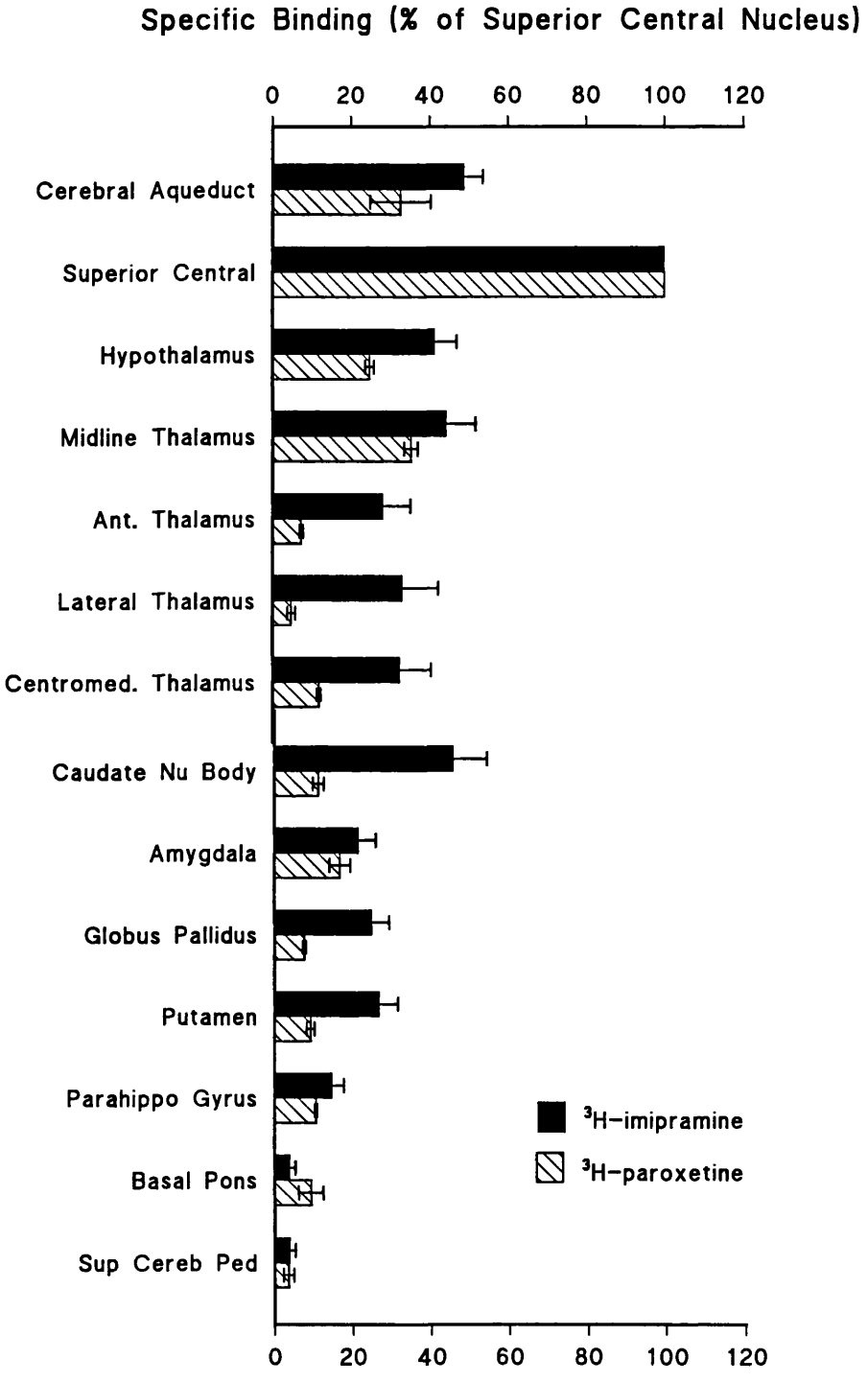


Fig. 5.10. A comparison of specific ^{3}H-imipramine (solid bars) and ^{3}H-paroxetine (hatched bars) binding in various regions of human brain determined by autoradiography in whole brain sections. The mean ± s.e.m. values obtained were normalised to a percentage of the superior central raphe nucleus (100%), the area of highest binding in each experiment. Three measurements were taken from each area in four consecutive sections. n = 3.

Region	^{3}H-imipramine		^{3}H-paroxetine	
	fmol/mg ± s.e.m.	% of S.C.N	fmol/mg ± s.e.m.	% of S.C.N.
Cerebral aqueduct	62.03 ± 6.16	48.76 ± 4.84	153.23 ± 31.75	35.65 ± 7.68
Superior central raphe	127.2 ± 14.31	100	429.80 ± 78.84	100
Basal pons	5.01 ± 1.8	3.93 ± 1.41	39.86 ± 10.35	9.27 ± 3.12
Superior cerebellar peduncle	4.97 ± 1.76	3.91 ± 1.38	15.74 ± 5.91	3.67 ± 1.33
Hypothalamus	52.59 ± 7.19	41.34 ± 5.65	107.87 ± 10.61	25.09 ± 1.11
Midline thalamic nuclei	56.26 ± 9.59	44.23 ± 7.54	151.67 ± 33.99	35.28 ± 1.76
Anterior thalamic nuclei	35.88 ± 8.89	28.21 ± 6.99	33.68 ± 8.59	7.84 ± 0.44
Lateral thalamic nuclei	42.14 ± 11.65	33.12 ± 9.16	21.63 ± 7.59	5.03 ± 1.01
Dorsomedial thalamic nuclei	41.32 ± 10.04	32.48 ± 7.89	50.46 ± 15.25	11.74 ± 0.52
Caudate nuclear body	58.12 ± 11.03	45.69 ± 8.67	48.07 ± 18.38	11.18 ± 1.42
Globus pallidus	31.74 ± 5.62	24.95 ± 4.41	32.88 ± 4.03	7.65 ± 0.39
Putamen	34.16 ± 5.95	26.86 ± 4.68	39.65 ± 13.2	9.22 ± 0.99
Amygdala	27.37 ± 5.65	21.52 ± 4.44	72.26 ± 18.81	16.81 ± 2.67

Table 5.4. Comparative distribution of ^{3}H-imipramine and ^{3}H-paroxetine specific binding to 5-HT re-uptake sites in human whole brain sections. Data are the means ± s.e.m. of specific binding. n = 3. S.C.N: superior central raphe nucleus.

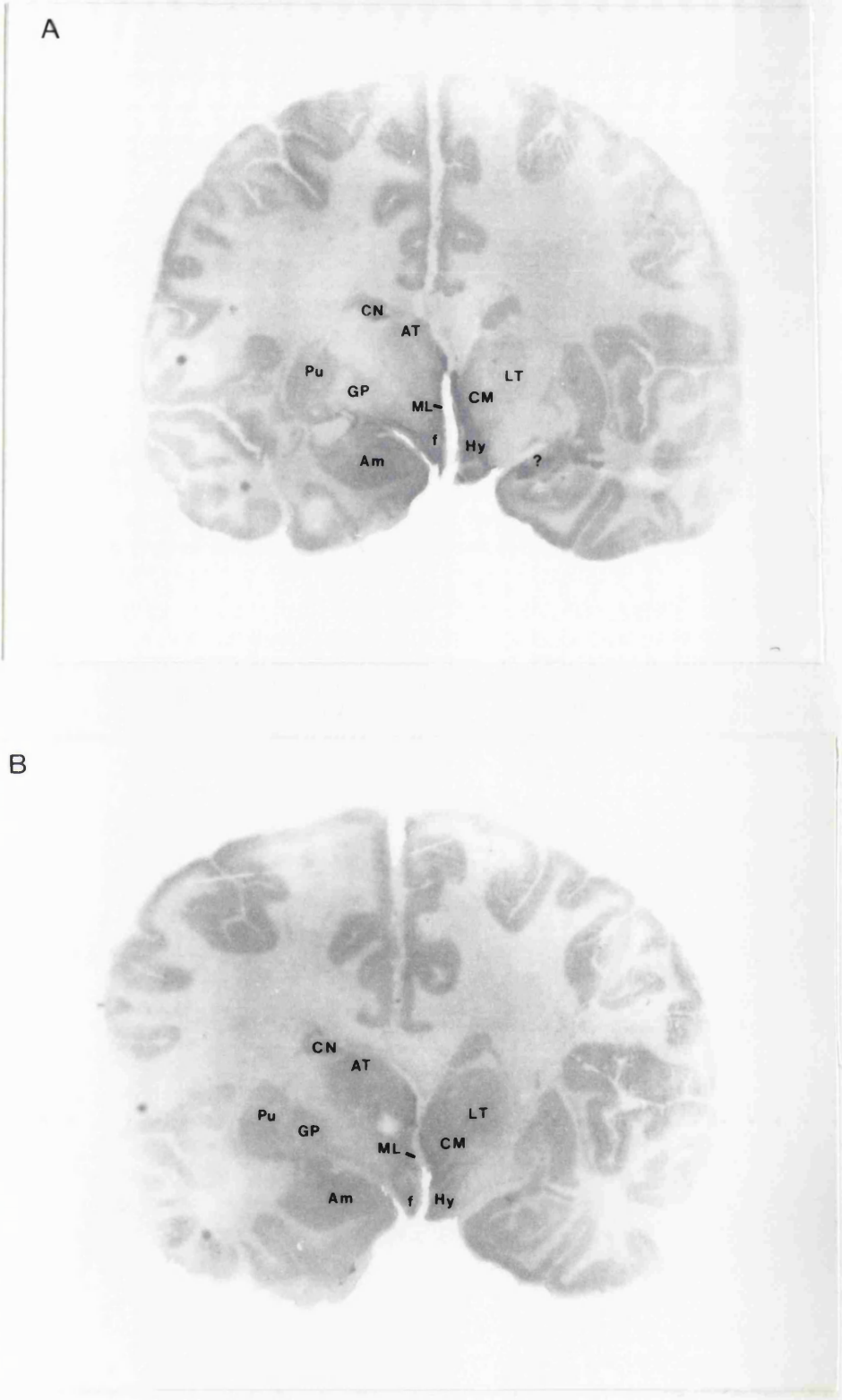


Fig. 5.11. Photomicrographs of the distribution of ^{3}H-paroxetine (A) and ^{3}H-imipramine (B) binding sites in the thalamus and hypothalamus of human whole brain coronal sections . For abbreviations see glossary

5.4.3. Inhibition of ^{3}H-paroxetine by imipramine.

Specific ^{3}H-paroxetine binding to human brain 5-HT re-uptakes sites, in the presence of 100μM imipramine, was measured using autoradiography (Table 5.5). Even at a concentration of 100μM, imipramine failed to completely inhibit ^{3}H-paroxetine binding, however in certain regions, specific ^{3}H-paroxetine binding was reduced by more than 80%. This reduction was noted in areas of high (superior central nucleus, ventromedial hypothalamus) and low (red nucleus, anterior thalamic nuclei) specific binding (Fig. 5.12). In the entorhinal cortex and cerebellum, specific binding was decreased by less than 25%.

5.5. EFFECT OF POSTMORTEM DELAY ON ^{3}H-PAROXETINE BINDING IN RAT BRAIN.

Because a large enough sample size of human brains was not available, it was not possible to perform the statistical analysis required to determine the effect of postmortem delay on ^{3}H-paroxetine binding sites in human brain tissue. Therefore experiments were carried out on rat brains under simulated postmortem conditions. Rats were sacrificed and their heads were kept in cold storage (+4^{0}C) for 0, 4, 24, 48, 72 and 96 hours before using the brains in homogenate binding experiments as described in Methods section 2.1.5.

Fig. 5.13. shows that ^{3}H-paroxetine binding was not affected by a postmortem delay of up to 96 hours at concentrations of 0.01, 0.1, 0.25 and 1nM. A gradual decrease in binding observed at a concentration of 1nM was not considered significant, neither was the slight increase in binding at 24 and 48 hours seen with 0.25nM ^{3}H-paroxetine. The visible quality of the brain tissue deteriorated with increasing time, at 72 and 96 hours the tissue had softened, in particular the brainstem and cerebellum.

Table 5.5. Inhibition of ^{3}H-paroxetine binding by 100μM imipramine.

Region	^{3}H-paroxetine bound (fmol/mg tissue)		
	Specific	+ 100μM imip.	%
Superior central raphe	430 ± 79	29 ± 8	93 ± 6
Cerebral aqueductal grey	153 ± 32	21 ± 14	86 ± 7
Central tegmental tract	266 ± 44	31 ± 3	88 ± 5
Basal pons	40 ± 10	24 ± 4	40 ± 13
Superior cerebellar peduncle	16 ± 6	10 ± 3	36 ± 8
Red nucleus	31 ± 6	3 ± 0.5	82 ± 7
Substantia nigra	122 ± 17	30 ± 5	76 ± 6
Amygdala	72 ± 19	42 ± 11	42 ± 10
Hippocampus	45 ± 10	23 ± 5	50 ± 15
Midline thalamic nuclei	152 ± 34	53 ± 13	65 ± 13
Centromedial thalamic nucleu	50 ± 15	18 ± 6	64 ± 11
Anterior thalamic nucleus	34 ± 9	7 ± 3	82 ± 6
Lateral thalamic nucleus	22 ± 8	9 ± 1	61 ± 5
Lateral hypothalamus	108 ± 11	47 ± 13	57 ± 16
Ventromedial hypothalamus	100 ± 18	10 ± 2	89 ± 6
Putamen	40 ± 13	13 ± 5	68 ± 10
Entorhinal area	59 ± 15	49 ± 10	16 ± 5
Cerebellum	20 ± 3	16 ± 2	22 ± 5

Data are expressed as the mean ± s.e.m fmol/mg tissue of 3 measurements per area per section. imip. - imipramine. n = 2, ^{3}H-paroxetine in the presence of imipramine; n = 3, ^{3}H-paroxetine in the absence of imipramine.

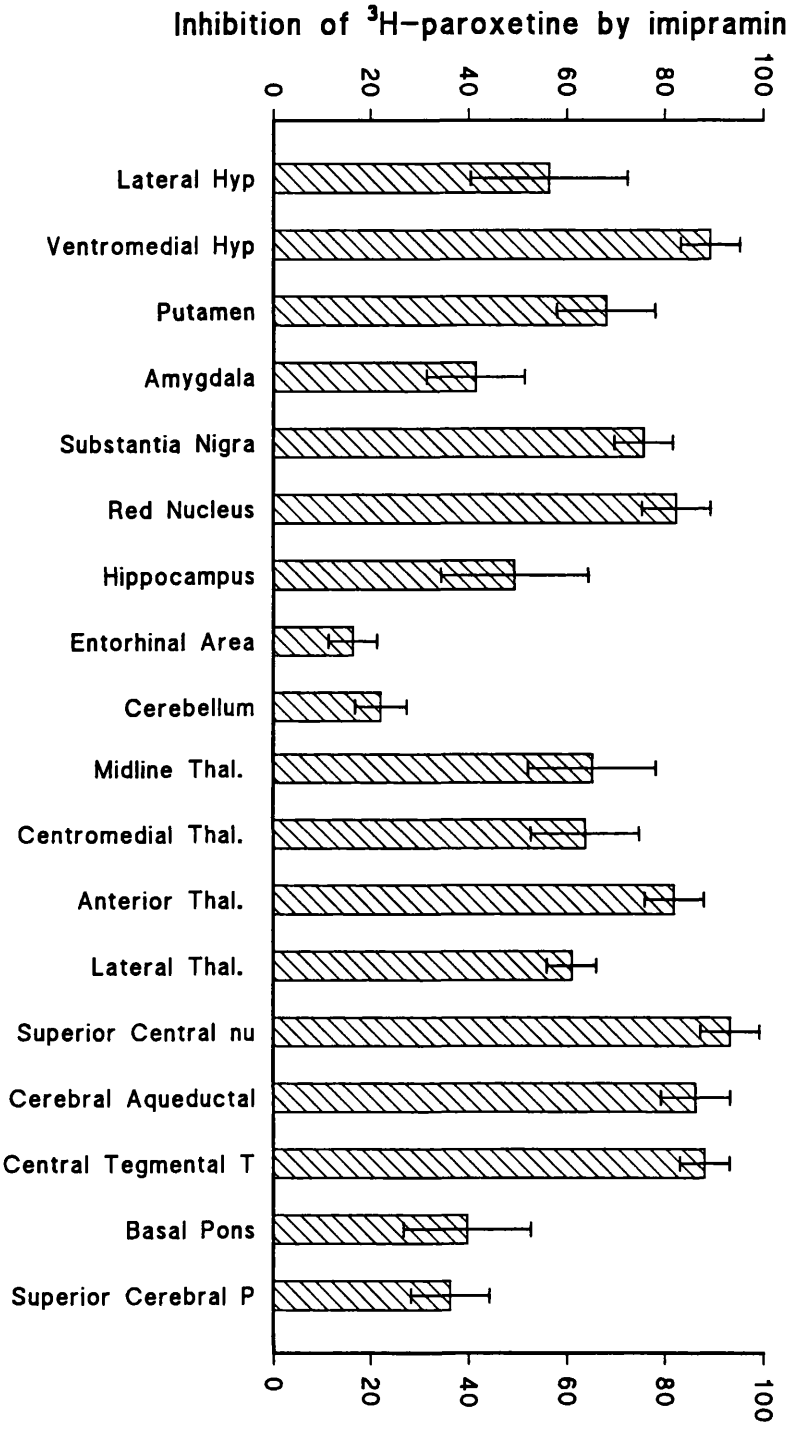


Fig. 5.12. Bar graph showing the percentage inhibition of specific ^{3}H-paroxetine binding by 100μM imipramine. Whole brain coronal sections were incubated with 5nM ^{3}H-paroxetine and 100μM imipramine in the absence or presence of 20μM citalopram, n = 2. Binding values for ^{3}H-paroxetine in the absence of 100μM imipramine were obtained from the distribution study (Table 5.2). n = 3.

