5HT RECEPTORS AND α_2-ADRENOCEPTORS; CLASSIFICATION, LIGAND BINDING, FUNCTIONAL CORRELATES AND ALTERATIONS IN CEREBRAL ISCHAEMIA.

A thesis presented for the degree
of Doctor of Philosophy

By

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FOR MY DAD

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Abbreviations

Term	Abbreviation
5-hydroxytryptamine	5HT
5-carboxamidotryptamine	5CT
Dipropyl 5-carboxamidotryptamine	DP-5CT
5,6 dihydroxytryptamine	5,6 OHT
8-hydroxy-2-(di-n-propylamino)tetralin	8-OH-DPAT
trifluromethylphenylpiperazine	TFMPP
Lysergic acid diethylamine	LSD
Cyanopindolol	CYP
[^{125}I]-Iodocyanopindolol	[^{125}I]-CYP
Adrenaline	A
Noradrenaline	NA
Dopamine	DA
N-ethylmaleimide	NEM
Phenylglyoxal	PGL
Desipramine	DMI
Guanosine 5-triphosphate	GTP
5-Hydroxyindole acetic acid	5HIAA
Human umbilical artery	HUA
Pig coronary artery	PCA
Concentration response curve	CRC
Cerebral spinal fluid	CSF
Electroencephalagram	EEG
Non-specific binding	nsb
Apparent dissociation constant of radioligand	Kd
Total concentration of binding sites	Bmax
Apparent dissociation constant of competitor	Ki
Concentration causing 50% inhibition of binding	IC_{50}
Rate constant of association	K_1
Rate constant of dissociation	K_{-1}
High performance liquid chromatography	HPLC

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Declaration

The experimental work and other research which is contained within this thesis was undertaken by myself. None of the material has previously been presented for any other degree. Some of the results have been published during the period of this study, details of which are given below.

Publications

Alps, B.J., Brown, C.M., Calder, C. and Kilpatrick, A.T. (1984). Brain dopamine levels following experimental cerebral ischaemia. Br. J. Pharmac., **84**, 449P.

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Brown, C.M., McGrath, J.C., Midgley, J.M., Muir, A.G.B., O'Brian, J.W., Thonoor, C.M., Williams, C.M. and Wilson, V.G. (1988). Activities of octopamine and synephrine stereoisomers on α-adrenoceptors. Br. J. Pharmac., **93**, 417-429.

Summary

This study investigates 5HT receptor and α_2-adrenoceptor subtypes. The principal technique used is radioligand binding. Where possible, functional correlates for the binding sites were examined. Once characterised the 5HT binding assays were used to examine the effect of cerebral ischaemia on 5HT neuropharmacology. Finally, preliminary investigations of a possible interaction of 5HT with α_2-adrenoceptors were undertaken.

1. Under carefully defined conditions, $5HT_1$ binding sites were identified on rat and gerbil brain membranes, using [^{3}H]-5HT as radioligand. $5HT_{1A}$ and $5HT_{1B}$ binding sites were characterised by 8 compounds that showed selectivity for the two subtypes (5HT, 5CT, 8-OH-DPAT, RU24969, buspirone, spiperone, mianserin and pindolol). 5CT and RU24969 yielded triphasic concentration response curves in the rat but not the gerbil cortex, providing evidence that [^{3}H]-5HT labels three distinct $5HT_1$ sites in these membranes. High affinity [^{3}H]-5HT binding to an uptake site or $5HT_{1D}$ binding site, was absent in the rat cortex. However, preliminary studies indicated that a fourth, distinct [^{3}H]-5HT binding site was present on bovine cortex.

2. More selective radioligands led to better definition of heterogeneity of $5HT_1$ binding sites. [^{3}H]-8-OH-DPAT and [^{3}H]-WB4101 were found to label $5HT_{1A}$ binding sites, predominantly in the hippocampus, characterised by high affinity for 5HT, 8-OH-DPAT and buspirone. The binding of [^{3}H]-8-OH-DPAT was sensitive to GTP, suggesting the ligand may act as an agonist. [^{125}I]-CYP (in the presence of 30μM isoprenaline) was found to label the $5HT_{1B}$ subtype showing high affinity for 5HT, cyanopindolol, and RU24969.

3. The pharmacological profile of $5HT_{1A}$ sites in the rat and gerbil appeared to be similar. Unlike human or pig, a $5HT_{1B}$ site was present on gerbil brain membranes, which was similar to the rat. A $5HT_{1C}$ binding site in the gerbil was not evident from these studies.

4. The $5HT_{1A}$ binding site in the hippocampus was significantly down-regulated following chronic dosing with an antidepressant, which suggests a possible role for this subtype in the aetiology of depression. 5HT release studies suggest the autoreceptor is likely to be a $5HT_1$ subtype as showed by high affinity for 5HT and 5CT whereas the $5HT_2$ ligand mianserin and the $5HT_3$ ligand MDL 72222 were inactive.

5. In peripheral tissue, a high affinity, low capacity [^{3}H]-5HT binding site was identified on rat parenchymal lung membranes, which showed high affinity for 5HT and 5CT. This binding site was distinct from the 5HT uptake site as it showed only low affinity for imipramine and desipramine. No specific [^{3}H]-5HT binding was found on guinea-pig or rabbit atrial membranes.

6. The kinetic and pharmacological characteristics of the binding of [^{3}H]-ketanserin, [^{3}H]-mianserin and [^{125}I]-LSD to brain membranes from rat and/or gerbil were studied. All three ligands labelled sites with the characteristics of the $5HT_2$ binding site, showing high affinity for $5HT_2$ antagonists and low affinity for 5HT.

7. [^{3}H]-LSD was found to label a $5HT_2$ receptor on human platelet membranes, the affinity of drugs to inhibit this binding correlated well with their ability to inhibit 5HT-induced platelet aggregation.

8. High affinity, saturable, reversible binding, which could be inhibited by $5HT_2$ antagonists, was identified in homogenates of pig coronary and human umbilical arteries, labelled by [^{125}I]-LSD. This binding site correlated well with the receptor mediating the response to 5HT in the isolated PCA.

9. A reliable, rapid and highly predictive method for selection of stroke-prone gerbils was developed, using a microscopic examination of the anastomosis between the anterior cerebral arteries in the circulus arteriosus. Isoelectric EEG recordings also proved useful for identifing stroke-prone animals.

10. Global unilateral ischaemia (up to 3h) and brief bilateral ischaemia with recovery was found to have pronounced effects upon the level of 5HT in two areas of the gerbil brain, frontal cortex and corpus striatum.

11. Unilateral cerebral ischaemia (3h) caused a significant ipsilateral decrease in the number of $5HT_2$ binding sites in the gerbil frontal cortex, labelled by [^{3}H]-ketanserin, with no significant effect on the affinity. However, unlike 3h postmortem tissue, the remaining binding sites showed high affinity for $5HT_2$ antagonists, and therefore were still viable.

12. Bilateral ischaemia (5min) with 72h recovery also caused a significant decrease in the number of $5HT_2$ binding sites. Quipazine-induced head shakes, mediated via a $5HT_2$ receptor, were observed in the gerbil. However, no significant decrease in head shakes was seen in animals 24 or 72h following 5min bilateral ischaemia.

13. Preliminary studies were undertaken to localise $5HT_2$ binding sites in the gerbil brain using autoradiographic techniques. The [^{125}I]-LSD binding was assessed to be specific as it could be displaced by 2μM methysergide.

14. Cerebral ischaemia (10min bilateral and 2h reperfusion or 5min bilateral and 72h recovery) was found to cause a significant increase in the number of $5HT_{1A}$ binding sites in the gerbil hippocampus labelled by [^{3}H]-8-OH-DPAT, [^{3}H]-WB4101 or [^{3}H]-5HT without an apparent change in affinity.

15. A number of $5HT_{1A}$ ligands (8-OH-DPAT, RU24969, methysergide and buspirone) showed higher affinity for the the α_2-adrenoceptor on rat hippocampus compared with rat cortex. The displacement curves had low Hill slopes.

16. No evidence of an interaction between α_2-adrenoceptor and $5HT_{1A}$ receptors could be found in the hippocampus. 5HT had no direct effect on the binding of [^{3}H]-yohimbine at concentrations up to $1x10^{-4}$ M and it had no modulatory effects at concentrations of 1 or 10nM.

17. Chemical modification experiments indicated that the amino acids, arginine and cysteine are essential for the binding of [^{3}H]-yohimbine to rat cortex and hippocampus α_2-adrenoceptors.

18. [^{3}H]-Yohimbine was found to label two binding sites in rat cortex and hippocampus, shown by the biphasic curves for prazosin, oxymetazoline, 8-OH-DPAT, methysergide and RU24969. Both binding sites appeared to be α_2-adrenoceptor-like. [^{3}H]-Idazoxan was found to label a subpopulation of the [^{3}H]-yohimbine sites, equivalent to the site with low affinity for prazosin (site B).

19. [^{3}H]-Yohimbine labelled only one site on human platelet membranes which showed some species differences to the rat brain α_2-adrenoceptor binding sites.

This study verifies and adds to the current knowledge of 5HT receptors, and shows that 5HT is implicated in alterations due to cerebral ischaemia. The study also adds to the increasing literature evidence for subtypes of α_2-adrenoceptors.

Chapter 1: INTRODUCTION

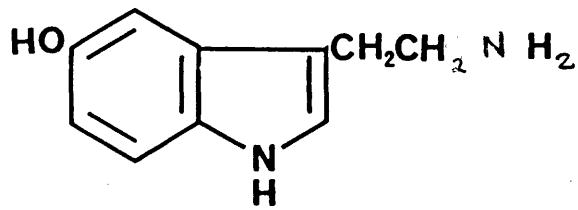
1. INTRODUCTION

1.1. General introduction on 5HT

1.1.1 Discovery and identification of 5HT

5HT was first identified as a vasoconstrictor agent present in clotted blood (Rapport et al, 1948). Originally termed serotonin, it is now generally referred to as 5-hydroxytryptamine (5HT;fig. 1.1) (Rapport, 1949).

Fig. 1.1 The chemical structure of 5HT



In mammals the main source of 5HT present in the body (about 90%) is found in the gastro-intestinal tract, where it was originally called enteramine (Erspamer and Asero, 1952; Stacey, 1966a). Although considerable amounts of 5HT are found in normal diet, the high level of 5HT in enterochromaffin cells does not arise as a consequence of 5HT uptake from the intestine but by "in situ" synthesis from tryptophan (Douglas, 1980). 5HT is continually released from enterochromaffin cells out into the gut lumen or into the portal circulation. The release into the circulation can be increased by either mechanical stimulation of the intestine or by pharmacological interventions, i.e. through the activation of serosal muscarinic receptors (for refs. see Forsberg and Miller, 1982).

1.1.2 Peripheral 5HT

Virtually all of the 5HT in the blood is contained in the thrombocytes (Stacey, 1966b). It has been estimated that 10^{12} platelets circulating in a 70 kg adult contain in total approximately 500μg of 5HT (Fozard, 1981). The total quantity of 5HT in an adult is approximately 10mg (Douglas, 1980). The quantity of 5HT in the blood represents only 5% of the total available 5HT. In addition to 5HT associated with thrombocytes, plasma also contains some free 5HT. This fraction has been calculated to account for 3-45μg/L (Crawford, 1963). Platelets do not synthesise 5HT but possess a high affinity uptake system, which allows a rapid and effective accumulation of 5HT from the surrounding plasma (Coppen et al, 1978; Pletscher, 1978).

The function of 5HT in platelets is obscure; one view is that platelets may simply remove the 5HT escaping from the enterochromaffin cells (Douglas, 1980). 5HT is released from platelets as a result of aggregation and promotes "platelet shape change" without inducing the "release reaction" (Douglas, 1980). The effect is small and reversible (Born, 1970 ; Graf and Pletscher, 1979) and is due to activation of specific receptors on the platelet surface ($5HT_2$) (Drummond and Gordon, 1975).

In addition to being in platelets and enterochromaffin cells 5HT is also present in the pineal gland, where it is mainly the percursor of melatonin. 5HT neurons have been identified in the peripheral nervous system, notably in plexuses innervating the gut. These neurons seem to have metabolic properties very similar to those observed in central 5HT neurons (Gershon, 1981).

1.1.3 Central nervous system

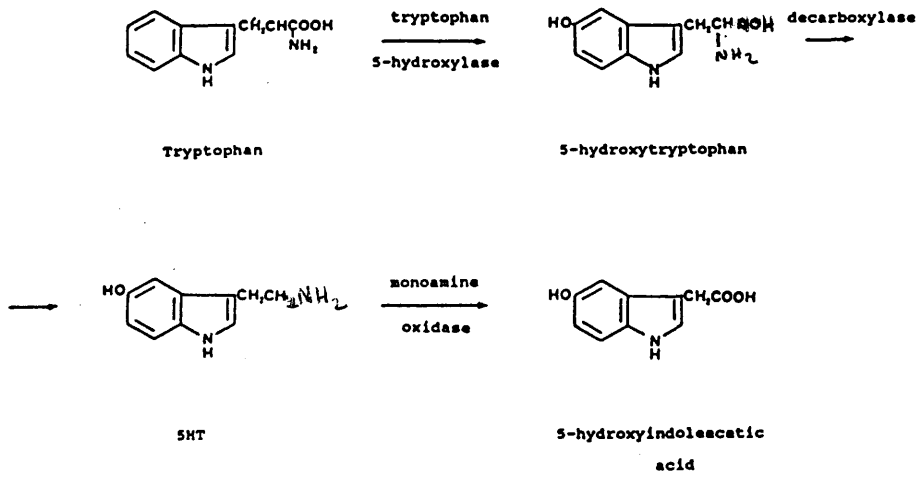
Considerable evidence exists to indicate that 5HT acts as a neurotransmitter.

1. 5HT is distributed heterogeneously within the brain (Dahlstrom and Fuxe, 1964).
2. The effect of iontophoretic injections of 5HT mimics the electrical stimulation of neurons containing 5HT

(Aghajanian et al, 1975).

3. 5HT is released from nerve endings in response to electrical stimulation (Gothert, 1982).
4. The monoamine is synthesised in nervous tissue and the main enzymes involved with its metabolism have been identified (see Green and Grahame-Smith, 1975), see fig. 1.2.

Fig. 1.2 5HT formation and metabolism



5. 5HT is inactivated by a high affinity neuronal uptake system (Shaskan and Snyder, 1970).
6. 5HT pathways have been described in the brain; they correspond to an organised neuronal system orginating principally from neurons in the raphe area and projecting to several brain regions (Fuxe et al, 1968; Kuhar et al, 1972 and Aghajanian et al, 1973).

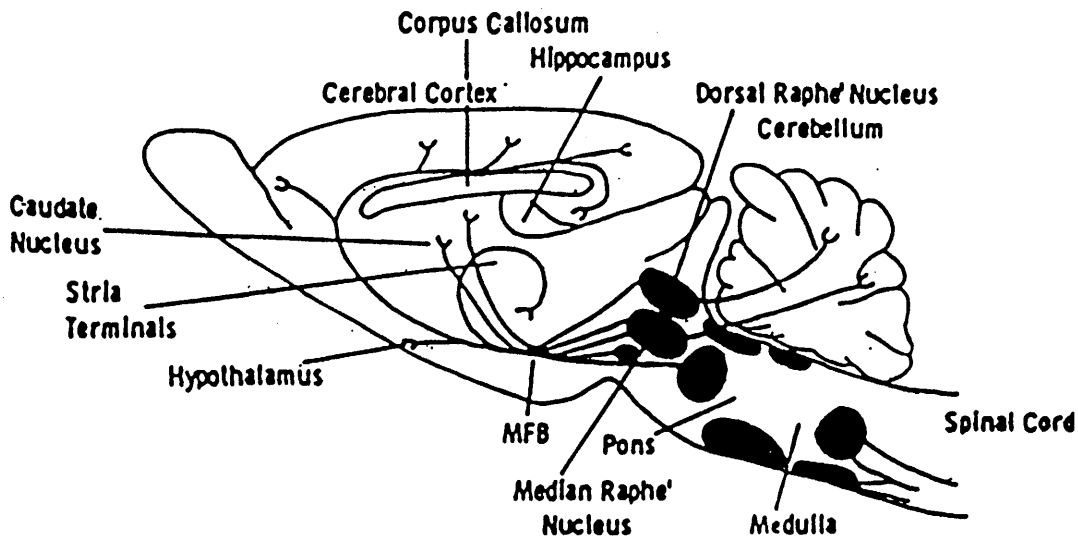
The density of 5HT terminals in the CNS is generally less than 5% and only a small percentage of these exhibit contact with other cells (Beaudet and Descarries, 1981). It has been suggested that the anatomical distribution of 5HT neurons in the CNS would be more consistent with 5HT being a neuromodulator than a neurotransmitter (Hamon et al, 1984a). Indeed 5HT neurons exert an influence on several central functions (appetite, memory,

thermoregulation, sleep, sexual behaviour, anxiety, depression and food consumption) without directly participating in the specific neuronal organisation responsible for the behaviour.

1.1.4 5HT pathways in the CNS

5HT is selectively contained in neurons with cell bodies mainly located in the midline area of the brainstem and terminals diffusely distributed throughout the CNS. The cell bodies of 5HT neurons supplying mainly the spinal cord are found in the posterior raphe nuclei, nucleus raphe obscurus, pallidus and magnus; whereas the projections innervating the forebrain regions i.e. striatum, hippocampus, hypothalamus, amygdala and cerebral cortex, belong to the anterior raphe nuclei, nucleus raphe dorsalis (DRN), centralis superior and linearis rosralis. A schematic representation of 5HT pathways is shown in fig. 1.3.