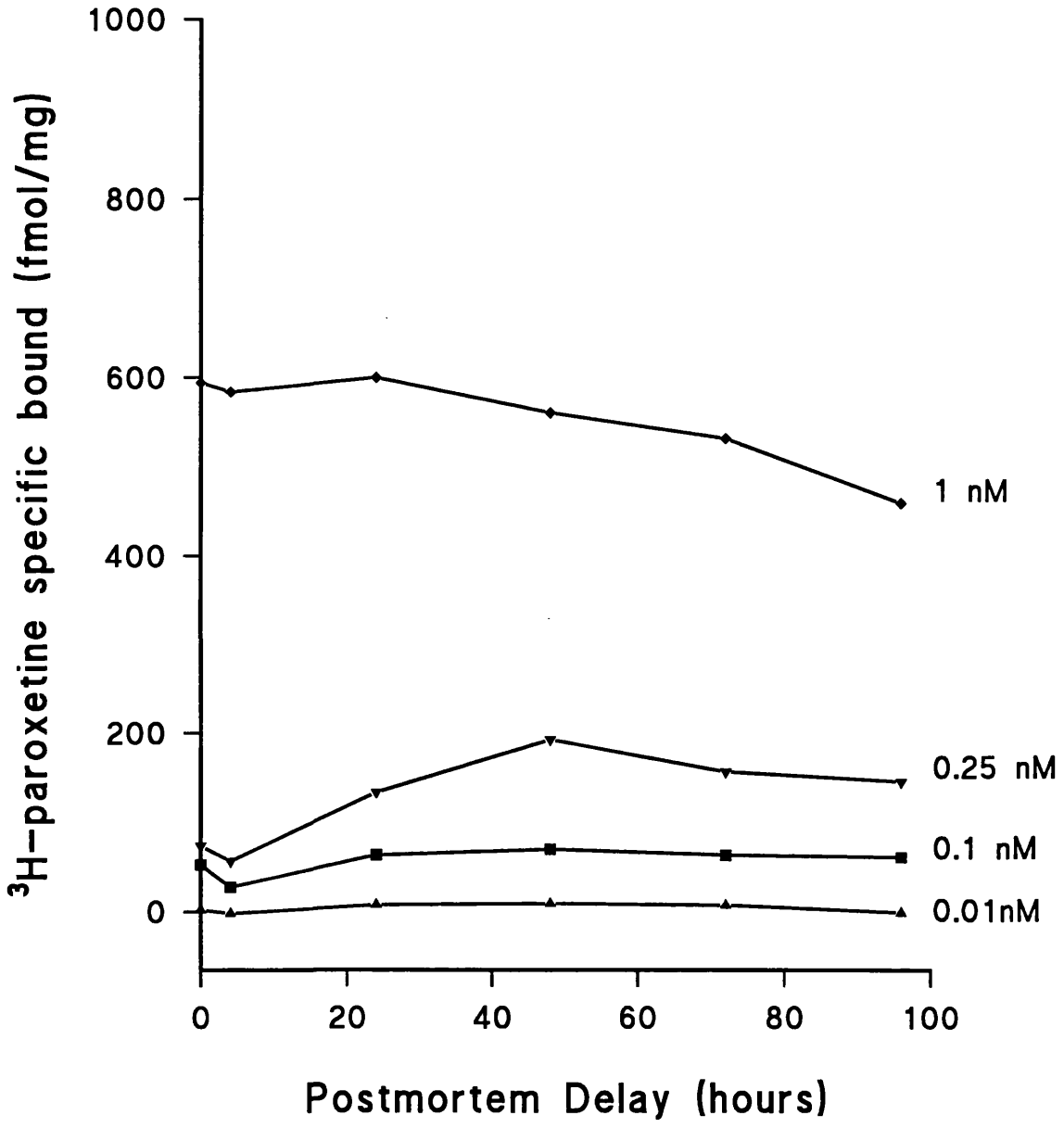


Fig. 5.13. Effect of postmortem delay on ^{3}H-paroxetine binding sites. Rat brains subjected to simulated postmortem conditions were left in cold storage for 0, 4, 24, 48, 72 and 96 hours. ^{3}H-paroxetine binding (0.01, 0.1, 0.25 and 1nM) in whole brain homogenates was calculated as the mean fmol/mg of 3 measurements taken from experiments performed in triplicate. n = 4 rats per time period.

DISCUSSION

The K_d value obtained (0.371 ± 0.067 nM) for saturable ^{3}H-paroxetine binding in human brain sections was similar to the dissociation constant reached in earlier experiments carried out in rat brain sections (0.329 ± 0.091 nM, see Chapter 3). This falls within the range of values obtained in human neuronal membrane binding studies in the cortex, although these vary widely from 0.038nM (Lawrence et al, 1990a) through 0.1nM (Arranz and Marcusson, 1994) to 0.6nM (Hrdina et al, 1993). Since this is the first time that saturation studies have been carried out in human brain sections using autoradiography, it was not possible to compare similar values in human brain sections. While the K_d of ^{3}H-paroxetine binding in rat brain membranes (Habert et al, 1985; Marcusson et al, 1988) was found to be higher (three to ten-fold) than in slide-mounted rat brain sections, the fact that the K_d values obtained in this study for ^{3}H-paroxetine binding in human and rat brain sections were similar, it would be reasonable to assume that in these experiments ^{3}H-paroxetine is binding to the same 5-HT re-uptake site in both species.

The B_{max} values of ^{3}H-paroxetine binding in human frontal cortex membranes range from 40 fmol/mg protein (Lawrence et al, 1990b) to 197 fmol/mg protein (Erreboe et al, 1995). The B_{max} value measured in the human frontal cortex in the current investigation appears to be 10-fold lower (18.78 fmol/mg). This is because the membrane binding values were expressed as fmol/mg protein whereas quantitative image analysis is measured in terms of nCi/mg tissue which is then converted to fmol/mg tissue. Assuming a 10:1 ratio of protein to tissue, this would mean that the comparative values of human frontal cortex binding levels measured in autoradiography are similar to those observed in homogenates.

The lack of viable IC_{50} values was due to a number of reasons; insufficient data points were measured, the lowest concentration of displacer was 1nM where inhibition was still apparent, specific ^{3}H-paroxetine binding in the frontal cortex is low and therefore any inhibition is likely to be masked by high levels of non-specific binding. This suggests that there is a degree of low affinity ^{3}H-paroxetine binding in human brain tissue which is not displaceable by the usual serotonin re-uptake inhibitors used to define non-specific binding. The ranking order of inhibition (paroxetine > citalopram > fluoxetine > sertraline) revealed that paroxetine has a greater affinity for the 5-HT re-uptake site than citalopram. This is confirmed by the higher K_d of ^{3}H-citalopram (1.5nM) in human brain and platelet membranes (Plenge and Mellerup, 1991) and the ranking order of inhibition of ^{3}H-citalopram binding in rat brain; paroxetine > citalopram > sertraline. Inhibition studies of ^{3}H-paroxetine in the rat brain followed a ranking order of citalopram > fluoxetine (Marcusson et al, 1988; Marcusson et al, 1992).

Laruelle et al (1988) found that levels of specific ^{3}H-paroxetine binding, as a percentage of total binding, were lower in human brain homogenates than in rats. This lack of high specific binding due to a fraction of non-displaceable binding, was highlighted by the low levels of ^{3}H-paroxetine binding exhibited in areas such as the frontal cortex. While non-displaceable binding was apparent to some extent in ^{3}H-paroxetine binding in all brain regions, what is of importance is whether this low affinity component is binding to pre-synaptic 5-HT re-uptake sites and if not, whether it has any effect on the high affinity binding site. When Raisman et al (1986) used 100µM chlomipramine to define ^{3}H-paroxetine binding in human putamen, a low affinity binding site was revealed and in 1989, Bäckström and colleagues described a non-specific ^{3}H-paroxetine binding site in human putamen which was displaced by desipramine, norzimeldine and fluoxetine but not by citalopram or 5-HT (Bäckström et al, 1988). In the frontal cortex this 5-HT insensitive binding represented as much as 60% of total binding, similar values to those shown in the current investigation.

Arranz and Marcusson (1994) showed that inhibition of ^{3}H-paroxetine and ^{3}H-citalopram binding by desipramine, norzimeldine and citalopram in human frontal cortex and hypothalamus revealed a 2-site binding model, with a high affinity (low nanomolar) and low affinity (micromolar) component. When a concentration of 1mM 5-HT was introduced into the incubation medium, the high affinity ^{3}H-paroxetine binding was abolished without affecting the low affinity fraction. This led them to suggest that the high affinity binding component, defined by inhibition with desipramine, norzimeldine and citalopram was 5-HT sensitive, whereas the low affinity fraction was not, and therefore was not binding to the 5-HT re-uptake site.

In contrast, a study by Marcusson, Andersson and Bäckström (1989) showed that when citalopram was incubated with human tissue at the same time as the radioligand, then competitive inhibition was displayed and ^{3}H-paroxetine binding to both affinity components was reduced. From these results they suggested that when citalopram is pre-incubated with the binding tissue prior to addition of ^{3}H-paroxetine (as performed in their earlier study, Bäckström et al, 1988), the 5-HT re-uptake site is altered in some way to affect the subsequent affinity of ^{3}H-paroxetine binding.

The complex action of citalopram on the affinity of ^{3}H-paroxetine binding was noted as early as 1985, when Plenge and Mellerup showed that citalopram and paroxetine increased the binding affinity for ^{3}H-paroxetine to rat platelets during dissociation rate studies. They suggested that the altered affinity was due to non-competitive inhibition by binding to separate sites on the serotonin transporter in platelets (Plenge and Mellerup, 1985). In 1990, Plenge, Mellerup and Nielson showed that the molecular weight of ^{3}H-citalopram and ^{3}H-paroxetine binding proteins of rat brain membranes was very similar (72.7 and 66.8 kDa respectively) and therefore they probably bound to the same protein molecule. A low affinity allosteric binding site was defined by the effect of 200μM citalopram on dissociation rates of ^{3}H-citalopram and ^{3}H-paroxetine, and increasing irradiation of the membranes reduced the low affinity site at the

same rate as the high affinity binding site. From these results they concluded that all three sites would be found on the same protein molecule (Plenge et al, 1990).

Affinity modulation by citalopram on ^{3}H-paroxetine, ^{3}H-citalopram and ^{3}H-imipramine binding to human putamen and platelets (Plenge et al, 1991) confirmed the presence of a low affinity allosteric site in human tissue. While citalopram attenuated the dissociation rate of ^{3}H-paroxetine and ^{3}H-citalopram binding in both human and rat brain, indalpine exhibited a species difference by increasing the dissociation rate of ^{3}H-citalopram binding in human putamen and platelets but not in rat brain.

All of the above inhibition experiments were carried out using membrane binding homogenates or platelets. This is the first time that ^{3}H-paroxetine binding inhibition experiments have been carried out using autoradiography in human brain sections. The fact that at 10μM, citalopram inhibition of the high affinity ^{3}H-paroxetine binding reached a plateau suggests that using a concentration of 20μM citalopram to define the specific binding of ^{3}H-paroxetine under the present autoradiography conditions will not affect the low affinity binding component. In the case of autoradiography or binding experiments using tritiated serotonin re-uptake inhibitors, it would be circumspect not to use high micromolar concentrations of serotonin or serotonin uptake inhibitors to define non-specific binding, in order to ensure only the high affinity binding component is measured. It will be interesting to investigate the inhibition constants in different brain regions in whole brain sections when a greater number of brains are obtained, as this type of experiment requires a large amount of tissue.

The distribution of ^{3}H-paroxetine binding sites in human brain, as determined in this study, exhibited a heterogeneity of binding site densities. These values were, in general, found to be in agreement with other autoradiographic studies of human 5-HT re-uptake binding sites labelled with ^{3}H-paroxetine, ^{3}H-citalopram and ^{3}H-imipramine (Table 5.6). While the distribution of binding sites

compared well with the regions measured in the study carried out by Cortés et al (1988), these authors concentrated on the brainstem and midbrain regions. No measurements were made in the hippocampus, amygdala, striatum or cortex, with the exception of the frontal cortex. The B_{max} values were up to 10-fold higher in some regions, than those obtained in the current investigation. These high binding values were probably the result of a shorter wash out time (2 x 20 minutes). It is also possible that a number of low affinity binding sites were labelled by their use of a 500nM ^{3}H-paroxetine concentration. Indications of this were shown in the good correlation ($r^2 = 0.737$, $p < 0.0001$) between ^{3}H-imipramine and ^{3}H-paroxetine specific binding densities where ^{3}H-imipramine is known to label a low affinity binding site which is not related to the 5-HT re-uptake site.

The distribution of ^{3}H-paroxetine binding sites in human brain was also compared with the distribution of 5-HT content in Table 5.6. (Bucht et al, 1981; MacKay et al, 1978). They defined measurements of 5-HT content as a percentage of the caudate content (100% by definition) which indicated a moderate concentration of 5-HT in this region. In the present study only low levels of ^{3}H-paroxetine binding were found in the caudate nuclear body. Bucht et al, (1981) and MacKay et al, (1978) also noted a very high concentration of 5-HT in the pons (> 300%) which was low in ^{3}H-paroxetine binding sites. They did not define any specific regions in the pons therefore it is possible that this would be taken to include the raphe nuclei which would explain the high levels of 5-HT present.

The distribution of ^{3}H-paroxetine binding sites does not always match the serotonin content within a given region. Parsons et al (1992) measured the distribution of 5-HT and 5-HIAA in the cerebral cortex and found a progressive increase in a rostro-caudal direction in 5-HT turnover. ^{3}H-paroxetine binding was distributed uniformly in this direction and binding levels did not correlate with either 5-HT or 5-HIAA in the 6 areas measured. In 1988, Laruelle and colleagues found the subcellular distribution of ^{3}H-paroxetine binding in the

human frontal cortex was located primarily in synaptosomal fractions. In addition, the vesicles and plasma membrane fractions of the striatum showed a high concentration of ^{3}H-paroxetine. They interpreted this ^{3}H-paroxetine presence as binding to nonfunctional 5-HT uptake carriers in vesicle and axons since there is an absence of ^{3}H-5HT uptake in this fraction (Laruelle et al, 1988). It is possible therefore, that the mismatch between 5-HT content and 5-HT re-uptake sites could be a result of ^{3}H-paroxetine binding to these non-functional carriers.

The converse nature of the assymetry of ^{3}H-imipramine binding seen in the frontal cortex of controls and psychiatric or depressed patients (B_{max} higher in the right hemisphere of controls, B_{max} higher in the left hemisphere of psychiatric subjects) suggests that there may be a role for the hemispherical distribution of antidepressant binding sites in affective disorders (Arato et al, 1987; Demeter et al, 1989). However, in the present study the hemispherical distribution of ^{3}H-paroxetine was measured in a wide range of brain regions and no significant difference in binding to the left or right hemisphere was found. This was in agreement with Lawrence et al (1990b) who found no hemispherical difference in ^{3}H-paroxetine binding to the putamen, substantia nigra or putamen and Plenge et al, (1990a) who found no difference in ^{3}H-paroxetine binding between the left and right hemisphere of the cortex. Furthermore, ^{3}H-citalopram binding displayed a uniform distribution between the left and right hemisphere of the frontal cortex (Leake et al, 1991).

The B_{max} values measured in the current investigation revealed a higher density of ^{3}H-paroxetine binding sites in rat brain than in human brain. A species difference in ^{3}H-paroxetine binding densities was also found in membrane binding studies (Laruelle et al, 1988; Marcusson et al, 1989) and Arranz and Marcusson (1994) found a 10-fold difference in the frontal cortex of rat and human brain. It is possible this could be attributed to ^{3}H-paroxetine binding with a greater affinity for low affinity binding sites in rat brain, however this is unlikely since the ^{3}H-paroxetine concentration used in this study was below the

K_d value. The similarity in K_d values obtained from rat and human brains and the strong correlation between specific binding levels in rat and human brain regions indicate that the ^{3}H-paroxetine binding site in human brain is very similar to the ^{3}H-paroxetine binding site in rat brain.

Immunohistochemical studies by Baker et al (1991) revealed a greater proportion of serotonin containing neurons in the dorsal raphe of human brain as compared with rats, however 5-HT cell bodies in the rat brainstem were tightly clustered around the midline with a more uniform cortical distribution. In the primate the serotonin cells are loosely arranged with a more restricted pattern of 5-HT fibre terminals in the cortex resulting in a greater independence of projections (Azmitia 1987). In addition, the increased appearance of myelinated serotonin fibres in human brain reduces the interaction of soma along the axon length and therefore it is likely that the reduction in maximal ^{3}H-paroxetine binding sites in human brain are a reflection of this arrangement of 5-HT cell bodies and fibres.

The very high density of binding sites observed in the superior central raphe (median raphe) nucleus (median raphe:dorsal raphe; 430:489) as compared to the lower levels found in the rat brain (208:580) suggest a greater involvement by the superior central raphe nucleus in the human. The median raphe does form the second largest group of serotonergic neurons in the human brainstem (Törk, 1990) and therefore it would be expected to have a very high level of 5-HT re-uptake sites. This is reflected in the similar proportion of ^{3}H-paroxetine binding site densities in dorsal and median raphe (1600:1991) found by Cortés et al (1988).

The most apparent species difference was observed in the human hypothalamus (lateral and ventromedial) which showed a high level of binding sites. Higher levels of binding were also noted in human hypothalamus as opposed to rat hypothalamus using ^{3}H-citalopram and ^{3}H-imipramine (Duncan et al, 1992). The feeding and satiety centres are located in the lateral and

ventromedia hypothalamic nuclei, and therefore it is possible that the appetite suppressant side-effect of SSRI's is in part mediated through binding sites in the hypothalamus. The influence of the hypothalamus on emotional well-being is unclear, however the physical expression of emotional states is modulated by the hypothalamic influence on the autonomic nervous system. Less obvious differences in ^{3}H-paroxetine binding to rat and human brain regions (substantia nigra, dentate gyrus and midline thalamic nuclei) were also noted.

These species different binding site densities suggest that the influence of serotonin re-uptake inhibitors could have a different functional effect in rat and human brain. Certainly it is worth bearing these differences in mind when interpreting data extrapolated from rat to human.

In the experiments carried out to determine the distribution of ^{3}H-imipramine binding sites in human whole brain sections, the specific binding of ^{3}H-imipramine in human whole brain sections was found to be the highest in the superior central raphe nucleus. It would be expected to be highest in the dorsal raphe nuclei, however the sections incubated did not contain the dorsal raphe nuclei. Therefore the specific binding in the superior central raphe nucleus was used as a reference value to normalise the data because it is known to contain a high density of 5-HT cell bodies and 5-HT reuptake sites.

The general distribution of ^{3}H-imipramine binding sites in human whole brain coronal sections correlates well with autoradiography performed on fragments of tissue (Duncan et al, 1992) and hemispheric sections (Gross-Isserhof et al,1989). Fuxe et al (1983b), demonstrated a close relationship between the quantitative autoradiographic localisation of ^{3}H-imipramine binding sites and the distribution of ascending 5-HT neuron systems in rat brain. The correlation of ^{3}H-imipramine binding sites with the distribution of 5-HT was substantiated by Hrdina et al (1990), however in comparison to ^{3}H-paroxetine the correlation was considered to be lower, despite still being significant.