Fig. 1.3 5HT pathways in the rat brain



Aghajanian et al, (1970) identified pacemaker cells in the DRN with a slow, 1-2 spikes/sec, and highly regular

discharge pattern which could be completely suppressed by systemic administration of low doses (10μg/kg) of lysergic acid diethylamine (LSD) (a 5HT ligand; Gaddum, 1953). This suggested that the slow regular firing cells were serotonergic, since this type of activity was encountered only in areas of the brain known to contain 5HT cells.

5HT neurons in the DRN are autoactive as they are not influenced by the forebrain or hindbrain but controlled by local homeostatic responses, i.e. negative feedback. Adrenergic neurons have been shown to provide a tonic excitatory input to 5HT neurons in the DRN, whereas other neurotransmitters GABA, histamine, glycine and 5HT itself are inhibitory.

5HT, in order to produce its diverse effects on a number of physiological functions, is thought to bind to a number of specific receptors on the plasma membrane of the cell. The following section describes the present classification of 5HT receptors but first outlines the origins of receptor theory and the role played by ligand binding assays in determining receptor classification.

1.2. Receptor classification

1.2.1 The origin of the receptor concept

The concept of biological active substances (neurotransmitter, hormone, drug, etc) producing an action on an effector cell through an interaction with a specific protein (receptor) located on or within the cell has become widely accepted since the turn of the century. This concept is generally attributed to the work of Paul Ehrlich (1854-1915) and J.N. Langley (1852-1926). However, the French physiologist Claude Bernard (1813-1878), although not using the terminology of receptors "per se", was the first to demonstrate that functional efficacy of a drug depends on its access to a particular location. While studying the action of curare he postulated that the site of action was positioned between the nerve endings and the

muscle cell. Later Langley studying the action of curare and nicotine on striated muscle concluded that these compounds acted directly on the muscle cell through a receptor ("receptive substance"). He furthermore stated that the occurrence of receptors was widespread and that it was through this site located within the effector cell that most compounds exerted their physiological actions. Erhlich, who concentrated on selectivity of chemotherapeutic agents concluded with his dictum "Corpora non agunt nisi fixata" (Agents cannot act unless they are bound).

Therefore early evidence indicated that receptors are specific for particular drugs or endogenous substances and that through this selectivity various biological systems are controlled. By 1970, using classical pharmacological techniques and neurophysiological methods the existence of specific receptors for a variety of endogenous compounds, including neurotransmitters, was established.

Chemical neurotransmission depends upon synaptic communication mediated via neurotransmitters acting at specific receptor sites. The study of neurotransmitters was greatly advanced during the mid 1950's by the introduction of sensitive and specific assays for biogenic amines and the availability of radiolabelled biogenic amines and their precursors. Biochemical studies characterised mechanisms for neurotransmitter biosynthesis, storage, release and inactivation. By 1970 considerable experimental data on the catabolism and pharmacological profiles of various neurotransmitters was available yet still very little was known about neurotransmission at the molecular level in relation to its interaction with its receptor.

Classical pharmacological methods adopted for determining the affinity of agonists and antagonists were indirect and plagued by numerous experimental and analytical artefacts. However, during the 1960's it was shown that radiolabelled compounds could be used to directly label and define receptor binding sites. These binding sites exhibited the same pharmacological profile as

that observed for specific receptors in interactive studies (Jensen and Jacobson, 1962; Paton and Rang, 1965). This ability to determine directly the affinity of agonists and antagonists of the receptor has established ligand binding as a useful additional technique in the process of receptor classification.

1.2.2 Receptor binding assays

Paton and Rang (1965) pioneered the first attempts to study a neurotransmitter receptor at the biochemical level. To measure direct binding to slices of guinea-pig ileum [^{3}H]-atropine was used. Unfortunately because of the low specific activity of the [^{3}H]-atropine and the high levels of non-specific binding, the binding site was not characterised in great detail. The application of binding techniques to the nicotinic cholinergic receptor also provided early success. [^{3}H]-Muscarone was shown to bind to nicotinic cholinergic receptors in electric organs of the electric fish using centrifugation (O'Brian et al, 1969) and equilibrium dialysis (O'Brian et al, 1974) separation techniques. Shortly afterwards several groups successfully demonstrated specific binding of [^{125}I]-alpha-bungarotoxin to nicotinic receptors on membranes of the electric organ.

In the early studies virtually all drug binding involved non-specific sites and not pharmacologically relevant receptors. To detect specific binding and minimise non-specific binding required technical innovations; including the development of high affinity ligands, labelled to high specific activity which favours binding to receptors rather than to non-specific sites, improved membrane preparations and vacuum filtration methods for separating bound from free radioactivity. Vacuum filtration permits extensive but rapid washing of the membrane to remove free radioactivity and radioactive drug loosely bound to non-specific sites while retaining tighter bound drug-receptor complexes.

In vitro binding assays have in the last fifteen years been established for many neurotransmitter receptor systems and are the most direct way to characterise a receptor.

The specificity of the receptor system is achieved if binding sites are saturable, reversible and show the same pharmacological characterisation as the receptor, and are stereoselective. Assays can be designed to make quantitative as well as qualitative assessment of the binding site and the use of radioligands also makes it possible to study ligand-receptor kinetics. Receptor binding assays have been used to study the receptor during solubilisation, purification and reconstitution and more recently, as appropriate radioligands become available, to label membrane receptors on intact cells and to study receptor internalisation and other regulatory processes. Ultimately these experiments may lead to better understanding of how receptor occupation leads to the observed biochemical and physiological response. The technique has also been used to successfully label ion channels, second messenger systems and specific enzymes.

Direct binding studies can provide an accurate measurement of agonist and antagonist affinities but considerable caution must be taken to eliminate experimental errors and, perhaps more commonly, analytical artefacts (see Limbird, 1986). The importance of ensuring receptor specificity of the binding assays is emphasised by the ever increasing number of reports of radioligands binding to non-receptor material; e.g. [^{125}I]-insulin binding to glass tubes and talc (Cuatrecasas and Hollenberg, 1975) and specific histamine H_1 receptors on glass fibre filters (Bielkiewicz and Cook, 1985). Therefore it is crucial to verify the specificity of the binding site by direct comparison with functional studies.

1.3. Classification of 5HT receptors

1.3.1 History

The first studies on 5HT receptors concerned their pharmacological characteristics on guinea pig ileum and led Gaddum and Picarelli (1957) to postulate the existence of two receptor types, M and D, based on their sensitivity to

morphine (M) and dibenzyline (phenoxybenzamine)(D). In the following 20 years or so this proposal remained unchanged despite reservations concerning the selectivity of the antagonists employed. Phenoxybenzamine has been shown to block α-adrenoceptors (Drew, 1976), histamine and acetylcholine responses on smooth muscle and M receptors (Day and Vane, 1963).

The introduction of a more diverse approach to the study of 5HT mediated responses including ligand binding, electrophysiology, behavioural observations as well as measurement of smooth muscle contractions, has led to the conclusion that several types of 5HT receptors exist in the CNS and periphery. With such a diverse experimental approach, a number of classifications of 5HT receptors have been proposed. This has both aided and hindered our understanding of the vast amount of data generated in the last 10 years. The origin and relevance of some of these classifications, shown in table 1.1, will be discussed further.

Table 1.1. Proposed classifications of 5HT receptors

1. M and D classification (Gaddum and Picarelli, 1957)
2. $5HT_1$, $5HT_2$ binding classification (Peroutka and Snyder, 1979)
3. S_1, S_2 binding classification (Leysen et al, 1981)
4. S_1, S_2 electrophysiology classification (Aghajanian, 1981)
5. $5HT_1$ subtypes; $5HT_{1A}$, $5HT_{1B}$ subtypes (Pedigo et al, 1981). $5HT_{1C}$ subtype (Pazos et al, 1984a). $5HT_{1D}$ subtype (Heuring and Peroutka, 1987).
6. $5HT_3$ subtypes (Richardson et al, 1985)
7. $5HT_1$-like, $5HT_2$ and $5HT_3$ functional receptor classification (Bradley et al, 1986)
8. 5HT-ETMIC, 5HT-ETMIF and 5HT-IL subtypes have been proposed for the peripheral nervous system (Wallis 1981).
9. A,A',B,C,alpha and beta 5HT receptor types have been identified in molluscs (Gerschenfeld and Paupardin -Tritsch, 1974).

1.3.2 Ligand binding assays

Initial attempts to observe 5HT receptors directly by binding studies were performed by Marchbanks (1966). Several classes of low affinity binding sites were shown to exist with association constants in the order of $2x10^{6}$ M and $5x10^{5}$ M. Binding studies continued with equilibrium dialysis filtration using [^{3}H]-LSD as the radioligand (Farrow and Van Vunakis, 1972). However, the use of [^{3}H]-LSD as a ligand to label 5HT sites was limited by its lack of selectivity; as it was also found to label dopamine sites (Burt et al, 1976). Bennett and Aghajanian, (1974), using rapid filtration techniques for seperating membrane bound and free radioactivity, showed [^{3}H]-LSD binding to be saturable, reversible and display high affinity (Kd 7.5nM) for a 5HT site in the brain. The binding of [^{3}H]-LSD was also shown to be stereospecific, D-LSD >>> L-LSD. Moreover, [^{3}H]-LSD binding displayed regional variations with the highest levels of binding observed in the brain regions known to have a large 5HT innervation. Lesions of the raphe nucei, where 5HT neurons originate, had minimal effects on [^{3}H]-LSD binding, suggesting that the receptor is associated with post-synaptic membranes.

Bennett and Snyder (1975, 1976) carried out 5HT receptor binding in mammalian brain tissue using both [^{3}H]-LSD and [^{3}H]-5HT and detected high affinity saturable binding sites with both ligands. These early studies revealed notable differences between the [^{3}H]-LSD and [^{3}H]-5HT binding sites with regard to the density of binding sites, their regional distribution and affinity for a number of compounds. Two interconvertible 'states' of the 5HT receptor were proposed with [^{3}H]-5HT labelling the agonist 'state' and [^{3}H]-LSD, a mixed agonist/antagonist labelling both the agonist and antagonist 'states'.

[^{3}H]-Spiperone (a neuroleptic used to label dopamine receptors) was found to label a population of 5HT binding sites in rat frontal cortex with the same pharmacological profile as those labelled by [^{3}H]-LSD in the cortex (Leysen and Laduron, 1977, Leysen et al, 1978), but

different to those labelled with [^{3}H]-LSD in other brain areas. In theory if all three ligands labelled the same population of receptors the cold ligands would in displacement studies give similar affinities and displacement slopes. In fact, antagonist affinity values derived for [^{3}H]-LSD were found to be intermediate between those for [^{3}H]-5HT and [^{3}H]-spiperone, suggesting that [^{3}H]-LSD binds to sites labelled by both [^{3}H]-5HT and [^{3}H]-spiperone. However, [^{3}H]-LSD binding sites, determined in the presence of 300nM 5HT, appear identical to the sites labelled by [^{3}H]-spiperone. As a result of these findings Peroutka and Snyder (1979) proposed subdivisions of the 5HT binding sites as an alternative to the two 'state' receptor model. Binding sites for [^{3}H]-5HT in membranes from rat cerebral cortex were designated as $5HT_1$, whereas binding sites for [^{3}H]-spiperone were designated $5HT_2$ (Peroutka and Snyder, 1979; Hamon et al, 1981; Leysen et al, 1981). This subdivision was therefore initially based on the distinct binding properties of the two sites.

Defining the $5HT_1$ binding site was originally based upon the order of potency of the 5HT indole related agonists (the usual order obtained being 5HT > 5-methoxytryptamine > 5,6-dihydroxytryptamine > tryptamine), or the weak inhibition by classical 5HT antagonists. Some antagonists, such as spiperone, show shallow Hill slopes which was interpreted by Pedigo, Yamamura and Nelson (1981) as evidence for multiple $5HT_1$ binding sites. This gave rise to the subdivision of $5HT_1$ binding sites into $5HT_{1A}$ sites and $5HT_{1B}$ sites.

$5HT_{1A}$ sites have high affinity for spiperone (2-3nM), predominate in the frontal cortex.

$5HT_{1B}$ sites have low affinity for spiperone (35000nM), predominate in the corpus striatum.

The existence of multiple $5HT_1$ binding sites was confirmed by other groups (Nelson et al, 1980; Cortes et al, 1984; Pazos et al, 1984a,b) and verified by the

discovery and introduction of the specific $5HT_{1A}$ ligand 8-OH-DPAT (8-hydroxy-2-(Di-n-propylamino)tetralin) (Middlemiss and Fozard, 1983).

The introduction of [^{3}H]-8-OH-DPAT as a specific radioligand (Gozlan et al, 1983) has greatly aided the study of $5HT_{1A}$ sites. Other ligands used include; [^{3}H]-LSD, [^{3}H]-metergoline [unsuitable because of its high level of non-specific binding (Hamon et al, 1981)] and [^{3}H]-WB 4101 (Norman et al, 1985).

Characterisation of $5HT_{1B}$ sites was aided by reports that some β-blocking agents have high affinity for 5HT recognition sites (Middlemiss et al, 1977; Nahorski and Willcocks, 1983). In 1985, Hoyer and colleagues introduced radiolabelled iodocyanopindolol ([^{125}I]-CYP) as a specific ligand for the $5HT_{1B}$ recognition site (Hoyer et al, 1985a). RU24969 (5-methoxy-3-[1,2,3,6-tetrahydropyridon-4-yl]1H-indole) (Euvrard and Boissier, 1980) and 5CT (5-carboxamidotryptamine) (Saxena and Verdouw, 1985) show high affinity for this site, although they are non-selective. The piperazine, TFMPP (1-(3-trifluoromethyl-phenyl)piperazine) exhibits some selectivity for the $5HT_{1B}$ binding site (Stills et al, 1984).

A third subtype, the $5HT_{1C}$ binding site, was first characterised in membranes from pig choroidal plexus and cortex (Pazos et al 1984a; Hoyer et al 1985b) and was labelled by [^{3}H]-mesulergine and [^{3}H]-5HT. Later the $5HT_{1C}$ binding site was identified in the rat choroidal plexus using [^{125}I]-LSD (Yagaloff and Harlig, 1985) and rat frontal cortex using [^{3}H]-5HT in the presence of 100 nM 8-OH-DPAT and 100nM RU24969 (Peroutka, 1986). This site displayed nanomolar affinity for mesulergine and mianserin, moderate affinity for ketanserin but was devoid of affinity for 8-OH-DPAT, RU24969, beta blockers and specific $5HT_{2}$ antagonists.

Recently a fourth subtype, the $5HT_{1D}$ binding site has been characterised in membranes from bovine brain (Heuring

and Peroutka, 1987) and pig and human brain (Hoyer et al, 1987a) using [^{3}H]-5HT in the presence of 100nM 8-OH-DPAT and 100nM mesulergine.

$5HT_2$ binding sites were first identified using [^{3}H]-spiperone in the frontal cortex tissue (Leysen and Laduron, 1977; Leysen et al, 1978). The sites are characterised by high affinity for the classical 5HT antagonists and low affinity for 5HT (2700μM) and 5HT-like agonists. Although 5HT only shows micromolar affinity for these sites it is 100 fold more potent than at other known neurotransmitter receptors.

New 5HT antagonist ligands have been developed which clearly label the same binding site as [^{3}H]-spiperone. These include [^{3}H]-mianserin (Peroutka and Snyder, 1981), [^{3}H]-mesulergine (Closse, 1983), [^{3}H]-methiothepine which, although it is a potent 5HT antagonist, has unacceptably high non specific binding and is therefore of little use as a radioligand (Nelson et al, 1979) and the most important [^{3}H]-ketanserin (Leysen et al, 1982a). [^{125}I]-LSD is a highly selective $5HT_2$ ligand (Hartig et al, 1983). It has been used successfully to label $5HT_2$ sites in peripheral tissues.

A more detailed examination of the characteristics of $5HT_1$ and $5HT_2$ binding sites is discussed in chapters 3 and 4.

The term "$5HT_3$ site" has been used to describe a number of novel 5HT binding sites (Todd and Ciarenello, 1985; Robaut et al, 1985). Including the pre-synaptic 5HT binding site in the rat striatum labelled by [^{3}H]-8-OH-DPAT which differs from the $5HT_{1A}$ binding site in the hippocampus (Hamon et al, 1984b and c). However, to maintain order the 'true' $5HT_3$ binding site must be reserved for the binding of $5HT_3$ ligands. [^{3}H]-ICS 205-930 (3α-tropanyl)-1H-indole -3-carboxylic acid ester) has recently been shown to bind to neuroblastoma cells (Hoyer et al, 1987b) and [^{3}H]-GR65630 (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone) has been reported to bind to rat brain membranes (Kilpatrick et al, 1987).

A summary of the major advances in binding to 5HT receptors is shown in table 1.2.

Table 1.2 Major developments in 5HT binding studies

1975/76 Bennett and Snyder first identified 5HT binding sites using [^{3}H]-LSD and [^{3}H]-5HT The differences in binding were explained as a two-state model of the receptor.

1977 Leysen and Laduron showed [^{3}H]-spiperone labelled a 5HT site

1979 Peroutka and Snyder suggested [^{3}H]-5HT labels a $5HT_1$ site and [^{3}H]-spiperone a $5HT_2$ site.

1981 Leysen's group introduced [^{3}H]-ketanserin, a potent/selective $5HT_2$ ligand

1981 Pedigo et al subdivided $5HT_1$ sites into $5HT_{1A}$ and $5HT_{1B}$ on the basis of biphasic displacement curves of [^{3}H]-5HT by spiperone

1983 Gozlan et al introduced [^{3}H]-8-OH-DPAT as a specific $5HT_{1A}$ ligand

1984 Pazos, Hoyer and Palacios identified a $5HT_{1C}$ subtype labelled by [^{3}H]-mesulergine

1985 Engel's group introduced [^{125}I]-cyanopindolol as a $5HT_{1B}$ ligand

1987 A fourth $5HT_{1D}$ subtype is identified in bovine (Heuring and Peroutka); pig and human brain (Hoyer et al, 1987a).