An autoradiographic comparison of ^{3}H-paroxetine and ^{3}H-imipramine in human brain was carried out by Cortés et al (1988) where they found that there was little difference in the distribution of binding sites. As discussed previously, a very high concentration of ^{3}H-paroxetine was used (500nM) and it is possible that both ligands were binding to a low affinity binding site. In that particular study the ^{3}H-imipramine incubation was carried out at room temperature. It has been shown that incubation at room temperature decreases the density of ^{3}H-impramine binding (Kinnier et al, 1981). However, in the present study, experiments were performed with an incubation at 4^{0}C, the binding values measured were still much lower than those reported in other autoradiography studies. The reason for this is unclear, the most likely explanation is that these low specific binding values are the result of high non-specific binding.

In this study the high non-specific binding was found to be a hindrance in quantifying the autoradiographic ^{3}H-imipramine binding sites (defined by inclusion of 100μM desipramine) as it distorts the visual depiction of binding density distribution. High non-specific binding is to be expected in human tissue (up to 40 % higher than in rat brain), however in this case non-displaceable binding was in excess of the amounts normally seen. It is suggested that this is due to a combination of binding to low affinity ^{3}H-imipramine recognition sites and non-displaceable backing membrane binding.

Studies of the characteristics of ^{3}H-imipramine binding in human brain membranes have shown a strong correlation with the binding profile of ^{3}H-imipramine in rat brain (Langer et al, 1981; Raisman et al, 1980) which describe a heterogeneity of receptor sites comprising low and high binding affinities. The high affinity, sodium-dependant ^{3}H-imipramine recognition sites were found to be functionally related to the serotonin reuptake process and effectively displaced by tricyclic and some other non-tricyclic antidepressants. The non-serotonergic low affinity binding site has a high capacity nature and is distributed across regions which include the serotonergic transporter site (Hrdina et al, 1990). This has been confirmed by finding that lesioning of

serotonergic neurons in rat brain with p-chloroamphetamine and 5,7-DHT (Dawson et al, 1983; Duncan et al, 1992) only partially abolished ^{3}H-imipramine binding.

Although the low affinity fraction is sensitive to desipramine, which was used in the current experiment to displace non-specific binding, Langer et al (1981) demonstrated in membrane preparations from different regions of human brain, that as the concentration of ^{3}H-imipramine increased in a saturable manner, the non-specific binding defined by 100μM desipramine increased in a linear fashion to exceed the specific binding at high ^{3}H-imipramine concentrations. The cross-over point varied from region to region. This corresponds with the data from the present study which showed an apparent positive correlation between specific binding (%) and density of binding sites in selected regions.

In addition to this low affinity binding, a significant artifact in the form of low affinity ^{3}H-imipramine binding to filters was seen by Moret and Briley (1986) and Williams and Phillips (1985). The section support structure used in the autoradiography method of the present study, was a membrane filter material, and could well be an additional factor in exhibiting non-displaceable binding.

Bäckström and Marcusson (1987) described the desipramine-sensitive ^{3}H-imipramine binding as having 2 components; protease sensitive and protease resistant. The protease sensitive fraction was found to be high-affinity, sodium dependant and able to be displaced by 5-HT and 5-HT reuptake inhibitors and was therefore very likely to bind to the serotonin transport site. The protease resistant fraction was low-affinity in the micromolar range and did not fit the above criteria despite being desipramine sensitive. ^{3}H-paroxetine binding in the human brain is protease sensitive and sodium dependant, therefore it is likely that the sites where both ligands bind with similar density levels are these protease sensitive, 5-HT displaceable recognition sites.

There is now no doubt that ^{3}H-imipramine binds to two different sites in the brain; a high affinity recognition site for the serotonin transporter and a non-serotonergic low affinity site, however the significance of these low affinity sites are unknown. The effective therapeutic plasma concentrations of many antidepressants are in the μM range, well within the limits of low-affinity binding concentrations, and as a result, the distribution of these sites should not be dismissed.

In experiments carried out in the present study, 100μM imipramine was capable of reducing ^{3}H-paroxetine binding by more than 80% in certain regions, however binding in some areas (amygdala, hippocampus, entorhinal area, cerebellum, basal pons and superior cerebellar peduncle) was inhibited by less than 50%. This would imply that there is a degree of ^{3}H-paroxetine high affinity binding (defined by 20μM citalopram) which is not displaceable by imipramine at high concentrations. Given that imipramine binds to both a high and low affinity site (Hrdina et al, 1984; Reith et al, 1983), this lack of inhibition suggests that ^{3}H-paroxetine is binding to sites within certain areas in the brain for which imipramine has little or no affinity.

When this investigation was carried out, the number of brain sections available for experimentation were limited due to a much reduced supply of brain tissue, and as a result the sections were only used for ^{3}H-paroxetine binding in the presence of 100μM imipramine or 20μM citalopram. In order to calculate the percentage of inhibition, specific ^{3}H-paroxetine binding values from the distribution study were used. This may have introduced an element of experimental error, however this is the first time the autoradiographic distribution of ^{3}H-paroxetine binding sites has been measured in the presence of imipramine. Therefore it is not possible to confirm whether this is artifact or indeed a reflection of alternate, discrete ^{3}H-paroxetine binding sites within certain brain regions. Since the lack of inhibition is only exhibited in specific brain regions, this effect would not have been observed in membrane binding studies. It is also possible that the concentration of ^{3}H-paroxetine used in these

experiments (5nM) reduces the affinity of imipramine for these binding sites. At present these suggestions are speculative and additional experiments would have to be carried out in order to confirm these results. It will be interesting to pursue these experiments further when a greater supply of brain tissue becomes available. Saturation experiments would have to be performed for each region in the presence of high concentrations of imipramine to determine whether this effect is the result of a change in apparent affinity of ^{3}H-paroxetine to discrete areas or an alteration of maximal binding sites. Increasing concentrations of imipramine would have to be added to determine whether this effect is apparent at high or low affinity imipramine binding concentrations.

^{3}H-imipramine binding sites in the basal ganglia, temporal and insula cortex, anterior thalamus, hypothalamus, frontal and parietal cortex have shown a positive correlation with age (Cash et al, 1987; Gross-Isserhof et al, 1989; Owen et al, 1986; Severson et al 1985) although Allen et al (1982) found that ^{3}H-5-HT uptake and ^{3}H-imipramine binding in the temporal lobe was not associated with age. In addition to this, a negative correlation has been shown with age and the number of 5-HT_{1A} (Dillon et al, 1991), 5-HT_{1D} (Arranz et al, 1993) and 5-HT_{2} receptor binding sites (Arango et al, 1990; Arranz et al, 1993; Cheetham et al, 1988; Gross-Isserhof et al, 1990; Lowther et al, 1994), thereby indicating the influence of age on serotonin receptor sites. Due to the limited sample number used in the current investigation, it was not possible to determine the effects of age on ^{3}H-paroxetine binding sites, however, previous results have shown that ^{3}H-paroxetine binding sites appear to remain stable with increasing age (Arranz et al, 1993; Dean et al, 1995; Hrdina et al, 1993).

A negative correlation between 5-HT and 5-HIAA concentrations with postmortem delay (4.5 -148 hours) was observed in the midbrain (Bucht et al, 1981). However, Gross-Isserhof et al (1989) found that there was no significant effect of a short (9.6 ± 3.2 hours) or long (31.3 ± 6.6 hours) postmortem delay on ^{3}H-imipramine binding in human brains. The present investigation of the effect of a postmortem delay of up to 96 hours in rat brains using ^{3}H-paroxetine,

showed that there was no significant change in binding densities. This is supported by evidence that no significant correlation was found between the density or affinity of ^{3}H-paroxetine binding sites and postmortem delay in human brain tissue (Dean et al, 1995; Hrdina et al, 1993). Lawrence et al (1990a) did find a significant negative correlation between the number of ^{3}H-paroxetine binding sites in the amygdala and postmortem delay, however, this effect was not seen in other brain areas.

Region	^{3}H-paroxetine[a]			^{3}H-citalopram[b]	^{3}H-imipramine[c]	5-HT[d]
	A[1]	B[2]	C[3]			
Dorsal Raphe	+++	+++	+++	+++	+++	
Median Raphe	+++	+++	+++	+++	+++	
Central Gray	+++	+++			++	+++
Cerebellum	+		+	+	+	+
Red nucleus	+	+			+	
Subs. Nigra	+++	+++	+++	+++	+++	+++
Amygdala	++		++		++	++
Hippocampus	+		+/++		++	+
Ammon's H	++			++		
Thalamus						++
Midline +++	+++	++	++/+++			
Anterior	+	++	+		++	
Ventral +				++		
Lateral	+	++			++	
Hypothalamus			+++		+++	++
Lateral	+++	+++			++/+++	
Ventromedial	+++	++			++	
Caudate nucleus	+	++	+/++	++	++	++
Putamen	+	++	++	++	++	++
Globus Pallidus	+	++	++	++	++	++
Claustrum	+			++	++	
Septal Nuclei	++					
Subs. Innomonata	+				+++	
Accumbens	++			++		+
Olfactory Area	++					++
Cingulate gyrus	+		++	+	++	+
Frontal Cortex	+	++	+	+	++	+
Temporal gyrus	+		+		++	+
Insula	+			+	++	

Table 5.6. A comparison of binding site densities of ^{3}H-paroxetine, ^{3}H-citalopram and ^{3}H-imipramine in human brain where the levels of binding are graded accordingly: +++ - high; ++ - moderate; + - low. A review of the 5-HT content is included. Where equally dissenting values are obtained, they are reflected as eg. +/++. [a1] - autoradiographic binding values obtained from this study. [a2] - autoradiographic binding values (Cortés et al 1988). [a3] - membrane binding values (Bäckström et al, 1989; Laruelle et al, 1988; Lawrence et al, 1990; Plenge et al, 1990a). [b] - Chinaglia et al (1993), Duncan et al (1992); [c] - Gross-Isserhof et al (1989), Langer et al (1981); [d] - Bucht et al (1981); Mackay et al (1978), Hardy et al, 1987.

CHAPTER SIX

^{3}H-FLUOXETINE AND ^{3}H-SERTRALINE BINDING IN RAT BRAIN.

INTRODUCTION

In 1974 D. Wong and colleagues described the selective serotonin uptake inhibitory activity of a new compound, Lilly 110140 (3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine (Wong et al, 1974a). This compound was found to be as active as chlorimipramine in inhibiting 5-HT uptake into rat brain synaptosomes, however, unlike chlorimipramine and imipramine, it had a 200 and 300-fold lower activity against noradrenaline and dopamine uptake (Wong et al, 1974b). Furthermore, in contrast to imipramine and chlorimipramine, Lilly 110140 did not inhibit the uptake of noradrenaline into rat heart in *in vivo* experiments (Wong et al, 1975). The selectivity of this compound which subsequently became known as fluoxetine, was confirmed in further experiments which showed that fluoxetine had little or no affinity for alpha$_1$-, alpha$_2$- and beta-adrenergic, 5-HT, dopamine, H_1 histamine, muscarinic acetylcholine, opiate and GABA receptors (Stark et al, 1985; Wong et al, 1983, Wong et al, 1991).

Any alterations in receptor-specific radioligand binding to the 5-HT$_1$ receptor (Fuxe et al, 1983b; Peroutka and Snyder, 1980); 5-HT$_2$ receptor (Beasley et al, 1992; Stoltz et al, 1983) and β-adrenergic receptors (Beasley et al, 1992; Byerley et al, 1988; Wamsley et al, 1987) were the result of chronic administration of fluoxetine as opposed to a direct inhibitory effect. It was therefore predicted that fluoxetine would bind exclusively to the serotonin transporter site.

In 1983, Wong and Bymaster described the binding of ^{3}H-fluoxetine in rat brain cerebral cortex membranes. IC_{50} values of chlomipramine, imipramine, nisoxetine and desipramine were found to be 3, 5, 240 and 110nM respectively. In these experiments synaptosomal membranes (P_2) were incubated with 1nM ^{3}H-fluoxetine for 90 minutes at 0^{0}C. 1μM imipramine was included to define non-specific binding.

In 1985, the use of ^{3}H-fluoxetine was once again described in studies on the inhibitory activity of the optical isomers of fluoxetine. The (-) isomer showed a significantly higher IC_{50} value than the racemate and (+) isomer, however the inhibition potency of these isomers were all within the low nanomolar range. The (+) isomer was slightly more potent than the (-) isomer in inhibiting 5-HT uptake *in vitro* (Wong et al, 1985). This appeared to be the last time that any studies with ^{3}H-fluoxetine have been published.

Another selective serotonin uptake inhibitor, sertraline (1S, 4S-N-methyl-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahyrdo-1-napthylamine), was first described in 1983. Sertraline exhibited a marked potency for the direct inhibition of 5-HT uptake in rat brain synaptosomes, as well as an *ex* vivo inhibition of 5-HT uptake into synaptosomal preparations from rats pre-treated with sertraline (Koe et al, 1983). *In vitro* receptor binding studies showed that sertraline had no significant affinity for muscarinic, histamine H_1, 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, dopamine D_2 and α_1-, α_2- or β-adrenoreceptors (Doogan and Caillard, 1988; Koe et al, 1990).

Binding studies in rat brain membranes revealed that ^{3}H-sertraline bound with high affinity (K_d 0.81nM) to a single site on the serotonin transporter. The ranking order of inhibition potency by other 5-HT re-uptake inhibitors was similar to that seen in experiments with ^{3}H-paroxetine, and PCA pre-treatment resulted in a loss of 57% and 31% of binding sites as compared to controls (Koe et al, 1990). These results confirmed that ^{3}H-sertraline was binding to the 5-HT re-uptake site in rat brain. This is the only publication found that refers to the use of ^{3}H-sertraline in rat or human brain studies.

Welch and Williams (1992) described the synthesis of 7-^{3}H-sertraline by reducing the 7-bromo sertraline precursor with tritium gas to produce a radiochemical purity of 99%, however no further information was given regarding the stability of the tritium labeled compound or use of the tritated ligand in biological studies.

Clinical trials have demonstrated that fluoxetine and sertraline are superior in efficacy to that of placebo, and comparable to that of the tricyclic antidepressants (Doogan and Caillard, 1988; Kasper et al, 1992; Laakman et al, 1988). In addition, preliminary data indicate that fluoxetine and sertraline are effective in treating panic disorder, obsessive-compulsive disorder, eating and personality disorders and substance abuse (Guthrie, 1991; Kasper et al, 1992). The selective serotonin re-uptake inhibitory activity is reflected in the side-effect profiles of fluoxetine and sertraline, which are not associated with the sedation, cardiotoxicity, weight gain and anticholinergic adverse effects commonly seen with the tricyclic antidepressants. The most frequently observed side effect of fluoxetine and sertraline is a high incidence of nausea, however this declines with continued therapy (Cooper, 1988; Doogan and Caillard, 1988; Guthrie, 1991; Zerbe, 1986).

The distribution of ^{3}H-paroxetine (De Souza and Kuyatt, 1987), ^{3}H-citalopram (Duncan et al, 1992), ^{3}H-cyanoimipramine (Kovachich et al, 1988), and ^{3}H-imipramine (Fuxe et al, 1983b) binding sites has been established using autoradiography, however no information is available as to the distribution of fluoxetine or sertraline binding sites in rat or human brain. In the present study, therefore, the intention was to map the distribution of ^{3}H-fluoxetine and ^{3}H-sertraline binding sites in rat and human brain and, on the basis of this, to detail the characteristics of ^{3}H-fluoxetine and ^{3}H-sertraline binding sites.

As very little is known about the stability of ^{3}H-fluoxetine and ^{3}H-sertraline, initial experiments such as light and temperature sensitivity, binding to glass and plastic and the influence of wash and incubation time parameters were carried out.

RESULTS

6.1. EVALUATION OF ^{3}H-FLUOXETINE AS A VIABLE LIGAND.

6.1.1. Light and temperature sensitivity.

The radioactive counts (dpm) of sections incubated with 1nM ^{3}H-fluoxetine under conditions of natural light or complete darkness showed that the amount of total and non-specific binding was unaffected by exposure to light (Fig. 6.1). Binding appeared to be non-saturable. Total and non-specific ^{3}H-fluoxetine binding increased with a rise in incubation temperature (Fig. 6.2). The amount of specific binding (< 10%) was unaltered by changing the incubation temperature, therefore all further experiments were carried out at room temperature.

6.1.2. Binding to glass and plastic.

Due to the lengthy incubation times used for binding of tritiated 5-HT re-uptake inhibitors, sections were required to be incubated in containers with incubation medium to prevent evaporation. Experiments were carried out to determine if there is a difference in ^{3}H-fluoxetine binding to sections incubated in glass or plastic containers.

Fig. 6.3. shows that total and non-specific ^{3}H-fluoxetine binding was higher in sections incubated in glass jars. The magnitude of the standard errors obtained when measured binding in the sections tended to obscure any differences observed between incubation in glass or plastic containers. Pieces of plastic container which were cut out and counted for radioactivity did reveal a high level (> 400 dpm) of ^{3}H-fluoxetine binding, whereas pieces of glass microscope slides did not (< 25 dpm). Due to the quantity of incubation medium used (7ml), it is unlikely that sufficient ^{3}H-fluoxetine binds to the plastic in order to reduce the amount of binding in the sections, however, all further experiments using ^{3}H-fluoxetine were carried out in glass jars.

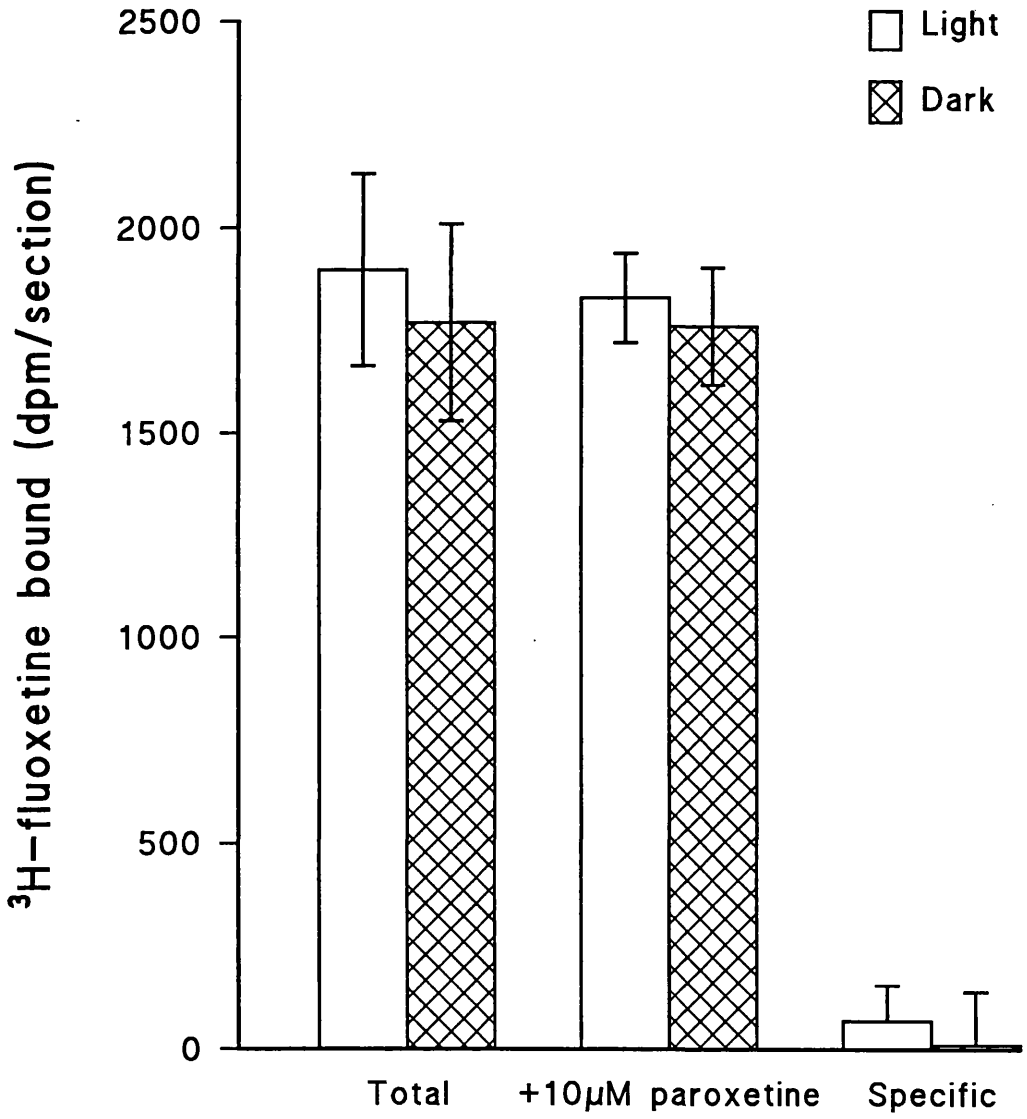


Fig 6.1. Light sensitivity of ^{3}H-fluoxetine binding in rat brain. Slide-mounted sections (10μm) were incubated with 1nM ^{3}H-fluoxetine for 90 minutes in glass Coplin jars which were either left in natural light (open bars) or kept covered (cross-hatched bars) for the duration of the incubation procedure. Results are the mean ± s.e.m. dpm of 3 sections per incubation jar. Experiments were carried out in duplicate. n = 3.

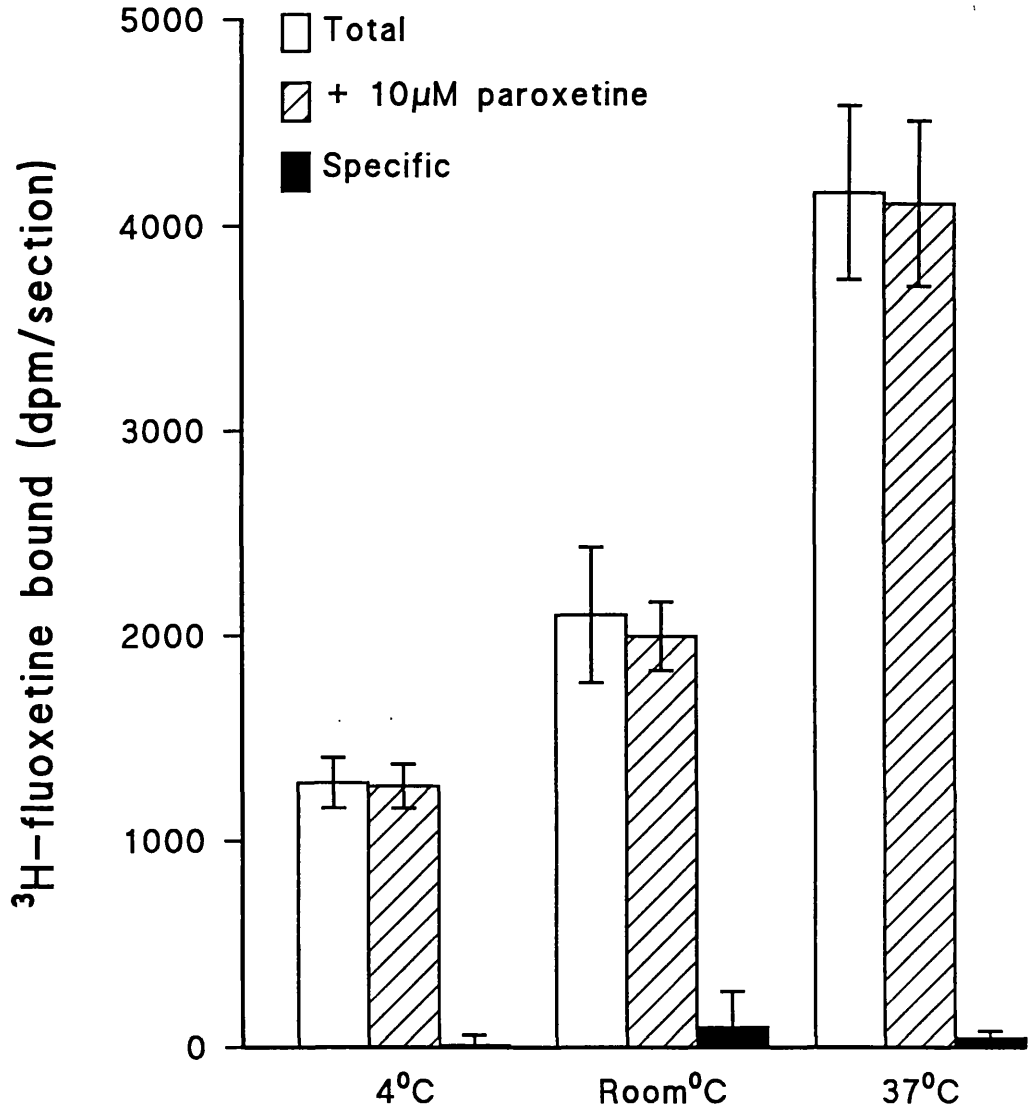


Fig 6.2. Effect of temperature on ^{3}H-fluoxetine binding in rat brain. Slide-mounted sections (10μm) were incubated with 1nM ^{3}H-fluoxetine for 90 minutes at 4°C, room temperature or 37°C. Results are the mean ± s.e.m. dpm of 3 sections per incubation jar. Experiments were carried out in duplicate. n = 3.

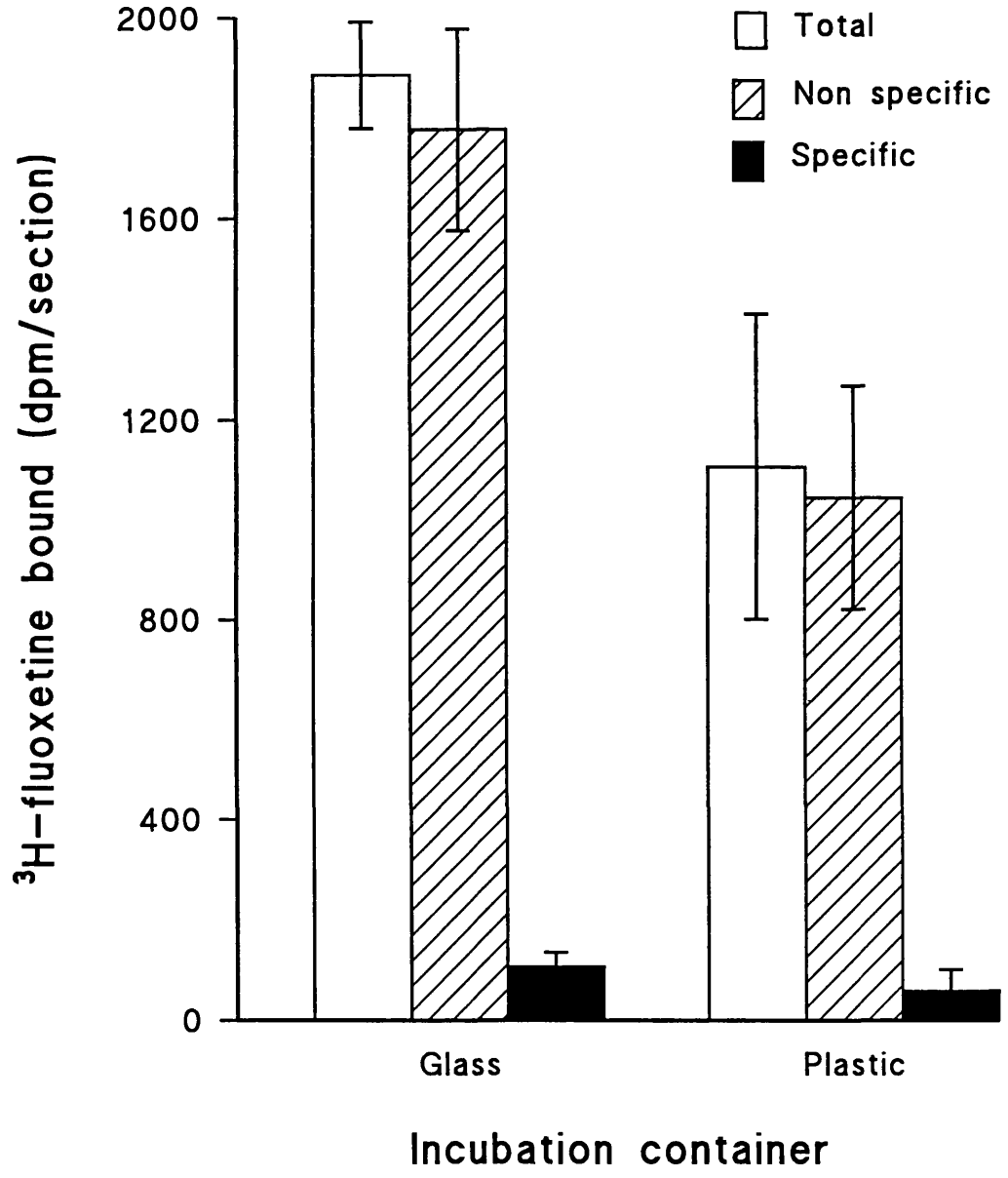


Fig 6.3. ^{3}H-fluoxetine binding to glass or plastic incubation containers. Rat brain sections were incubated with 1nM ^{3}H-fluoxetine for 90 minutes in plastic containers or glass Coplin jars. Results are the mean ± s.e.m. dpm of 3 sections per incubation jar. Experiments were carried out in duplicate. n = 3.

6.1.3. Membrane binding assay.

Binding of ^{3}H-fluoxetine to rat brain membranes was examined using a filtration assay. Initial experiments were performed to determine whether ^{3}H-fluoxetine bound to the filters used in the Brandell Cell Harvester. Pre-soaking filters in a 0.3% solution of polyethylenimine increased ^{3}H-fluoxetine binding in GF/F and GF/B filters (Fig. 6.4). The least amount of binding was found in GF/C and GF/B (untreated) filters, however the amount of radioligand binding to these filters in the absence of membrane samples was still high (approximately 4000 dpm).

Different tissue fractions of rat brain homogenates were tested to determine the subcellular localization of ^{3}H-fluoxetine binding (Fig. 6.5). In all fractions, the level of specific binding was low, with the exception of the whole brain (WB) samples. These were prepared by simply homogenising the whole rat brain in Tris-HCl buffer and then incubating samples with 1nM ^{3}H-fluoxetine. The nuclear fraction (P_1) appeared to have the highest levels of total and non-specific binding, however the values obtained from these samples varied widely in radioactivity content. It was decided therefore to use the P_2 (crude membrane) fraction prepared as described by Wong et al (1985).

Initial tests carried out using increasing protein concentrations of the P_2 membranes, showed that the amount of specific binding was similar in concentrations of 0.1, 0.3, 0.75 and 1mg/ml protein (Fig 6.6). In the 1.5mg/ml sample, the mean of the non-specific binding was higher than the total. As the 1mg/ml sample indicated a tendency towards a higher specific binding, this concentration of protein was used in subsequent experiments.

A saturation analysis of ^{3}H-fluoxetine binding to P_2 membrane showed that total and non-specific binding increased slightly from 0.1 to 2nM and then rose sharply to 10nM (Fig 6.7). This was not reflected in the specific binding however, which showed only a marginal increase with an increase in ^{3}H-fluoxetine concentration.

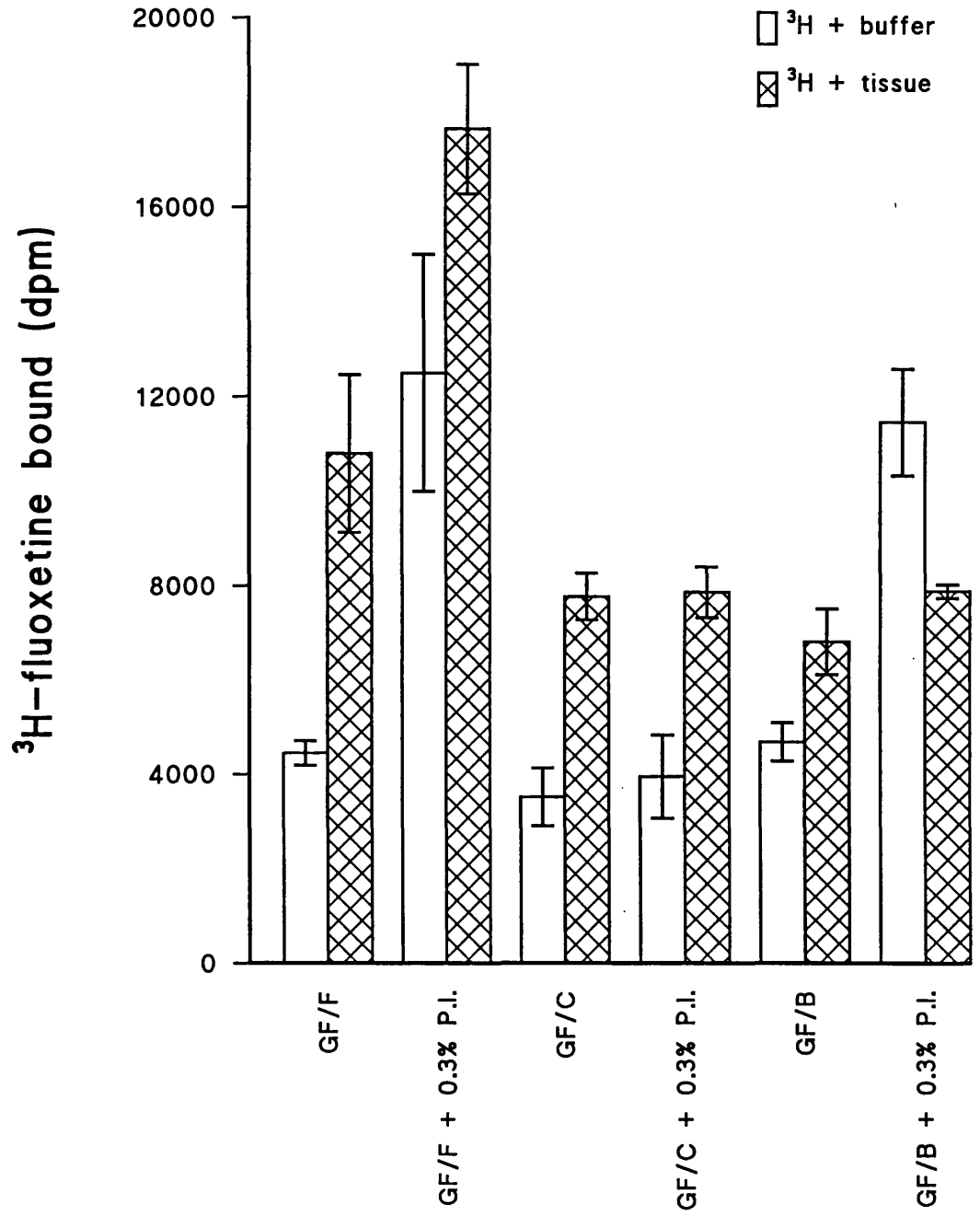


Fig 6.4. ^{3}H-fluoxetine binding to filters used in membrane binding experiments. 1nM ^{3}H-fluoxetine in the absence and presence of tissue homogenates (0.5mg/ml protein) was filtered through 3 different Whatman glass fibre filters using a Brandell Cell Harvester. The filters were either pre-soaked in 0.3% P.I. or left untreated. Results are the mean ± s.d. dpm of 6 circles of each filter type. Experiments were carried out in duplicate.