1987 A $5HT_3$ binding site is identified in rat brain using [^{3}H]-GR65630 (Kilpatrick et al, 1988) and on neuroblastoma cells using [^{3}H]-ICS 205-930 (Hoyer et al, 1987b)

1.3.3 New tools in 5HT research

The major advances in 5HT receptor classification have arisen directly from the development of drugs with high affinities and, in some cases, selectivity for individual receptor sites. These include ketanserin for the $5HT_2$ site (Leysen et al, 1981), 8-OH-DPAT for the $5HT_{1A}$ subtype (Middlemiss and Fozard, 1983). 8-OH-DPAT enantiomers show

stereoselectivity at the $5HT_{1A}$ site, in binding and functional tests (Fozard et al, 1987). Selective ligands for the $5HT_{1B}$ subtype include CGS 12066B (7-trifluoromethyl -4(4-methyl-1-piperazinyl)-pyrolo(1,2-a)quinoxaline) (Neale et al, 1987). Up to now no selective compounds have been developed for the $5HT_{1C}$ and $5HT_{1D}$ subtypes. In contrast selective agonists and antagonists are available for the $5HT_3$ receptor. Table 1.3 lists the most selective drug tools for 5HT receptor pharmacology, at the present time.

$5HT_3$ ligands listed in table 1.3, which have not so far been referred to in the text, include DOB (4-bromo-2,5-dimethoxyphenyl-isopropylamine); MDL 72222 (1αH, 3α, 5αH-tropan-3-yl)3,5-dichlorobenzoate) and BRL 43694 (endo)-N-[9-methyl-9-azabicyclo(3,3,1)non-3-yl]-1-methyl-indazole-3-carboxamide).

1.3.4 Functional receptors for 5HT

Several subtypes of 5HT receptors have been discussed above and as stated, the first subdivision of 5HT receptors stems from Gaddum and Picarelli, (1957). In the guinea-pig ileum 5HT causes excitatory responses due to two mechanisms: 1. Stimulation of neuronal M (morphine) receptors which result in acetylcholine release from post-ganglionic cholinergic neurons of the intramural plexus. 2. Stimulation of D (dibenzyline) receptors on smooth muscle of the guinea-pig ileum. Gaddum and Picarelli did substantiate their results using LSD and dihydroergotamine (LSD was a particularly potent antagonist of the D receptor) and cocaine which selectively antagonised the M receptor. This latter finding has since been confirmed (Fozard et al, 1979). The conclusion reached that two receptors for 5HT exist in the guinea-pig ileum has not been invalidated (see Feniuk, 1984; Fozard, 1984a; Humphrey, 1984), and this original proposal is used as the basis for the present functional classification.

Guinea-pig ileum M receptors have properties in common with rabbit heart pre-junctional excitatory receptors (Fozard and Mobarok Ali, 1978a), and are similar to the 5HT receptor subtype that initiates the Bezold-Jarisch reflex

Table 1.3 New tools in 5HT research

Drug	site(s)-action	receptor affinity
8-OH-DPAT	$5HT_{1A}$ agonist	
DP-5CT	$5HT_{1A}$ agonist	very selective
buspirone	$5HT_{1A}$ partial agonist	D_2
ipsapirone	$5HT_{1A}$ partial agonist	D_2
spiperone	$5HT_{1A}$ antagonist	D_2, $5HT_2$, α_1
TFMPP	$5HT_{1B}$ agonist	
iodocyano-pindolol	$5HT_{1B}$ antagonist	β_1 β_2
mesulergine	$5HT_{1C}$ antagonist	$5HT_2$, D_2
mianserin	$5HT_{1C}$ antagonist	$5HT_2$, H_1, α_2
yohimbine	$5HT_{1D}$ ligand	α_2
rauwolscine	$5HT_{1D}$ ligand	α_2
pindolol &	$5HT_{1A}$ antagonist	β_1 β_2
cyanopindolol	$5HT_{1B}$ partial agonist	
RU24969	$5HT_{1B}$, $5HT_{1A}$ agonist, $5HT_{1D}$ antagonist?	
5CT	$5HT_{1A}$, $5HT_{1B}$ & $5HT_{1D}$ agonist	
DOB	$5HT_2$ agonist	
methysergide	$5HT_2$ antagonist	$5HT_1$-like agonist
cyproheptadine	$5HT_2$ antagonist	muscarinic
ketanserin	$5HT_2$ antagonist	$5HT_{1C}$
ritanserin	$5HT_2$ antagonist	
2-methyl-5HT	$5HT_3$ agonist	
MDL 72222	$5HT_3$ antagonist	
ICS 205-930	$5HT_3$ antagonist	
BRL 43694	$5HT_3$ antagonist	
Ondanetron	$5HT_3$ antagonist	

This table is based on that shown used by Fozard, (1987) and has been modified to include the majority of the compounds used in this thesis.

(Fozard and Host, 1982), now termed $5HT_3$. However, close analysis of the neuronal excitatory responses induced by 5HT in guinea-pig enteric neurons (Buchheit et al, 1985) reveals that Gaddum and Picarelli's M receptor is not a $5HT_3$ receptor. The guinea-pig D receptor is very similar to the $5HT_2$ receptor found on vascular smooth muscle (Humphrey et al, 1982; Maayani et al, 1984) although this was disputed by Leysen et al, (1982a). The $5HT_2$ receptor antagonist ketanserin is ineffective at the D receptor in guinea-pig ileum (Van Neuten et al, 1981 and 1983).

A third type of 5HT functional response which mediates the actions of 5HT via receptors distinct from $5HT_2$ and $5HT_3$ receptors, is more difficult to define. It may in some cases be similar to the heterogeneous group of $5HT_1$ binding sites. Criteria was proposed for classification of these receptors as "$5HT_1$-like" by Bradley et al, (1986) (table 1.4).

Table 1.4. Summary of proposals for classification and nomenclature of functional 5HT receptors

proposed receptor nomenclature	Typical response	agonist	antagonist	binding site
$5HT_1$-like	Autoreceptor, smooth muscle (sm) relaxation, contraction of some vascular sm tachycardia in cats	5CT	Methysergide Methiothepin	$5HT_1$?
$5HT_2$	GI tract and vascular sm contraction, platelet aggregation, neuronal depolarisation		Ketanserin Methysergide Cyproheptadine	$5HT_2$
$5HT_3$	Depolarisation of peripheral neurons	2-methyl-5HT	Cocaine MDL 72222 ICS 205-930	?

The classification of Bradley et al, (1986) emphasized the co-identity of Gaddum and Picarelli's D receptor and the $5HT_2$ binding site, conceded the likely heterogeneity of "$5HT_1$-like" receptors and introduced a new category, $5HT_3$, which superceded the M receptor of the functional classification. Although a useful reference the Bradley et al classification is rapidly becoming out of date. It is now abundantly clear that subtypes of "$5HT_1$-like" receptors are very much a reality (Glennon, 1987).

Functional correlates of $5HT_1$ binding sites

Until recently the lack of specific and selective antagonists for the $5HT_1$ binding sites has hindered the search for functional correlates (see Bradley et al, 1986). However, a classification based on functional data for "$5HT_1$-like" receptors was proposed for the following reasons:-

1. The carboxamide analogue of 5HT, 5CT was found to have a greater affinity for the $5HT_1$ binding site than for the $5HT_2$ binding site (Engel et al, 1983).
2. In functional studies 5CT was found to mimic with equal or greater potency, the response of 5HT in tissues where the receptor did not fit the D or M classification (Feniuk et al, 1983, 1985).
3. 5CT was less potent at $5HT_2$ receptors (Feniuk et al, 1985), and at M receptors (Humphrey, 1984).

"$5HT_1$-like" receptors are associated with pre-junctional inhibition of neuronal transmitter release, smooth muscle relaxation and contraction of some cardiac and vascular smooth muscle. Some peripheral "$5HT_1$-like" receptors have been identified with $5HT_1$ binding subtypes; inhibition of transmitter release from guinea-pig cholinergic neurons is via a $5HT_{1A}$ receptor (Fozard and Kilbinger, 1985) whereas inhibition of noradrenaline (NA) release in the rat vena cava is via a $5HT_{1B}$ receptor (Molderings et al, 1987). The response to 5HT in the mouse bladder is via a $5HT_{1B}$ receptor (Holt et al, 1986), whereas in the rat fundus (Cohen and Fludzinski, 1987) and rat perfused kidney (Charlton et al, 1986) the response is mediated via a

$5HT_1$ receptor which is not $5HT_{1A}$, $5HT_{1B}$ or $5HT_{1C}$. This receptor type shows moderate affinity for yohimbine so it could belong to the $5HT_{1D}$ subtype, although has yet to be shown.

Peroutka has suggested the existence of a $5HT_1$ receptor mediated contraction on canine and human basilar arteries (Peroutka et al, 1983; Peroutka, 1984).