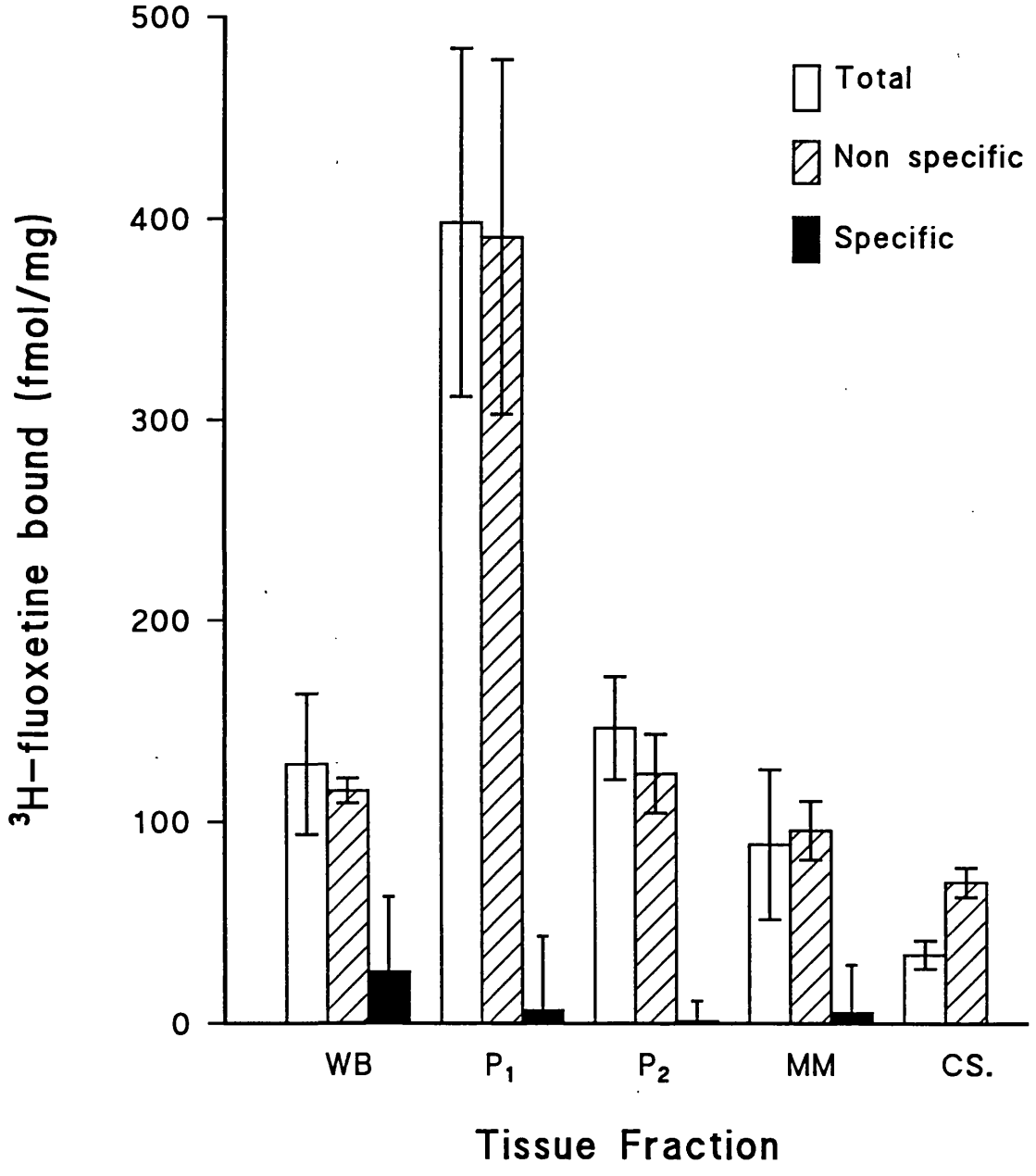


Fig 6.5. ^{3}H-fluoxetine binding in different brain tissue fractions. Total and non-specific binding was measured in whole brain (WB), mitochondrial/myelin membrane (MM), crude nuclear (P_1), crude membrane (P_2) and crude synaptic membrane (CS) fractions. Results are mean ± s.e.m. fmol/mg of 3 measurements per fraction. Experiments were carried out in triplicate. n = 6.

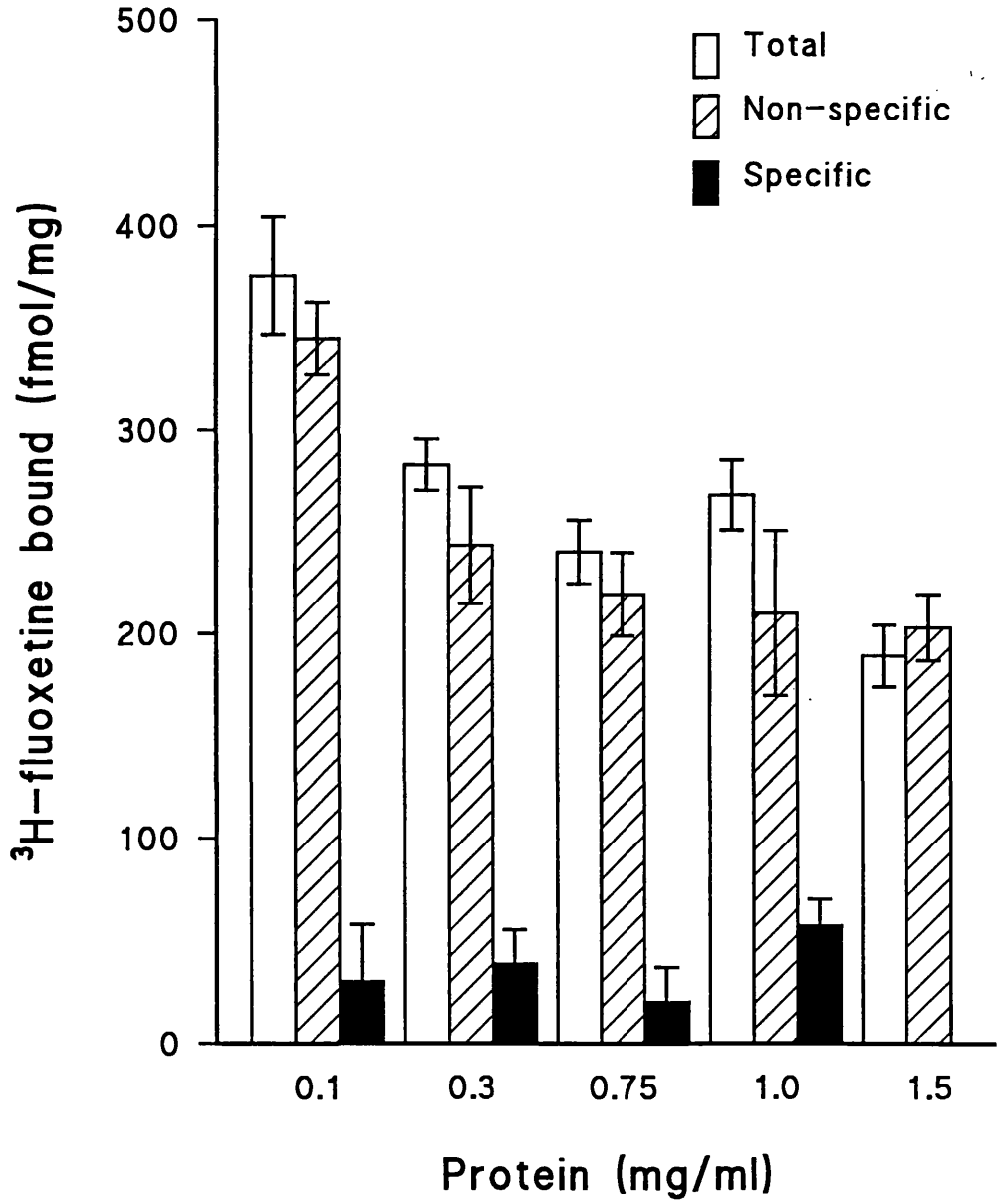


Fig 6.6. Effect of protein concentration on ^{3}H-fluoxetine binding. Increasing concentrations of crude membrane (P_2) aliquots were incubated with 1nM^{3}H-fluoxetine. Results are the mean ± s.e.m. fmol/mg of 3 measurements per protein concentration. Experiments were carried out in triplicate. n = 2.

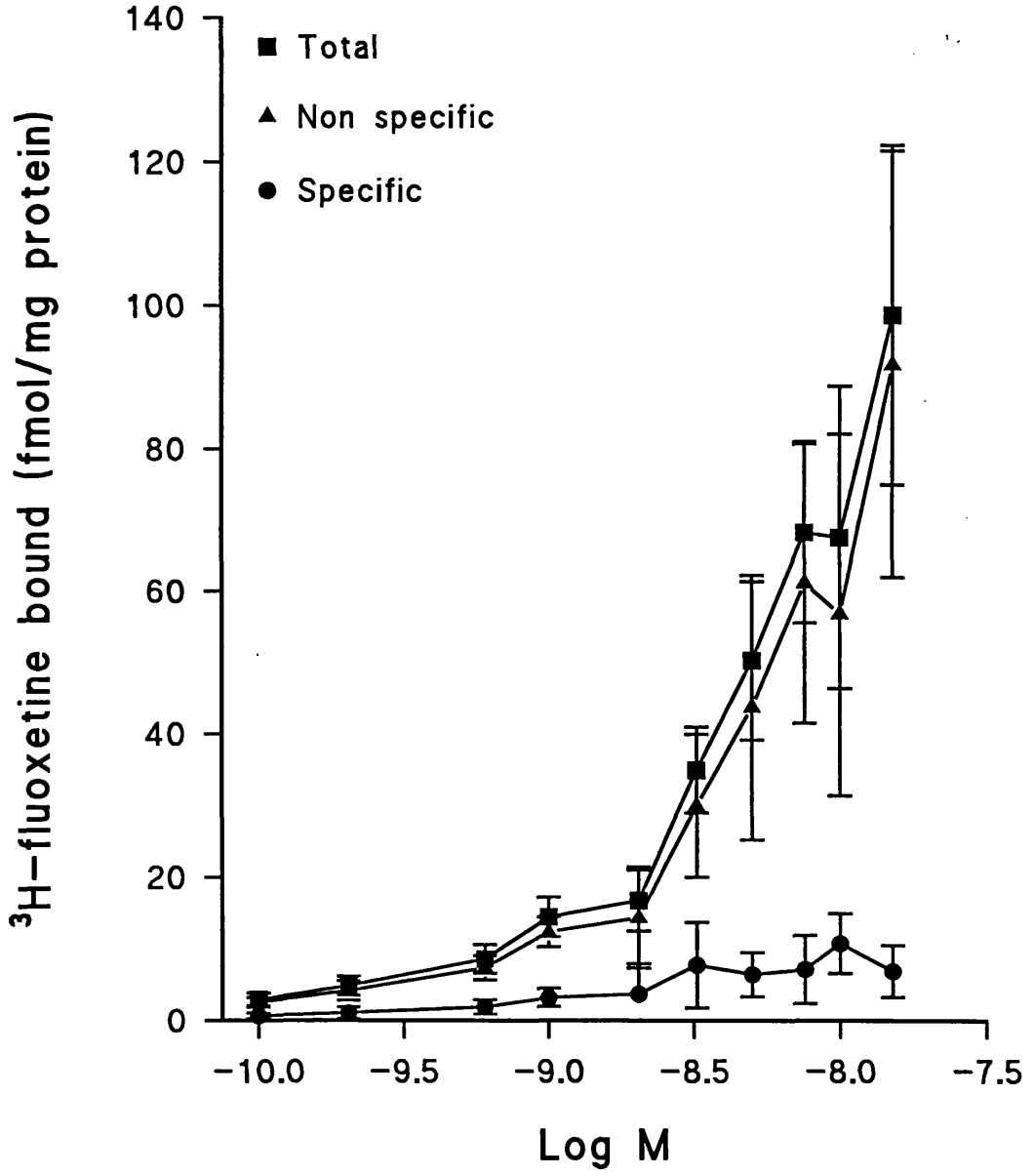


Fig 6.7. Saturation curve of ^{3}H-fluoxetine binding in rat brain membranes. P_2 membranes were incubated with increasing concentrations (0.1 - 15nM) of ^{3}H-fluoxetine. Results are mean ± s.e.m. fmol/mg protein of 3 measurements. Experiments were carried out in duplicate. n = 3.

6.1.4. Autoradiographic binding parameters.

Following a 90 minute incubation time, sections were washed twice during each time period (5 minutes to 4 hours). Regardless of the wash time, the total and non-specific binding was very similar and in some cases the non-specific binding measured was higher than the total binding (Fig. 6.8A). The only wash time which revealed a measurable specific binding (~ 20%) was at 5 minutes (2 x 2.5 minutes) and therefore additional experiments were performed with a range of wash times from 5 seconds to 5 minutes (Fig. 6.8B). In these experiments the specific binding originally noted was not present and once again there was no significant difference between total and non-specific binding.

Variations in incubation times were carried out, even though it was not possible achieve any specific binding, as it was conceivable that an incubation time of 90 minutes prevented excess ^{3}H-fluoxetine from dissociating from non-saturable sites even after 4 hours wash. Total and non-specific binding increased to similar extents throughout the range of incubation times without reaching any apparent equilibrium (Fig. 6.9). Taking into account the range of standard errors, however, saturation may have been achieved after 60 minutes. Specific binding levels were extremely low.

6.1.5. Saturation and inhibition in rat brain sections.

For all subsequent experiments, parameters of 15 minute pre-incubation, 90 minutes incubation and 2 x 60 minute washes were chosen.
Rat brain sections were incubated with increasing concentrations (0.01 - 10nM) of ^{3}H-fluoxetine. 2 slides per concentration were used for immediate measurement of bound radioactivity, while the other slides were apposed to film for 6 weeks. The saturation curve of ^{3}H-fluoxetine binding to brain sections showed that a small increase in specific binding was achieved with an increase in ^{3}H-fluoxetine concentration, however this was still only 10% of the total binding measured (Fig 6.10).

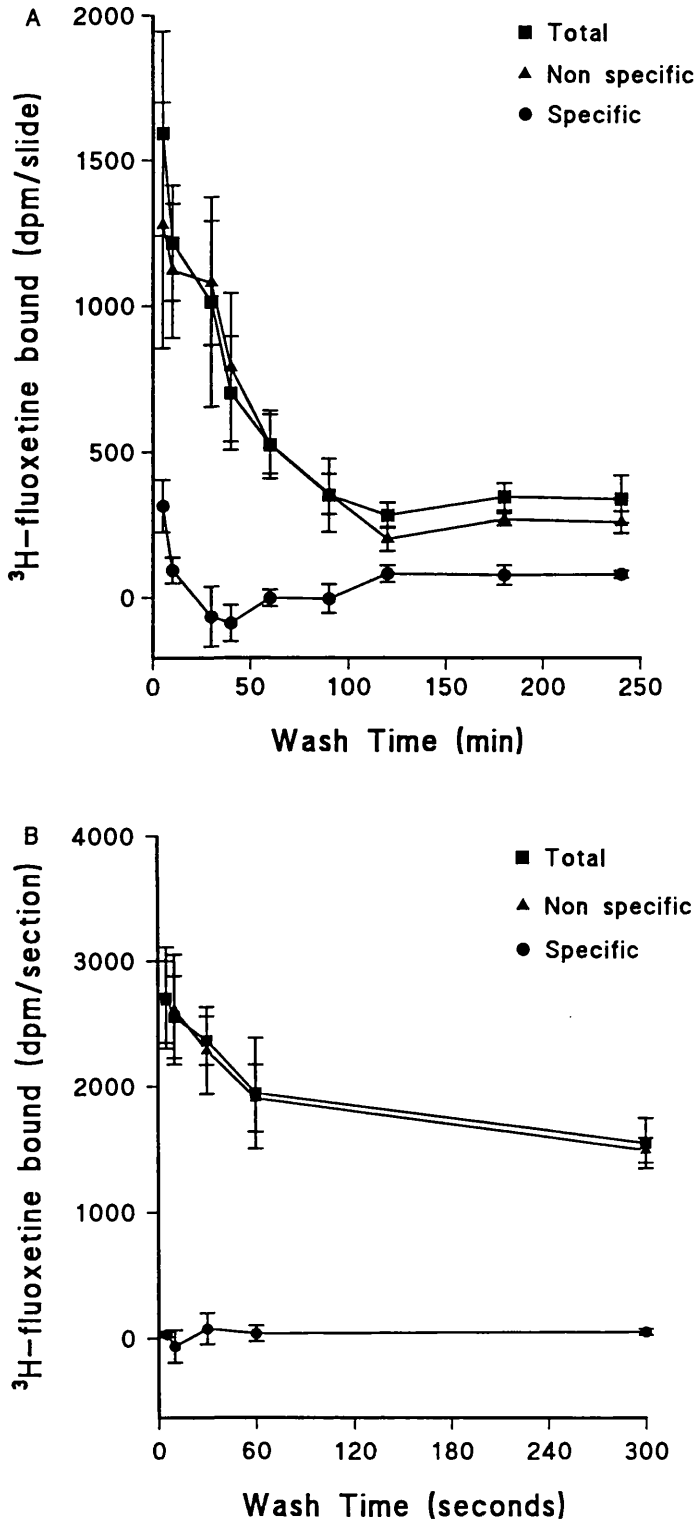


Fig 6.8. Optimum wash times for ^{3}H-fluoxetine binding in rat brain. Sections (10µm) were incubated with 1nM ^{3}H-fluoxetine for 90 minutes and then washed twice over a range of 5 to 240 minutes (A). Short wash times (5 - 300 seconds) were also carried out (B). Results are the mean ± s.e.m. dpm of 3 sections per incubation jar. Experiments were carried out four times. n = 6.

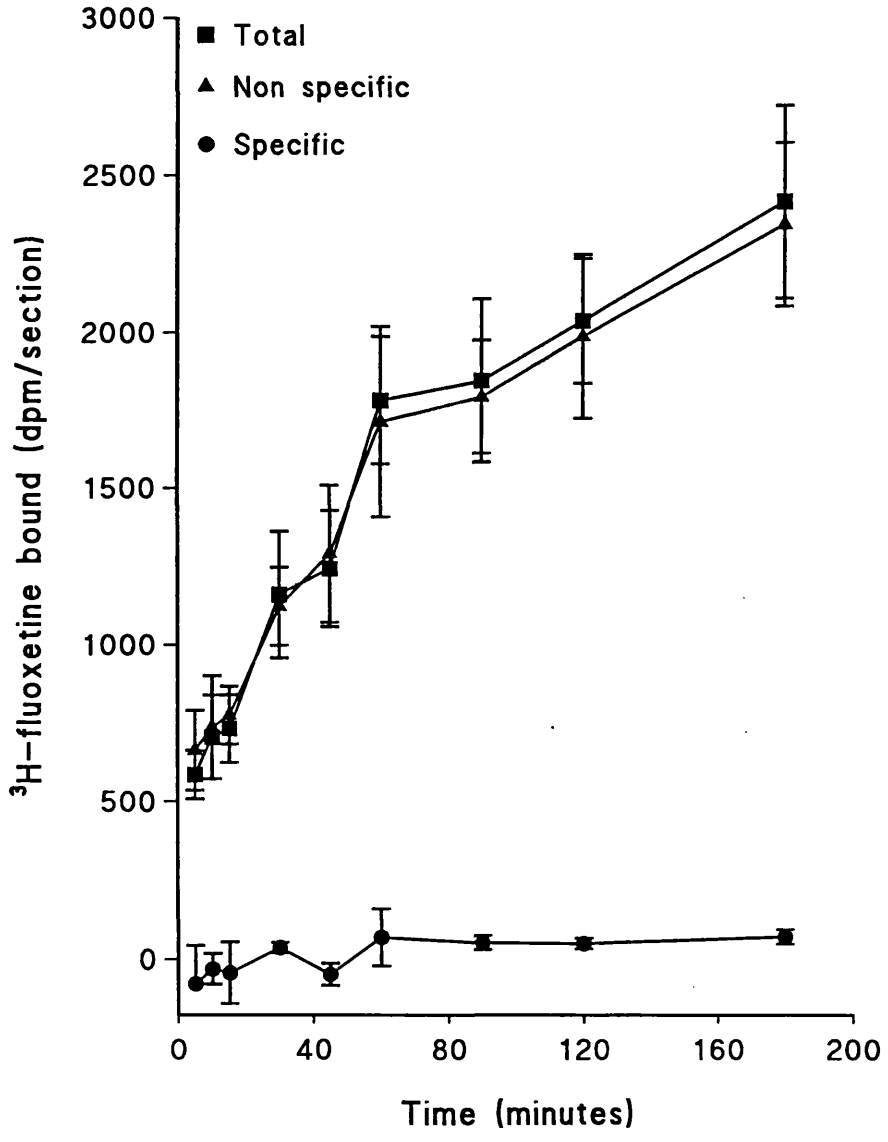


Fig 6.9. Optimum incubation times for ^{3}H-fluoxetine binding in rat brain. Sections (10μm) were incubated for 5 minutes to 3 hours with 1nM ^{3}H-fluoxetine and then washed for 2 x 60 minutes. Results are the mean ± s.e.m. dpm of 3 sections per incubation jar. Each experiment was carried out four times. n = 3.

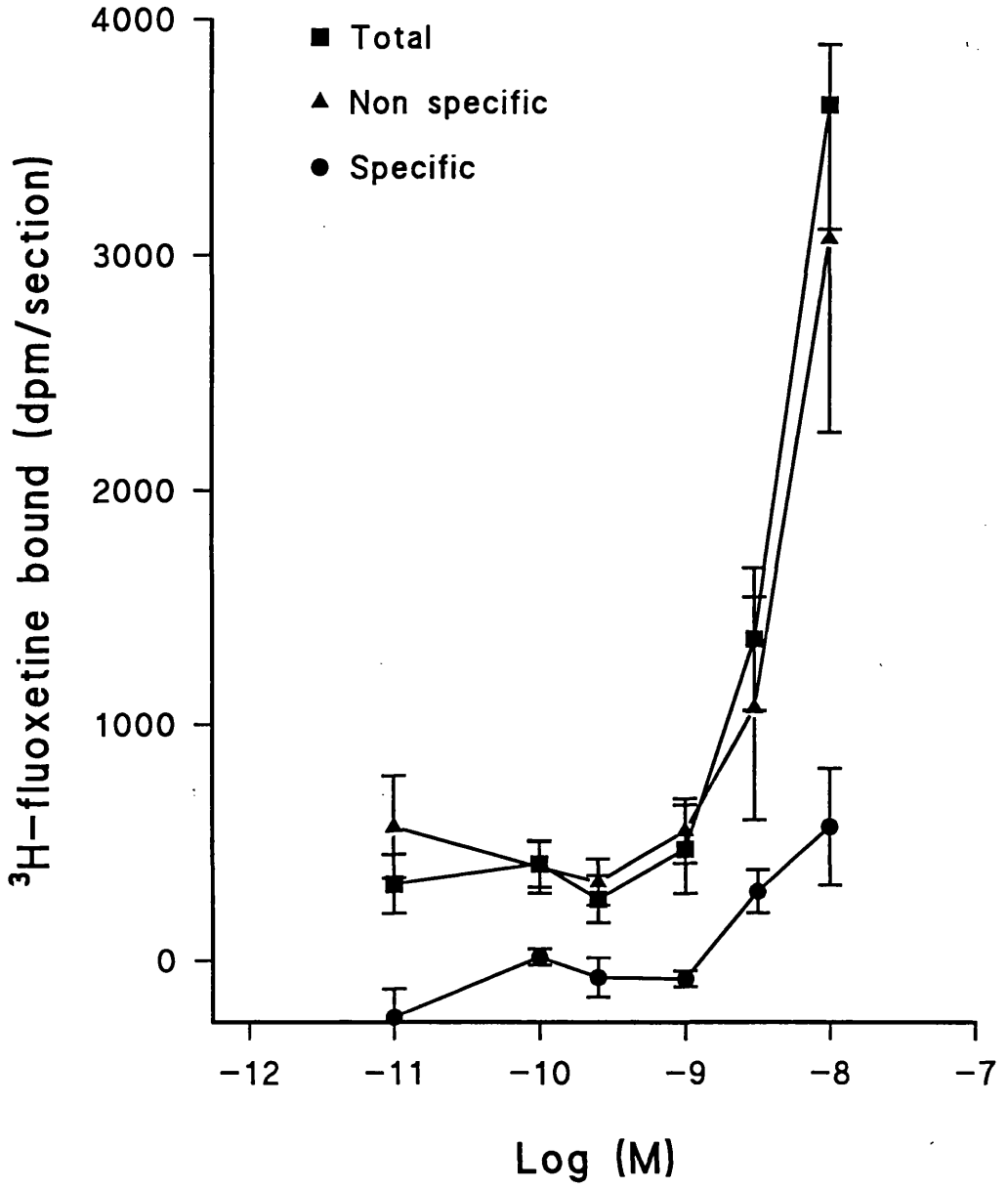


Fig 6.10. Saturation curve of ^{3}H-fluoxetine binding in rat brain sections. Sections were incubated with increasing concentrations (0.01- 10nM) of ^{3}H-fluoxetine. Results are mean ± s.e.m. dpm of 2 sections per incubation jar. Experiments were carried out in triplicate. n = 3.

Based on the assumption that fluoxetine, paroxetine, citalopram and sertraline will inhibit each other with an IC_{50} in the nanomolar range and therefore micromolar concentrations should completely abolish ^{3}H-fluoxetine binding, inhibition experiments were carried out to determine whether a high concentration (1 - 500 µM) of cold 5-HT re-uptake inhibitor was able to displace the high levels of non-specific binding. Only sertraline was capable of inhibiting ^{3}H-fluoxetine binding by more than 50% at a concentration of 100µM, while citalopram failed to produce an inhibition of more than 20%, regardless of the concentration used (Fig. 6.11).

6.1.6. Autoradiographic distribution.

Despite the negative results obtained thus far, an attempt was made to visualize the distribution of ^{3}H-fluoxetine binding in rat brain by autoradiography. Two different batches of ^{3}H-fluoxetine were used in these experiments.

^{3}H-fluoxetine binding to rat brain sections displayed a random pattern of distribution in experiments carried out using the first batch of ^{3}H-fluoxetine (Fig. 6.12A). The results of autoradiography carried out using the second batch of ^{3}H-fluoxetine did not show the "mottled" distribution of the earlier batch, however the distribution of binding sites was uniform across the entire brain section, with no selective binding seen in any brain areas (Fig. 6.12B). In particular, the cerebellum which is known to contain very few 5-HT re-uptake sites showed as many high density binding sites as areas which do contain a high density of 5-HT re-uptake sites.

Results of radiochemical purity analysis carried out on samples of ^{3}H-fluoxetine showed that they had become contaminated with impurities (purity <75%). At this point, experiments using ^{3}H-fluoxetine were suspended.

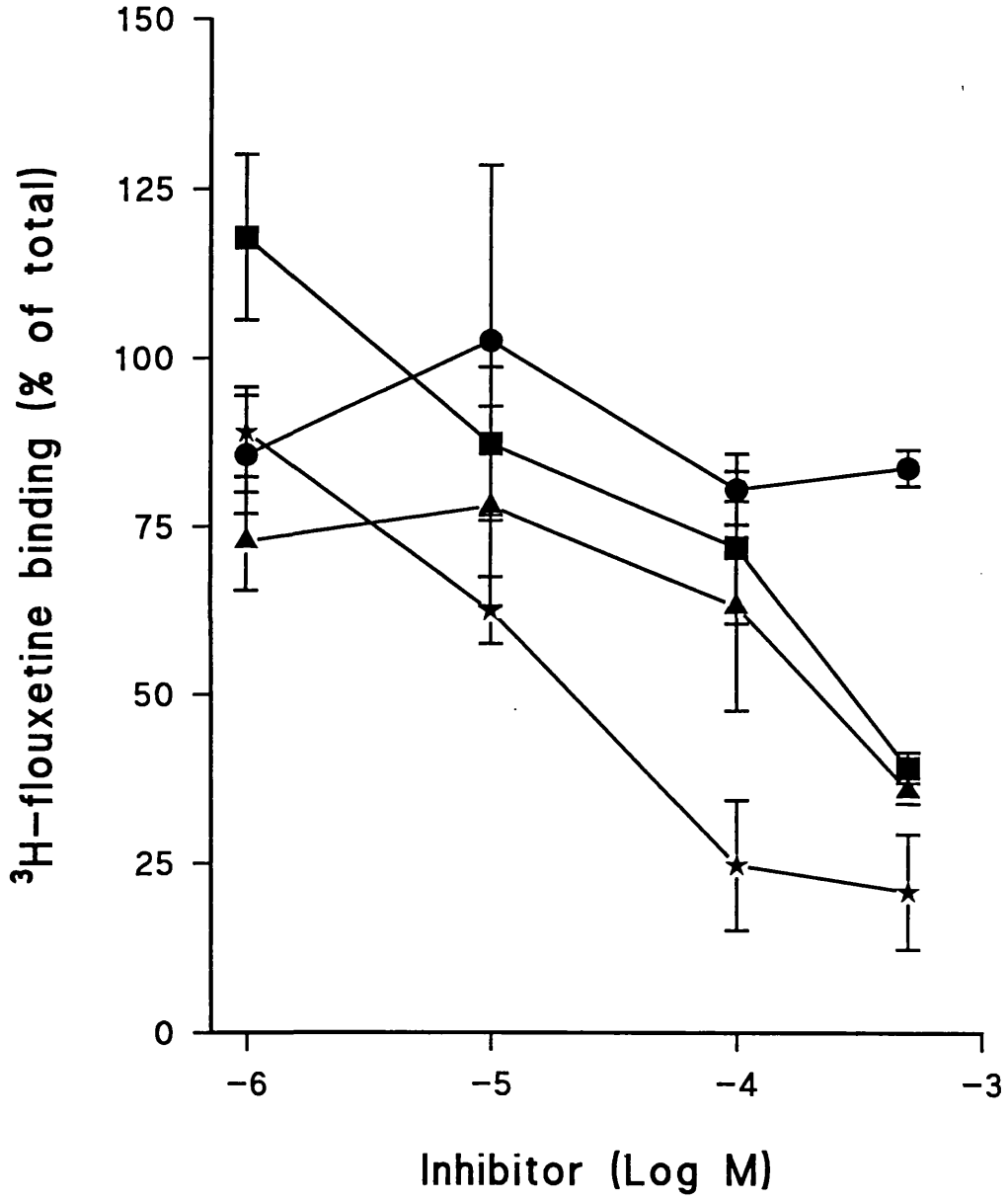


Fig. 6.11. Inhibition of ^{3}H-fluoxetine binding in rat brain. Sections were incubated with 1nM ^{3}H-fluoxetine for 90 minutes in the absence and presence of increasing concentrations (1 - 500μM) of cold fluoxetine (■), paroxetine (▲), citalopram (●) and sertraline (★). Results are expressed as the mean ± s.e.m. percent of total binding of 3 sections per incubation jar. Experiments were carried out in duplicate. n = 3.

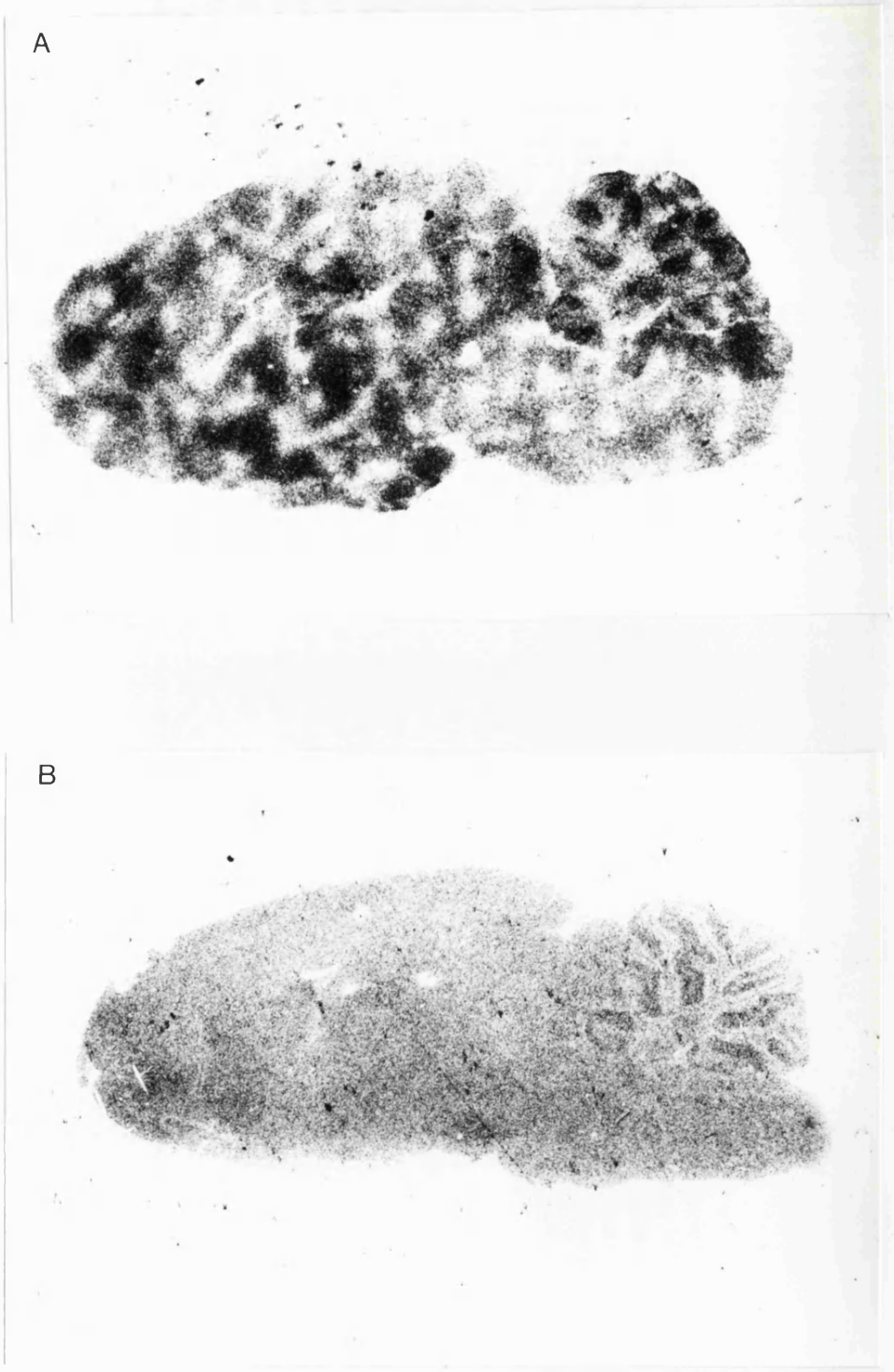


Fig. 6.12. Photographic visualization of ^{3}H-fluoxetine binding sites in rat brain sections. A - autoradiography performed using the first ^{3}H-fluoxetine batch. B - autoradiography performed using the second ^{3}H-fluoxetine sample.

6.2. EVALUATION OF ^{3}H-SERTRALINE AS A VIABLE LIGAND.

6.2.1. Light and Temperature sensitivity.

Total and non-specific ^{3}H-sertraline binding was unaffected by exposure to natural light (Fig. 6.13). Specific binding was found to be approximately 15% of total binding in natural light.

Incubation at 4^{0}C showed no specific binding (Fig. 6.14). Total and non-specific binding (defined by the inclusion of 10μM paroxetine) increased with an increase in temperature and the calculated specific binding was similar at both temperatures. Therefore all further incubations were carried out at room temperature.

6.2.2. Autoradiographic binding parameters.

A time course of increasing washes showed that from 5 to 40 minutes the non-specific binding was not significantly different to the total binding. Specific binding became measurable at 60 minutes and remained stable until 150 minutes and then decreased to zero (Fig. 6.15). The buffer was replaced half way through each wash. A wash time of 2 x 60 minutes was chosen.

A similar incubation profile was exhibited with total binding apparently higher than the non-specific binding at 5 minutes and then again from 30 to 120 minutes (Fig. 6.16). The highest specific binding was observed at 60 minutes and therefore this was chosen as the optimum incubation time.

At no time point was the difference between the total and non-specific binding statistically different. The lack of consistent total and non-specific binding levels led to the conclusion that the batch of ^{3}H-sertraline contained impurities and requests were made for re-testing.