$5HT_1$ receptors are also involved in the 5HT behavioural syndrome (Lucki et al, 1984) providing evidence for functional central $5HT_1$ receptors. The effect is most characteristically seen in rodents, but also observed in other animals and consists of; hindlimb abduction, forepaw treading and Straub tail. Tricklebank, (1984a,b) has shown that agonists selective for $5HT_{1A}$ and $5HT_{1B}$ subtypes, induce different behavioural patterns.

Functional correlates for $5HT_2$ binding sites

A $5HT_2$ mechanism may also be involved in certain central behavioural effects produced by 5HT agonists (Glennon, 1986; Tricklebank, 1985). $5HT_2$ agonists induce head twitches (Green and Heal, 1985).

Various groups have pointed out the similarity of the $5HT_2$ binding site and the vascular D receptor in the rabbit aorta (Humphrey et al, 1982; Maayani et al, 1984), guinea-pig ileum (Engel et al, 1984a and b) and in other vascular tissues from the rat (Bradley et al, 1983; Cohen et al, 1981). The affinity of a number of 5HT antagonists was similar when determined by ligand binding or in vitro isolated tissue experiments. Since ketanserin differentiated the $5HT_1$ and $5HT_2$ binding sites it has been used widely as a probe in $5HT_2$ receptor characterisation. The vascular $5HT_2$ (D) receptor has been identified in many tissues (see table 1.4, Humphrey, 1984). However, ketanserin is ineffective at the D receptor on guinea-pig ileum (Van Neuten et al, 1981 and 1983) which has been clearly identified as a $5HT_2$ receptor; a highly significant correlation was found for the potency of a series of antagonists (except ketanserin) to inhibit the contraction of the guinea-pig ileum and for their affinity at the $5HT_2$

binding site (Engel et al, 1984a and b). In addition the rank order of potency of a series of agonists for causing contraction of guinea-pig ileum and of the rabbit aorta, which is undoubtedly $5HT_2$, was the same (Humphrey et al, 1982; Maayani et al, 1984; Feniuk et al, 1985).

Thus the D receptor classification was becoming confused and has now been broadly replaced by the $5HT_2$ receptor terminology. Dibenzyline has been shown to have only weak affinity for the $5HT_2$ binding site on rat cortex membranes (Leysen et al, 1981).

Another difficulty in classifying 5HT receptors in this group occurs through the fact that 5HT at higher concentrations can act through other receptor types particularly α-adrenoceptors. Many α-adrenoceptor and 5HT antagonists have some affinity for both receptor types. 5HT has been shown to act both directly (Apperley et al, 1976; Fozard, 1976) and indirectly (Fozard and Mwaluko, 1976) on α-adrenoceptors. In the rabbit ear artery no difference is seen in the response to NA and 5HT when blocked by either phentolamine or cyproheptadine. Therefore, NA and 5HT are both acting through a common α-adrenoceptor (Apperley et al, 1976). In the rabbit aorta phentolamine and cyproheptadine have been shown to act through different receptors so that both 5HT receptors and α-adrenoceptors are distinguishable (Apperley et al, 1976).

Methysergide (developed by Doepfner and Cerletti, 1958) may be an important drug for classifying 5HT receptors on smooth muscle. It has a 300-400 fold higher affinity for 5HT receptors than for α-adrenoceptors. The nature of action of methysergide differs between smooth muscle preparations. It acts as a highly specific antagonist of the contractile action of 5HT in smooth muscle when this response is seen ($5HT_2$) and it acts as a partial agonist in other smooth muscle preparations where 5HT is inhibitory ("$5HT_1$-like").

Functional $5HT_3$ receptors

The term 'M receptor' has been synonymous with 'peripheral neuronal receptor' no matter the anatomical location or the functional response which it mediates (see Wallis, 1981). This is despite criticisms of the basis of the classification and no doubt reflects in part the initial lack of selective antagonists of these neuronal receptors.

Neuronal receptors have been reviewed from two main approaches; from functional pharmacological studies (Fozard, 1984a) and from electrophysiological studies (Wallis, 1981). Each has suggested a tentative classification.

Two "functional" preparations have been the focus of attention in the study of peripheral neuronal receptors for 5HT. In the guinea-pig ileum the 5HT-induced contraction is mainly indirect and due to the release of acetylcholine from pre-junctional neurons. Stimulation of acetylcholine release was shown to be mediated by specific receptors since $5HT_2$ (D) receptor antagonists and antagonists of other receptor types could not selectively block the response to 5HT (Day and Vane, 1963). However, the receptors could be rapidly and selectively desensitised by 5HT (Gaddum, 1953; Brownlee and Johnson, 1963). These receptors were designated as M receptors (Gaddum and Picarelli, 1957). The receptor in the guinea-pig ileum was insensitive to blockade by phenylbiguanide, an effective antagonist at neuronal receptors for 5HT in the mouse duodenum (Drakontides and Gershon, 1968) and which first indicated a difference between these M receptors.

The indirect positive inotropic effect of 5HT on the isolated rabbit atrium was antagonised by morphine and cocaine, and abolished by prior reserpinisation of the animal. The receptor mediating this response via release of neurotransmitter (NA) from sympathetic neurons was also described as the M receptor (Trendelenburg, 1960).

However, it has been demonstrated that differences exist between the M receptors in the guinea-pig ileum and rabbit heart. In the Langendorff perfused rabbit heart the

positive chronotropic effect of 5HT was not selectively antagonised by morphine (Fozard and Mobarok Ali, 1978a). Furthermore the concentration of morphine which gave a non-selective inhibition (10μg/ml) of the 5HT response was 100 times greater than the concentration of morphine which blocked 5HT-induced transmitter release from cholinergic neurons in the guinea-pig ileum (Day and Vane, 1963).

Characterisation of the $5HT_3$ receptor(s), was facilitated by the identification of a selective antagonist, MDL 72222 (Fozard, 1984b). At nanomolar concentrations MDL 72222 blocked the 5HT-induced release of transmitter from sympathetic neurons of the rabbit heart (Fozard, 1984b). Several other M receptors were identified on post-ganglionic sympathetic neurons, parasympathetic neurons and on afferent neurons. All of these receptors were susceptible to blockade by MDL 72222, and in each case were excitatory in nature (see Fozard, 1984a). MDL 72222 also confirmed earlier findings that the M receptor in the guinea-pig ileum was different from the other excitatory M receptors. MDL 72222 has negligible affinity for the M receptor in the guinea-pig ileum (Fozard, 1984b). Metoclopramide a competitive antagonist at excitatory 5HT receptors on post-ganglionic sympathetic nerves and on afferent neurons (Fozard and Mobarok Ali, 1978b; Fozard and Host, 1982) is a partial agonist at M receptors in the ileum (Kilbinger et al, 1982). The selective agonist 5-methoxytryptamine similarly shows a difference between the M receptor in the guinea-pig ileum and the other excitatory neuronal receptors; in the rabbit heart 5-methoxytryptamine is inactive while in the ileum it is a partial agonist (Fozard and Mobarok Ali, 1978a).

In a separate but almost parallel study selective agonists and antagonists were identified which pointed to subtypes of M receptors in different M receptor systems (Richardson et al, 1985). The agonist 2-methyl-5HT was equipotent at M receptors in the rabbit heart, guinea-pig ileum and rabbit vagus nerve (although approximately 2 fold less potent than 5HT) while α-methyl-5HT was less potent than 2-methyl-5HT, but to a different degree, in each

preparation.

A series of highly potent and selective Sandoz compounds confirmed that the M receptors in the three systems were different. ICS 205-930 differentiated between the receptors in the rabbit vagus nerve (pA_2 10.2), rabbit heart (pA_2 10.8) and in the guinea-pig ileum (pA_2 7.8). In receptor binding studies ICS 205-930 was shown to be highly selective as it lacked affinity for the receptor types tested: $5HT_1$, $5HT_2$, α, β, dopamine, histamine, muscarinic.

These excitatory receptors which are blocked by either MDL 72222 or ICS 205-930 now make up the category of receptors known as $5HT_3$ receptors. (Bradley et al, 1986). A number of new $5HT_3$ antagonists, which are also very selective, have been introduced; Ondanetron (GR38032F) (Brittain, et al, 1987) and BRL 43694 (Fake et al, 1987).

1.3.5 Second messenger systems linked to 5HT receptors

The biochemical mechanisms involved in receptor effector coupling have been characterised for the $5HT_{1A}$, $5HT_{1B}$, $5HT_{1C}$, $5HT_{1D}$ and $5HT_2$ receptor subtypes.

1. Adenylate cyclase

Barbaccia et al, (1983) first suggested that a $5HT_1$ receptor as defined by Peroutka and Snyder, (1981) might activate adenylate cyclase in the rat hippocampus. Adenylate cyclase coupled to a number of receptors, catalyses the formation of cyclic AMP from ATP. 5HT was later shown to activate adenylate cyclase in the guinea-pig hippocampus through a receptor which, based on the potency of 5CT, was suggested to be the $5HT_{1A}$ site (Shenker et al, 1985). This was questioned by De Vivo and Maayani, (1986) who suggested that 8-OH-DPAT was less potent than would be expected and acted as only a partial agonist. Markstein et al, (1986) confirmed that the action was through a $5HT_{1A}$ site by examining the rank order of potency of 13 agonists for stimulation of adenylate cyclase activity and showed there was good agreement with the rank order of affinity for the $5HT_{1A}$ binding site but not for the $5HT_{1B}$, $5HT_{1C}$ or $5HT_2$ sites.