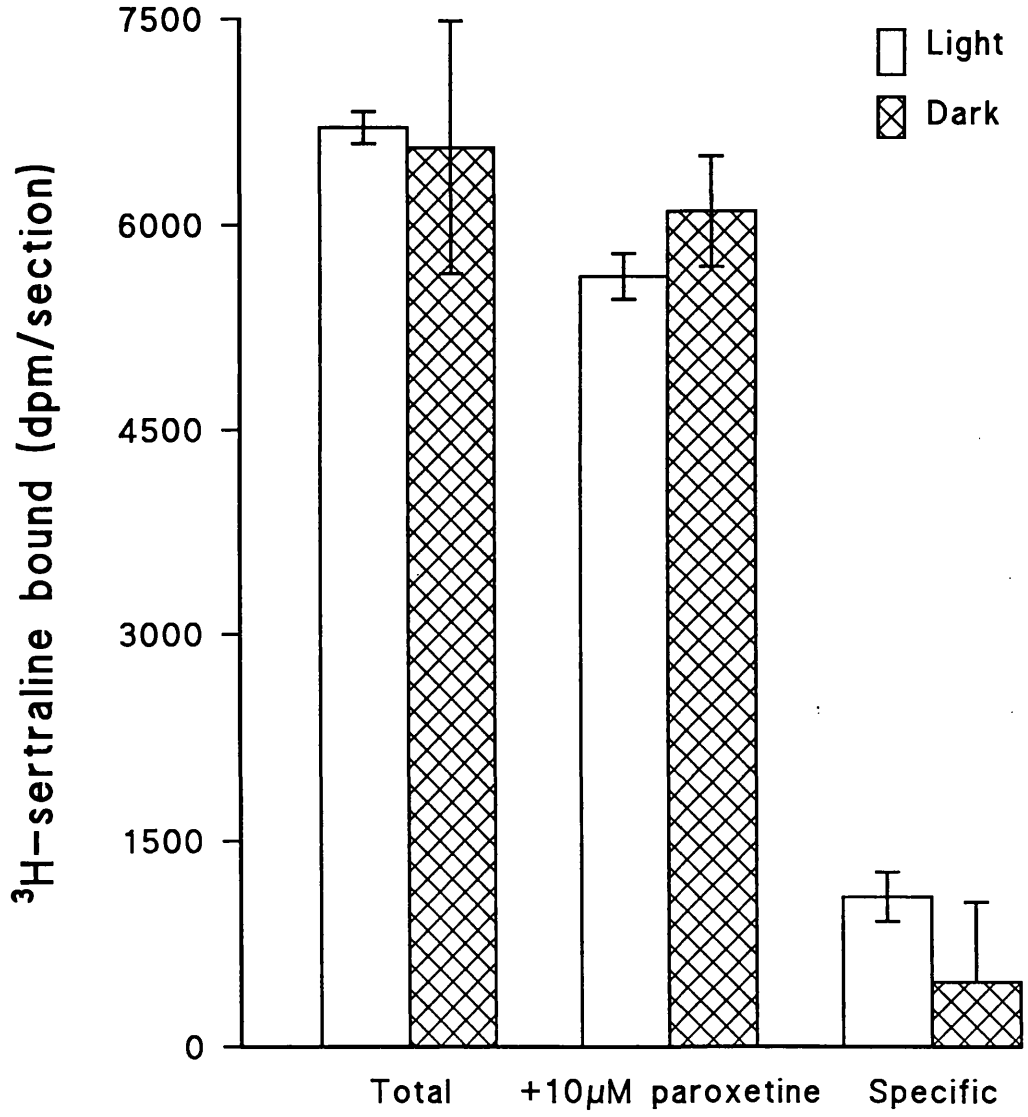


Fig 6.13. Light sensitivity of ^{3}H-sertraline binding to rat brain sections. Incubation with 1nM ^{3}H-sertraline was carried out under conditions of natural light or total darkness. Results are the mean ± s.e.m. dpm of 3 sections. Experiments were carried out in duplicate. n = 3.

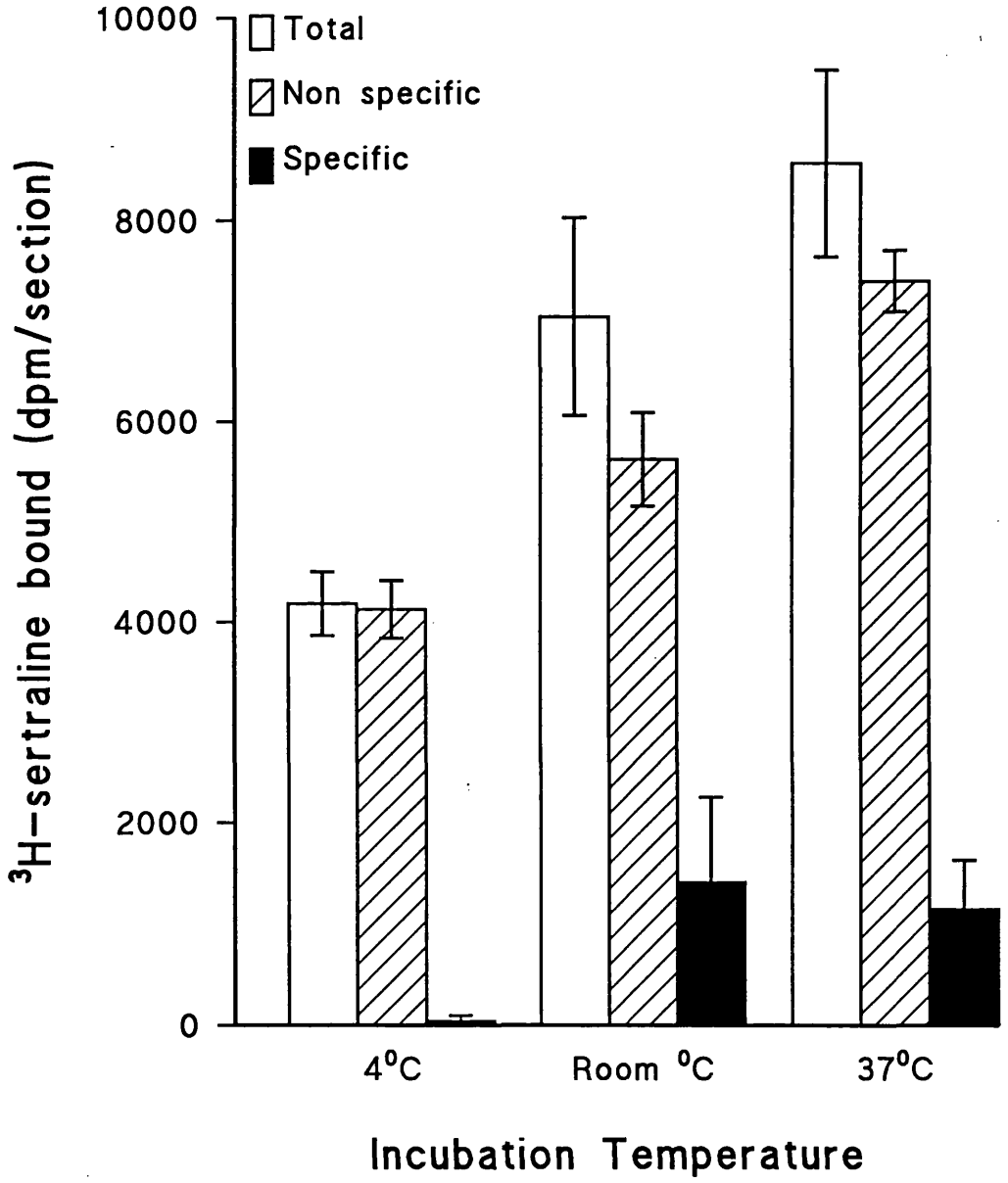


Fig 6.14. Optimum incubation temperature for ^{3}H-sertraline binding to rat brain sections. Incubations with 1nM ^{3}H-sertraline were carried out at 4^{0}C, room temperature and 37^{0}C for 1 hour. Results are the mean ± s.e.m. dpm of 3 sections. Experiments were carried out in duplicate. n = 3.

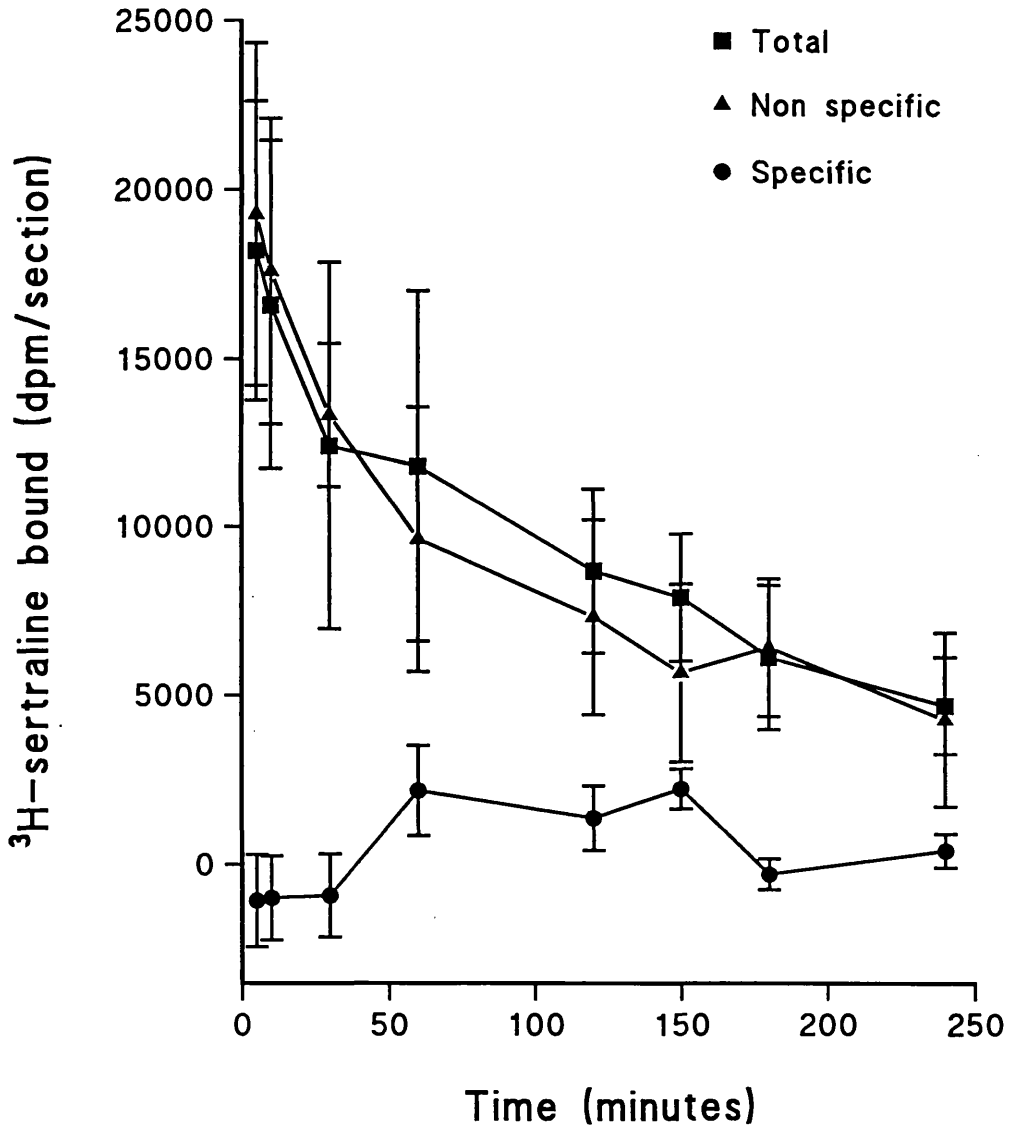


Fig 6.15. Optimum wash time for ^{3}H-sertraline binding. Rat brain sections were incubated with 1nM ^{3}H-sertraline for 1 hour and then washed for 5 to 240 minutes with a buffer change half-way through each wash. Results are the mean ± s.e.m. dpm of 3 sections. Experiments were carried out four times. n = 3.

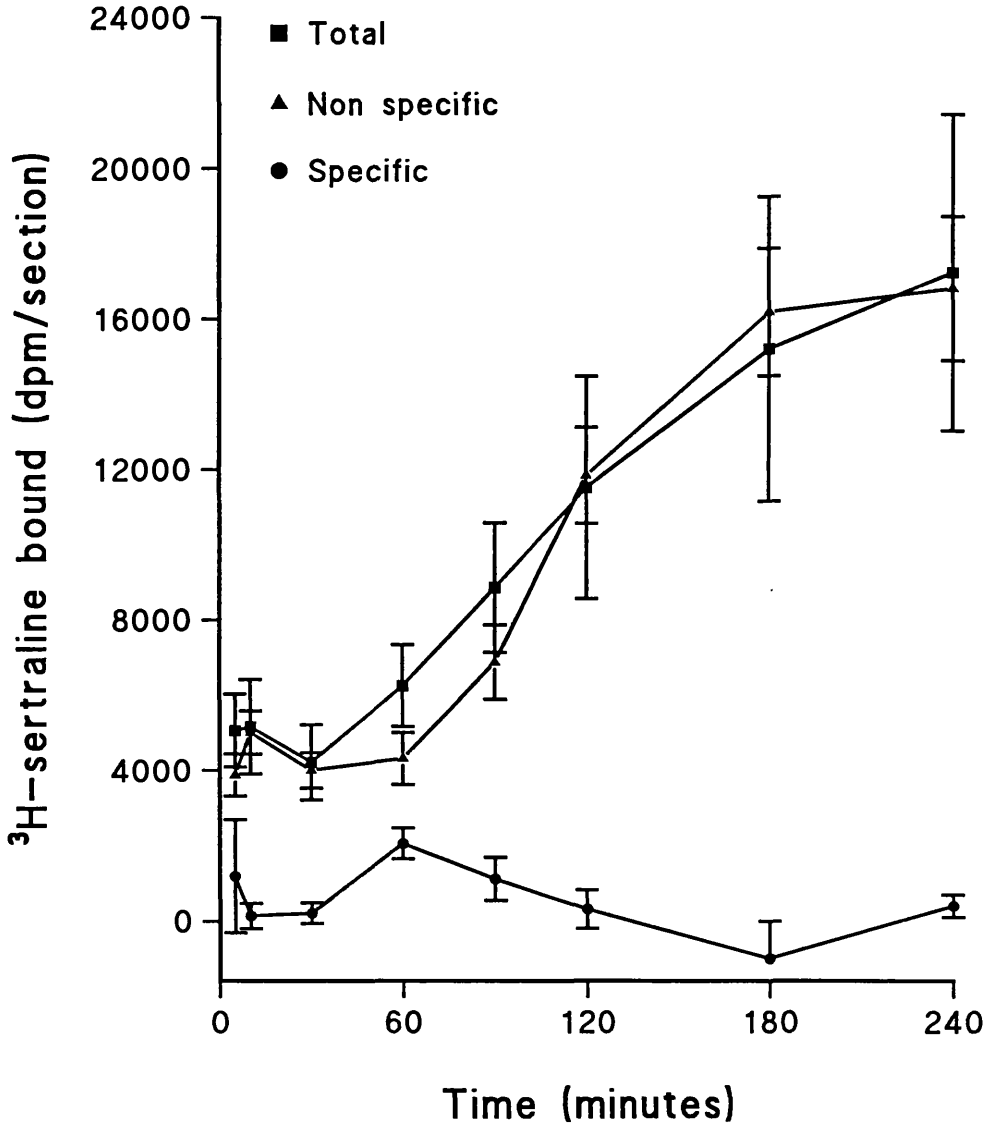


Fig. 6.16. Optimum incubation time for ^{3}H-sertraline binding. Rat brain sections were incubated for increasing time periods (5 - 240 minutes) and then washed for 2 x 60 minutes. Results are the mean ± s.e.m. dpm of 3 sections. Experiments were carried out in triplicate. n = 3.

6.2.3. Inhibition of ^{3}H-sertraline binding in rat brain membranes.

In the interim, experiments were carried out using increasing concentrations (1nM to 10μM) of cold sertraline, fluoxetine, paroxetine and citalopram to inhibit ^{3}H-sertraline binding in crude membrane homogenates (Fig. 6.17). At a displacer concentration of 10μM, inhibition of ^{3}H-sertraline was less than 40%. Only unlabelled sertraline appeared to inhibit ^{3}H-sertraline in a dose dependent manner.

6.2.4. Autoradiographic distribution.

The distribution of ^{3}H-sertraline binding sites was uniform throughout the rat brain and no regional selectivity of binding sites could be observed. In some sections a variation in binding levels could be observed, however the areas of high binding did not appear to correlate with known areas of high density 5-HT re-uptake sites and this was not consistent from section to section.

Purity testing of the original ^{3}H-sertraline sample, stored under optimum conditions, revealed that the radiochemical purity of the batch was 88.1%. 2 major impurities were observed, however these were not identified.

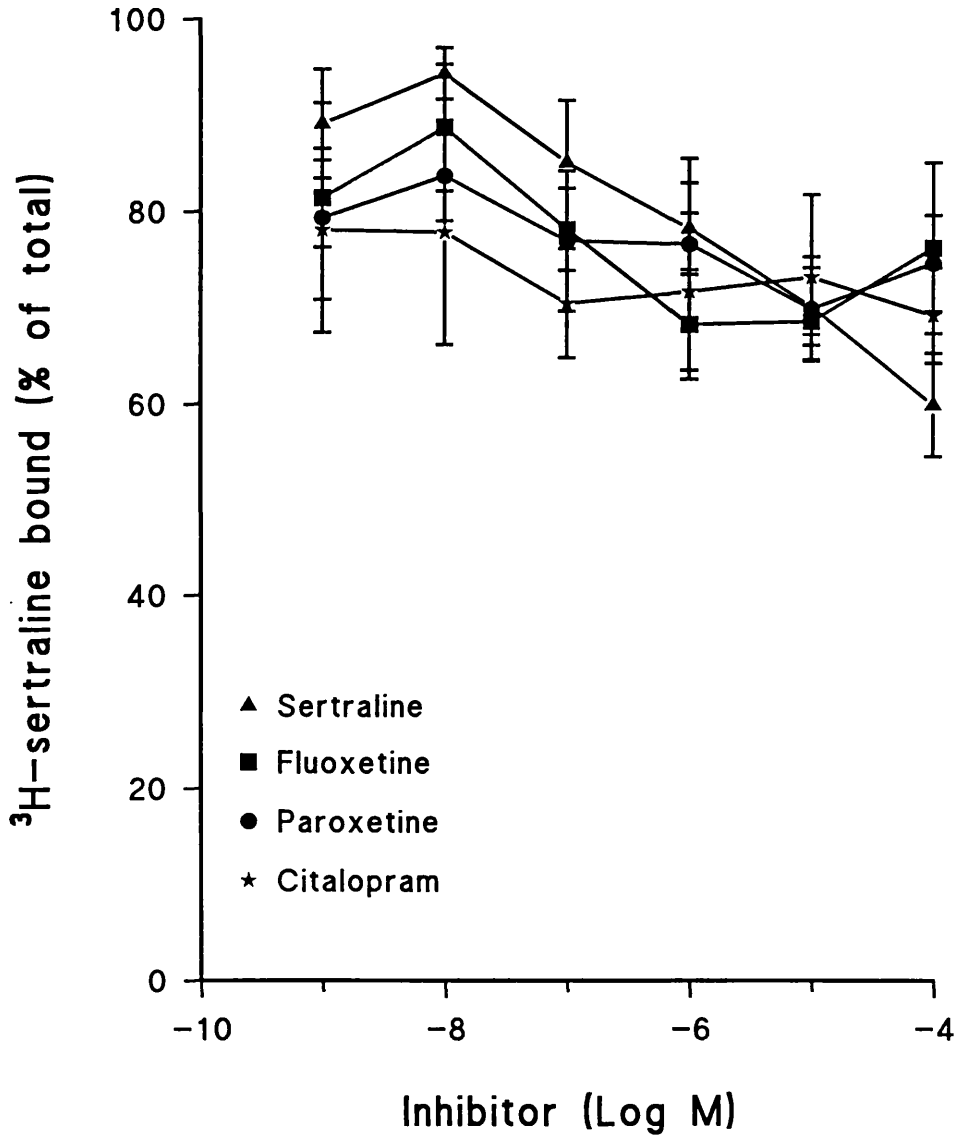


Fig 6.17. Inhibition of ^{3}H-sertraline binding to rat brain membranes. P_2 membrane homogenates were incubated with 1nM ^{3}H-sertraline in the absence and presence of increasing concentrations (1nM to 10μM) of cold sertraline, fluoxetine, paroxetine and citalopram. Results are the mean ± s.e.m percentage of total binding in the absence of inhibitors. Experiments were carried out in triplicate. n = 3.

DISCUSSION

When radioligand binding is measured by counting the radioactivity present in sections on slides, a small difference between total and non-specific binding is not unusual, as the average distribution of binding sites across a single brain section is being measured. If there are only a few areas of high density binding these values become masked by the general low levels of binding which may also be present in sections used to detect non-specific binding. Not every autoradiographic experiment works the first time and therefore this initial lack of measurable specific binding did not cause undue alarm.

A number of assumptions were made regarding the binding conditions of each ligand based on previous published reports using ^{3}H-fluoxetine (Wong et al, 1985) and ^{3}H-sertraline (Koe et al, 1990). These were:
a) The use of Tris-HCl (50mM, pH 7.4) buffer for initial experiments was acceptable as it is commonly used for binding to the 5-HT re-uptake site (D'Amato et al, 1987; Habert et al, 1985; Kovachich et al, 1988).
b) binding of ^{3}H-fluoxetine and ^{3}H-sertraline was sodium-dependent (neccessary for serotonin re-uptake; Mann and Hrdina, 1992; Wood 1987) and therefore 120mM NaCl was included in the Tris-HCl buffer.
c) the absence of a monoamine oxidase inhibitor (eg. pargyline) or other antioxidant in the buffers used by Wong et al (1983) and Koe et al (1990) indicated that ^{3}H-sertraline and ^{3}H-fluoxetine were chemically stable.

A 10μM concentration of paroxetine was used to define non-specific binding as it is considered to be the most potent of the SSRI's (Tulloch and Johnson, 1992) and was found to be most potent at inhibiting ^{3}H-sertraline binding (IC_{50} 0.70±0.07nM; Koe et al, 1990). It was concluded that a 10μM concentration would be sufficient to isolate any non-specific binding.

With an increase in washing time, the amount of total and non-specific binding should decrease to a point where any excess radioligand has been removed

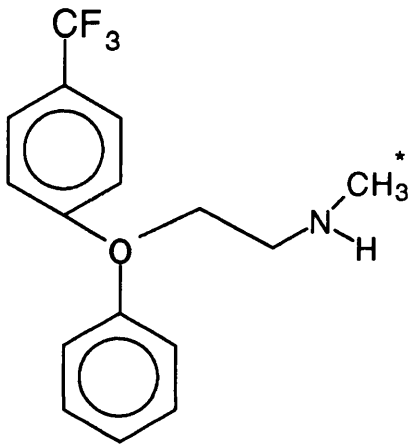
and the only remaining radioactivity is that which is bound to the 'receptor' sites. If the radioligand is prevented from binding to its recognition site by a high concentration of cold ligand, it will be washed away and the non-specific binding should reach a minimum level. The lack of any significant difference between the total and non-specific binding seen in the above experiments therefore appeared to be due to the inability of 10μM paroxetine to inhibit ^{3}H-fluoxetine or ^{3}H-sertraline binding within the brain section. This would suggest that the two radioligands were binding to sites other than the serotonin transporter.

Despite repeated attempts to produce workable experiments using ^{3}H-fluoxetine and ^{3}H-sertraline, the persistant lack of feasible results eventually led to the conclusion that this was the case. Since both drugs bind selectively to the serotonin re-uptake site, this would only be possible if the tritium label had become uncoupled from the ligands but was still binding to a recognition site, or if the original drugs had undergone some sort of degradation process. In both cases, synthesis of ^{3}H-fluoxetine and ^{3}H-sertraline was achieved by reduction of the N-methyl group with tritium gas (Fig 6.18). The primary metabolic step of fluoxetine and sertraline occurs via an N-demethylation process to form norfluoxetine and desmethylsertraline respectively (Doogan and Caillard; Murdoch and McTavish, 1992; Wong et al, 1975; Wong et al, 1993), which suggests that demethylation of either compound would be a fairly easy process. If this were to happen to ^{3}H-fluoxetine or ^{3}H-sertraline, the tritium label would be removed with the methyl group, leaving the parent compound as an unlabelled entity. This suggestion, however, is purely speculative, since no indication was given as to the structure of the impurities found during radiochemical purity testing.

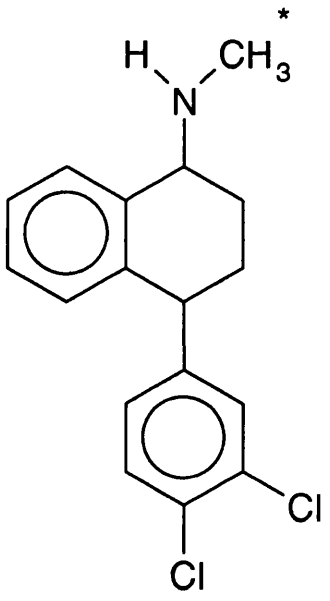
Since incubation with ^{3}H-fluoxetine and ^{3}H-sertraline was carried out in glass containers in 20ml of incubation medium, only a minimal proportion of the total ligand available would be bound to the sections at equilibrium, therefore it is very important to maintain a radiochemical purity of at least 95% and the

presence of any impurities would have a major effect on the binding site distribution. This was apparent in the photomicrographs of sections prepared for autoradiography.

The fact that so few published reports are available regarding the use of ^{3}H-fluoxetine and ^{3}H-sertraline, when both drugs are currently being prescribed for the treatment of depression in the United Kingdom, suggests that this problem with the stability of these two ligands is not a unique one.



Fluoxetine



Sertraline

Fig. 6.18. Site of tritium-labelling on the molecular structure of fluoxetine and sertraline. * indicates position of tritium label.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE DIRECTIONS

GENERAL DISCUSSION AND FUTURE DIRECTIONS

In the present study saturation and inhibition experiments of ^{3}H-paroxetine binding in rat brain have shown that ^{3}H-paroxetine binds with high affinity to a single binding site on the serotonin transporter. A detailed map of the distribution of ^{3}H-paroxetine binding sites was produced using quantitative autoradiography. Lesion experiments with 5,7-DHT have confirmed the selectivity of ^{3}H-paroxetine for the serotonin transporter in neuronal tissue. These results confirm that ^{3}H-paroxetine is a viable marker of 5-HT re-uptake sites.

The pattern of reduction in ^{3}H-paroxetine binding sites following direct injection of 5,7-DHT into the dorsal raphe indicated that 5-HT re-uptake sites were regionally affected within the brain. An explanation for this, is that innervation from the median raphe (spared from destruction by 5,7-DHT) will result in the presence of ^{3}H-paroxetine binding sites in areas which are affected by decreased dorsal raphe innervation. It is also possible that ^{3}H-paroxetine binding sites are selectively resistant to 5,7-DHT, as evidence of selective sensitivity of the 5-HT re-uptake sites to serotonergic neurotoxins has been suggested (Gobbi et al, 1990; Hensler et al, 1994).

In order to determine whether 5-HT re-uptake sites in certain brain structures are selectively resistant to 5,7-DHT, experiments would have to be carried out to compare the effects of site-specific injections of 5,7-DHT into the median raphe, dorsal raphe or both nuclei together. Further experiments comparing the effect of selective lesioning in the caudal and dorsal raphe on ^{3}H-paroxetine binding throughout the brain would provide greater detail as to the nature of the selective serotonergic innervation of these two areas. A comparative study using selective neurotoxins such as MDMA, PCA and 5,7-DHT under identical conditions of ^{3}H-paroxetine binding in autoradiography will be needed for further investigation into the sensitivity of ^{3}H-paroxetine binding sites to different neurotoxins.

The use of quantitative autoradiography as a means of determining alterations in ^{3}H-paroxetine binding sites has a number of advantages over membrane binding studies. Under procedure-induced circumstances, it is possible to measure differences in specific brain regions which are not apparent in membranes. Such is the case of tetanus toxin, where specific reductions in ^{3}H-paroxetine binding sites demonstrated the need for further investigation into the diffusion ability of tetanus toxin within the ventral hippocampus. It also indicated that tetanus toxin cannot be used as a region-selective neurotoxin for serotonin transport sites.

Mapping the distribution of ^{3}H-paroxetine binding sites in brain tissue using autoradiography does not allow for the discrimination between neuronal binding sites and binding to 5-HT uptake sites on astrocytes (Amundson et al, 1992; Whitaker et al, 1983). By using electron microscope autoradiography, Aghajanian and Bloom (1967) estimated that only about 5% of the grain clusters produced by i.v. injection of ^{3}H-5-HT were localised over glial cells, however Katz and Kimelberg (1985) showed that incubation with ^{3}H-5-HT produced grain clusters which were present above virtually all the cells in primary astrocyte cultures prepared from the cerebral cortex of neonatal rats. Therefore, given the widespread presence of glial cells within the brain (Young, 1994) it is entirely possible that the presence of ^{3}H-paroxetine binding sites on astrocytes during autoradiography contributes to at least some of the optical densities observed on the autoradiograms. Certainly, further investigation into the nature of ^{3}H-paroxetine binding sites on astrocytes in primary cell cultures is warranted. By comparing the distribution of ^{3}H-paroxetine binding sites with the *in situ* distribution of immunocytochemically stained glial cells using glial fibrillary acidic protein (GFAP) in brain tissue (Anderson et al, 1992), it would be possible to determine where astrocytes may play a role in ^{3}H-paroxetine binding site densities.

By developing a method of performing autoradiography in human whole brain sections, it was possible able to map the distribution of ^{3}H-paroxetine binding

sites in detail throughout the human brain. This creates a basis for further experiments comparing the distribution of ^{3}H-paroxetine binding sites in the brains from suicide victims with controls in order to ascertain whether the serotonin transporter is altered in some way in depression or suicide. However, to date, it appears that ^{3}H-paroxetine binding sites on the serotonin transporter are not altered in depression or in suicide (Andersson et al, 1992; D'haenen et al, 1988; Hrdina et al, 1993; Lawrence et al 1990a; Lawrence et al, 1993). These results are supported by recent evidence that the primary structure of the serotonin transporter is not altered during unipolar depression and manic-depressive illness (Lesch et al, 1995).

Since alterations in the B_{max} and K_d of ^{3}H-paroxetine during depression have only been studied in membrane binding experiments (Andersson et al, 1992; Hrdina et al, 1993; Lawrence et al, 1993), there still remains the possibility that changes do occur which can only be detected in selective brain regions through the use of autoradiography which allows a closer examination of the receptor site. This has been demonstrated in quantitative autoradiographic studies of the 5-HT_2 receptor and β-adrenoreceptor distribution in brains of suicide victims/depressives where anatomically selective alterations in radioligand binding sites have been observed in the cortex of suicide victims or depressives (Arango et al, 1990; Biegon and Israeli, 1987; Gross-Isserhof et al, 1990; Yates, et al, 1990).

Investigating depression with the use of postmortem brain tissue from suicides is complicated by the fact that subjects may have a history of drug treatment and not all suicide victims are depressed prior to death. Schizophrenia, personality disorder, drug abuse or a major grief event may result in suicide where depression is not a pre-disposing condition. In studies where postmortem tissue selection has been carefully monitored to include a large sample number of subjects with a confirmed retrospective diagnosis of depression and no history of recent antidepressant treatment, no difference has been found in either the 5-HT re-uptake site or 5-HT_2 receptor sites in the brains of the

suicide victims (Andersson et al, 1992; Lawrence et al, 1990a; Lowther et al, 1994). This is supported by evidence that ^{3}H-paroxetine binding was not altered in tissue from subjects who had committed suicide, yet a reduction in ^{3}H-paroxetine binding was observed in the hippocampus of subjects who had committed suicide and had schizophrenia (Dean et al, 1995)

Even though the ^{3}H-paroxetine binding site may not be altered during depression, the relationship between the distribution of ^{3}H-paroxetine binding sites and the distribution of other serotonin receptors has a direct bearing on the antidepressant action of paroxetine and other serotonin re-uptake inhibitors. If the consequence of inhibiting 5-HT re-uptake at the serotonin transport site is to enhance overall serotonergic transmission, then the question of how important the location of the serotonin transporter site is to the ultimate antidepressant action of these drugs is raised. It will be of interest therefore, to compare the distribution of ^{3}H-paroxetine binding sites with that of 5-HT_{1B} and 5-HT_{2} receptor binding sites in consecutive brain sections from controls and suicide victims. This should provide further information on; 1) the role of the 5-HT_{1B} receptor in depression, and 2) whether alterations in these receptors are confined to similar brain regions.

Evidence of low density ^{3}H-paroxetine binding sites in regions which are highly innervated by serotonergic projections, such as the frontal cortex, hippocampus and hypothalamus, raises the issue of a mismatch between the distribution of receptor sites and related neurotransmitters in the brain. A discrepancy in the distribution of post-synaptic 5-HT receptors and serotonin is to be expected as neurotransmitter molecules are most likely to be located on different neurons to post-synaptic receptor molecules. While both receptor sites and neurotransmitter molecules are found throughout the neuron (cell body, axon and terminals), the variability in neurotransmitter concentrations within the neuron may result in a mismatch between the density of receptor sites and content of neurotransmitter. Although a good match should be found between the distribution of pre-synaptic receptors and neurotransmitter, a mismatch may

occur when only one subtype of receptor is labelled. This becomes apparent when examining the distribution of 5-HT_1 receptors, where the rich serotonergic innervation of the hippocampus correlates well with a high density of 5-HT_{1A} receptors but not with the low density of 5-$HT_{1B/1D}$ receptors (Bruinvels et al, 1993; Hoyer et al, 1986a; Lowther et al, 1993; Waeber et al, 1988a). The mismatch between the distribution of pre-synaptic 5-HT re-uptake sites and 5-HT may therefore be the result of ^{3}H-paroxetine only labelling one type of 5-HT re-uptake site. However, a more likely explanation is that the method used in this study only investigates the distribution of high affinity ^{3}H-paroxetine binding sites, thus excluding the presence of low affinity binding sites in specific 5-HT re-uptake site binding measurements.

Although the serotonin transporter does not appear to be altered in depression or suicide, it has been implicated in a number of neurological conditions with a related incidence of depression. A reduction in ^{3}H-paroxetine binding to 5-HT re-uptake sites has been observed in postmortem brain tissue from patients with Parkinson's disease (Raisman et al, 1986); schizophrenia (Dean et al, 1995; Laruelle et al, 1993), alcoholism (Chen et al, 1991), and Alzheimer's disease (Chen et al, awaiting publication). By determining the distribution of ^{3}H-paroxetine binding sites in brains from patients with these disorders who do or do not present with concurrent depression, it may be possible to ascertain whether the relationship between the serotonin transporter and depression is dependent on prevailing neurological conditions.

While the method of autoradiography developed in the present study has been shown to be a successful and viable method of mapping the distribution of ^{3}H-ligand binding sites in human whole brain sections, the development of the technique raised a number of concerns which require further investigation and improvement. As discussed previously, it is essential to maintain the original structure of the brain during freezing. Once the brain is removed from the confines of the cranium, it tends to lose its shape and become distorted and therefore it is necessary to freeze the brain whole, in a "brain-shaped

container". Since brains vary in size and shape, it is possible that smaller brains will become more distorted during the freezing process. Furthermore, the potential for ice-crystal formation during freezing is much greater in whole brains as opposed to smaller separate brain sections. The use of cryoprotectant such as glycerol-DMSO is not possible as it requires prior fixation to allow sufficient time for the cryoprotectant to penetrate the tissue (Rosene et al, 1986). However, by pre-chilling the brains to 0°C and then immersing them in iso-pentane in a liquid nitrogen bath at a rate of 1cm per minute it is possible to minimize the risk of tissue fracture from ice-crystal formation (Toga et al, 1994).

Maintaining the symmetrical structure of the brain is essential for determining the distribution of binding sites in regions in the left and right hemisphere on an identical plane. It also facilitates the identification of brain regions from human brain atlases. It was found that there was no single brain atlas which provided the depth of detail required for this study, therefore a variety of atlases and references were used (see Section 2.2). By using a bandsaw to cut the frozen brain into 1.5 - 2cm slabs it will become much easier to cut symmetrical slices as discussed previously. Although many of the sections used in this study were not absolutely symmetrical, it was still possible to gain a far more accurate assessment of the spatial resolution of the areas being studied by using this method, than if isolated blocks of tissue were used.

Advances in computer-based 3-dimensional (3D) anatomic digital atlases of the human brain, using magnetic resonance imaging (MRI) or photographic images of histological sections, mean that determining the spatial resolution of a brain structure is possible. However these computerized atlases are still restricted by low resolution and lack of differentiation between anatomic subtypes (Hohne and Hanson, 1992; Kretschmann, 1989). Even with the development of high-resolution computerized serial imaging taken from the surface of consecutive brain sections (Toga et al, 1994), these 3D computerized human brain atlases are more suited to neurosurgical applications or analysis of techniques such as

PET. Furthermore, it will be some time before these facilities are commonplace in the laboratory.

In conclusion, in the present study a detailed map of the distribution of ^{3}H-paroxetine binding sites in both rat and human brain tissue was provided and ^{3}H-paroxetine was shown to bind to a single site on the serotonin transporter with high affinity. On the basis of this the effects of a direct injection of 5,7-DHT into the dorsal raphe on ^{3}H-paroxetine binding in the rat brain was determined. The lack of complete abolition of ^{3}H-paroxetine binding sites following 5,7-DHT injections raises questions as to whether the remaining ^{3}H-paroxetine binding sites are entirely due to innervation from the remaining portion of the dorsal raphe and median raphe or whether certain brain regions are resistant to the neurotoxic effects of 5,7-DHT. Investigations into the possible use of tetanus toxin as a site-specific serotonergic neurotoxin revealed that 5-HT re-uptake sites were reduced following injection of tetanus toxin into the ventral hippocampus, however the mechanism whereby this occurs is unclear. The intention of developing a novel method of using human whole brain sections for use with autoradiography was accomplished. By utilizing this method it was possible to determine the symmetry and distribution of ^{3}H-paroxetine binding sites in human whole brain coronal sections as well as establish the regional species differences of these binding sites in rat and human brain. Furthermore, development of this method of autoradiography provides the basis for a host of future radioligand binding and *in situ* hybridization experiments in both control and diseased human brains.

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