Under appropriate assay conditions $5HT_1$ receptors might

inhibit adenylate cyclase. De Vivo and Maayani, (1985) demonstrated that $5HT_{1A}$ agonists selectively inhibited adenylate cyclase stimulated by forskolin in the guinea-pig and rat hippocampus. Weiss et al, (1986) indicated that in intact murine cortical and striatal neurons, $5HT_{1A}$ receptor agonists inhibit cAMP accumulation induced by vasoactive intestinal polypeptide. Therefore, present results, suggest that $5HT_{1A}$ receptors are linked to adenylate cyclase although activation (via Gs) or inhibition (via Gi) of the enzyme depends on the pre-exsisting state of activation.

It has been suggested that the $5HT_{1B}$ binding sites in rats are presynaptic autoreceptors (Hibert and Middlemiss, 1986; Engel et al, 1986). However, selective lesioning (Verge et al, 1985, 1986) as well as controversy over the species location of the binding site has failed to support this. The $5HT_{1B}$ sites have been shown to be linked in an exclusively negative way to adenylate cyclase.

$5HT_{1D}$ receptors have recently been shown to be linked to adenylate cyclase in the calf substantia nigra (Hoyer and Schoeffter, 1988).

2. Alteration in K^+ conductance

Berridge (1981) suggested that the hyperpolarizing response to 5HT is probably via a $5HT_1$ receptor and mediated by cyclic AMP. Vandermaelen and Aghajanian, (1983) proposed that 5HT may alter the activity of calcium-activated K^+ channels in the dorsal raphe nucleus. However, Andrade et al, (1986) recently demonstrated that 5HT, possibly via $5HT_{1A}$ receptors, increases K^+ ion conductance in hippocampal CA1 neurons and that this response was unaffected by application of cyclic AMP or by chelation of intracellular Ca^{2+}. The 5HT response was abolished by prior treatment with pertussis toxin which suggests that hippocampal $5HT_{1A}$ receptors may be directly coupled to neuronal K^+ channels via pertussis toxin-sensitive G proteins (Andrade et al, 1987).

3. PI metabolism

Jafferji and Michell, first demonstrated 5HT activated PI metabolism in mammalian tissues in 1976. However, it was nearly ten years before preliminary evidence for the

coupling of $5HT_2$ receptors to PI metabolism was shown in brain (Conn and Sanders-Bush, 1984), vascular smooth muscle (Roth et al, 1984) and platelets (Leysen et al, 1984). The ability of 5HT to mobilise intracellular calcium in platelets is related to an interaction of 5HT with $5HT_2$ receptors (Affolter et al, 1984). Conn and Sanders-Bush (1985 and 1986) later showed that a good correlation existed between the Kd value determined for ketanserin inhibition of 5HT-stimulated turnover of phosphoinositides, and its affinity for the $5HT_2$ binding site in the rat frontal cortex. However, the affinities of other antagonists were not correlated in the two measurements (Kendall and Nahorski, 1985).

$5HT_1$ receptors in the hippocampus may also be coupled to PI metabolism. Janowsky et al, (1984) showed that the 5HT-stimulated PI hydrolysis was attenuated by metergoline but not significantly by the $5HT_2$ antagonist mianserin. Additionally the response was less sensitive to the $5HT_2$ antagonist ketanserin in the hippocampus than in the cortex (Conn and Sanders-Bush, 1985).

Activation of the $5HT_{1C}$ receptor in the rat choroidal plexus has also been shown to increase phosphatidylinositol turnover providing a possible functional correlate for this binding site (Conn and Sanders-Bush, 1986).

Activation of the $5HT_3$ receptor results from a fast depolarisation which may be very tightly coupled to ion channels (Fozard 1984a).

1.3.6 Classification of 5HT receptors using electrophysiological techniques

Aghajanian, (1981) described three types of 5HT receptors using electrophysiological techniques:

S1 A receptor facilitating excitation which is blocked by cinanserin.

S2 A receptor decreasing neuronal excitability which is not blocked by classical 5HT antagonists. However, 5HT is ten times more potent than LSD and $5HT_{1A}$ selective agonists are effective.

S3 An autoreceptor which is not blocked by classical 5HT antagonists and where LSD is more potent than 5HT.

The S2 receptor, characterised in the dorsal raphe nucleus of the rat has been shown to be homologous with the $5HT_{1A}$ high affinity binding site (De Montigny et al, 1984) and does not resemble the S2 ($5HT_2$) binding site.

A number of anomalies in the classification of 5HT receptors have been highlighted in this introduction, clearly some degree of uniformity is needed between binding, functional and electrophysiological studies. A summary of the current classification of 5HT receptors, adopted for this thesis is shown in table 1.5.

1.4 Clinical significance of advances in 5HT research

As knowledge of 5HT subtypes and their functions increase then the scope for selective modulation of 5HT mechanisms for therapeutic benefit to man also increases. $5HT_{1A}$ partial agonists, such as buspirone, have been shown to reduce the elevated neuronal activity associated with anxiolysis. This could, theoretically, be achieved by depressing 5HT neuronal firing (Andrade et al, 1987), decreasing 5HT turnover (Hjorth and Carlsson, 1982) or blocking $5HT_{1A}$ post-synaptic receptors (Andrade and Nicoll, 1987). A central antihypertensive activity is also associated with $5HT_{1A}$ partial agonists. $5HT_2$ antagonists are being investigated for psychiatric conditions such as schizophrenia, depression and anxiety. The recently introduced $5HT_3$ compounds are also being investigated for anxiolytic and antipsychotic properties, as well as for the treatment of migraine.

Table 1.5 Receptors for 5-hydroxytryptamine

	$5HT_1$				$5HT_2$	$5HT_3$
	1A	1B	1C	1D		
Agonists	5HT, DP-5CT 8-OH-DPAT, 5CT (LSD)	5HT, RU24969 5CT, TFMPP, (LSD)	5HT, quipazine	5HT, 5CT	DOB, quipazine (LSD)	5HT, 2methyl-5HT quipazine
Partial Agonists	buspirone, ipsapirone, WB4101			yohimbine? rauwolscine?		
Antagonists	spiperone	cyanopindolol propranolol	mesulergine mianserin ketanserin	RU24969?	ketanserin, LSD, ritanserin methysergide cyproheptadine	MDL 72222 ICS 205-930 BRL 43694 ondanetron
Location	CNS pre- & post-synaptic	rodent autoreceptor mouse bladder	choroid plexus	substantia nigra globus pallidus rat fundus/kidney?	post-synaptic blood vessels platelets, CNS	nerve endings
Agonist effect	anxiety, pain, hyperphagia, hypotension	hypophagia			vasoconstriction hallucinogens?	sensory nerve activation
Therapeutic area	anxiolytics antidepressants anorexic agents mild analgesics	obesity			antihypertensives antipsychotics anxiolytics	anxiolytics antipsychotics anti-emetics

Chapter 2: METHODS

2. METHODS

2.1. Binding Methodology

2.1.1a Membrane preparation

Preparation of rodent membranes

A washed, total membrane fraction of the rat or gerbil brain regions was used in the majority of binding studies. Male Sprague Dawley rats (CD[SD] Charles River UK LTD) (150-250g) were killed by cervical dislocation. Mongolian gerbils (Shamrock farm, England) (60-80g) were killed by decapitation following 6mg pentobarbital sodium (i.p.) anaesthesia. The brains were rapidly removed and the appropriate areas, cerebral cortex, frontal cortex (anterior to the optic chiasma), neostriatum, hippocampus or cerebellum carefully dissected on ice. These and all subsequent operations were performed at 4°C. Heart and lung tissue was removed when required.

Tissue was homogenised in 25 vol ice cold Tris HCl buffer (50mM Tris HCl; pH 7.4), using a polytron PT10 tissue disrupter (setting 5, 2 x 10s bursts). The homogenate was centrifuged at 30000xg in a refrigerated centrifuge (Sorvall RC-5B) (4°C) for 10min. The supernatant was discarded and the crude membrane pellet washed by centrifugation at 30000xg and resuspended in 25 vol ice cold Tris HCl buffer. After the third wash the resuspended pellet was incubated in 25 vol Tris HCl buffer (pH 7.4 at 37°C) at 37°C for 10min to remove endogenous 5HT (Nelson et al, 1978), before being recentrifuged at 30000xg for 10min at 4°C. The final crude membrane pellet was resuspended in the 3ml buffer and stored under liquid nitrogen. Membranes could be stored for up to 6 weeks without significant changes in receptor number or other binding characteristics.

Preparation of human platelet membranes

The membranes were prepared essentially as described by Cheung et al, (1982). Blood was collected from male or female volunteers, with 3.9% (w/v) sodium tricitrate as

anticoagulant. Platelet rich plasma (PRP) was obtained by centrifugation at 200xg for 10min. The PRP was then centrifuged at 25000xg for 10min at 4°C and the resultant pellet resuspended in ice cold lysing buffer (5mM Tris HCl, 5mM EDTA, pH 7.4), homogenised using a polytron PT10 tissue disrupter (10s) and again centrifuged at 25000xg for 10min. The pellet was washed once more with lysing buffer by gentle resuspension and then centrifugation before a final wash in Tris buffer (50mM Tris HCl; pH 7.4) followed by centrifugation at 25000xg for 10min at 4°C. The final pellet was resuspended in assay buffer for immediate use in the binding assay or stored under liquid nitrogen.

Preparation of vascular smooth muscle membranes

Pig hearts were collected from the abattoir and the left descending coronary artery removed and cleaned of any fatty adventitious tissue. The arteries (10-12/preparation) were then finely chopped and homogenised in 25ml of ice cold 0.25M sucrose. A ground glass-teflon homogeniser was found to be the most efficient method. Membranes were prepared from an initial centrifugation at 1000xg for 10min at 4°C followed by a second centrifugation of the supernatant at 39000xg for 15min at 4°C. The membranes were then washed twice by resuspension in assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) followed by centrifugation at 39000xg for 15min at 4°C. The final pellet was resuspended in assay buffer for direct use in the binding assay.

Human umbilical arteries (HUA) were collected in liquid nitrogen, then thawed and cleaned of small amounts of fatty adventitious tissue. The arteries (6/preparation) were finely chopped and homogenised in 25ml of ice cold 0.25M sucrose. Membranes were prepared from an initial centrifugation at 15000xg for 10min at 4°C and then respining the supernatant at 60000xg for 30min at 4°C, in a Beckman L8-M ultracentrifuge. The final pellet was then resuspended in assay buffer for direct use in the binding assay.

2.1.1b Equilibrium binding studies

Assays were carried out in a total volume of 1 or 2ml in polypropylene disposable assay tubes (Sterlin 4.9ml). The membrane suspension was allowed to thaw at room temperature and diluted with the assay buffer (50mM Tris HCl; pH 7.4 at 37°C containing 5.7mM ascorbate, 4mM $CaCl_2$ and 10μm pargyline) unless otherwise stated, before being added to the assay. Reagents were stored at 4°C before use and preincubated at 37°C for 10min before binding was initiated by adding the membrane preparation (routinely 150-500μg protein) with the radioligand and other assay additions. Tubes were rapidly vortexed and incubated to equilibrium in a thermostatically controlled water bath maintained at 37°C. Non-specific binding (nsb) was defined as the amount of radioactivity bound in the presence of a very large excess of the neurotransmitter itself or a highly specific drug. Separation of bound from free ligand was achieved by diluting samples with 2ml of ice cold assay buffer to slow down the reaction and allow easy transfer from the assay tube, followed by vacuum filtration (22mm Hg) through wet Whatman GF/B glass fibre filters mounted either on a millipore 12 port filtration manifold (Millipore 1225) or on a Brandel (M-24R) cell harvester. Filters were washed with 15mls of room temperature assay buffer to remove trapped free ligand, dried under reduced vacuum and then transferred to scintillation vials and the radioactivity determined by liquid scintillation spectrometry. In all experiments total ligand concentration was determined by counting aliquots of the radioactive stock or aliquots taken from two additional "unfiltered" total tubes. Free ligand was estimated by subtracting the concentration of total bound ligand from the total ligand concentration and specific binding was determined by subtracting nsb from total binding.

2.1.1c Separation of bound from free ligand

Separation of bound from free ligand was carried out under constant vacuum (22mm Hg). The optimal number of washes was determined 1-5 x 5ml washes with room temperature buffer or ice cold buffer. Later studies were

carried out using a Brandel M24 cell harvester. Again a constant vacuum pressure of 22 mm Hg was used and the optimum washing conditions determined, 10,20 or 30s at a constant flow rate of 1.5L/min.

2.1.1d Filter binding of ligands

Aliquots (1ml) of assay buffer containing either a saturable concentration of the radioligand or a concentration close to its Kd were diluted with 2ml of ice cold buffer and filtered under vacuum over Whatman GF/B glass fibre filters, which had been pretreated with inactivated (boiled) membranes to simulate the retardation of the filtration process produced by the presence of membrane suspension in binding assays. The filters were then washed with 3 x 5ml aliquots of room temperature buffer, and the radioactivity retained on the filters determined by liquid scintillation spectrometry. To assess the effect of non labelled compounds on radioligand filter binding, some of the routinely used competitors were included in parallel assays at the highest concentration used in binding studies.

2.1.1e Measurement of radioactivity

The glass fibre filters or samples containing radioactivity were added to 8ml of LKB optiphase 'MP' liquid scintillation fluid and shaken for 1-2h. This was necessary to ensure elution of filter-bound radioactivity. The radioactivity present in the samples was subsequently determined by counting for 3min in a Packard Tricarb 460C liquid scintillation counter. The counts per minute (cpm) were converted to disintegrations per minute (dpm) using the Packard quench indicating parameter (QIP). A typical quench curve, produced in the presence of glass fibre filters is shown in fig. 2.1. The QIP values for samples ranged from 550-650, an efficiency range of 35-45%.

Iodinated samples were counted in an LKB 1282 gamma counter. Filters were counted directly for 1min.

2.1.1f Protein assay

Membrane protein was determined using a protein assay kit, Pierce BCA protein assay (Smith et al, 1985). This method utilises the interaction of Cu^{2+} with proteins in

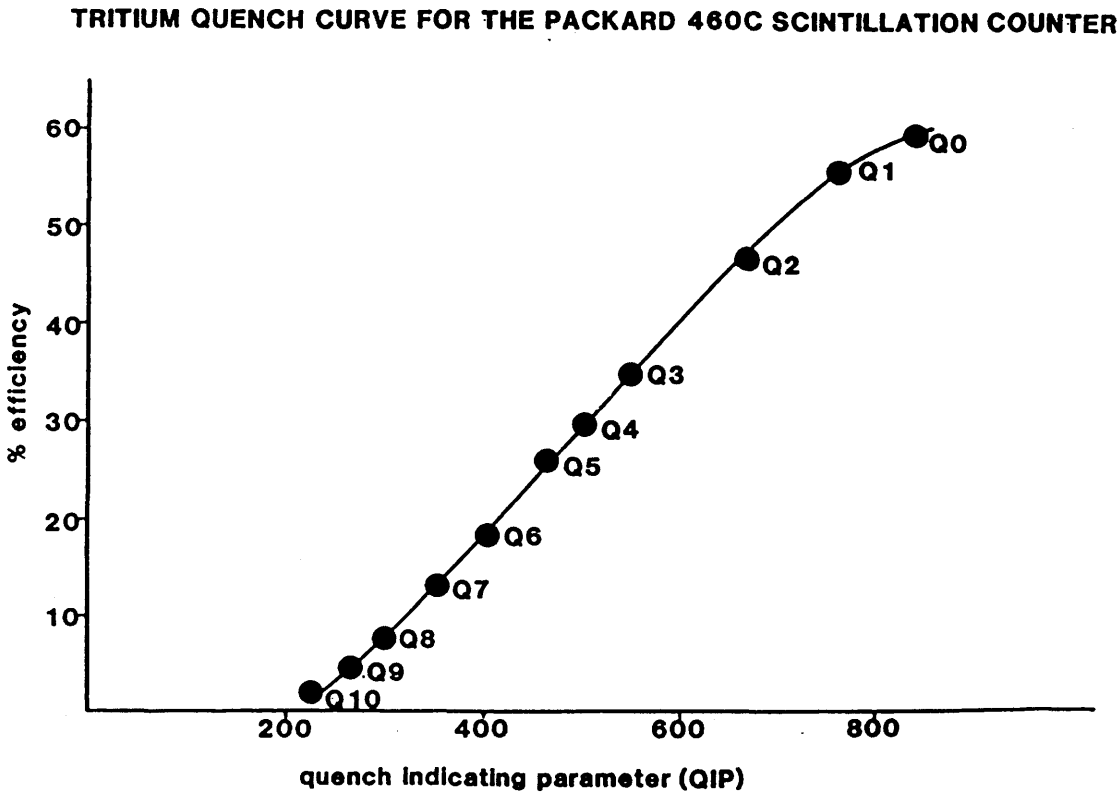


Fig. 2.1 The quench curve was constructed using a tritium standard, absolute activity 1.44×10^{-5} DPM/cm^3, and a series of 10 quenched samples. Each vial contained 8ml scintillant and 1ml of the standard ^3H solution. After counting and discarding any vials not falling within a 95% confidence limit, the quenching agents were added: Q0 unquenched, Q1 absolute alcohol, Q2 2% acetone, Q3 8% acetone, Q4 20% acetone, Q5 2% CCl_4, Q5 3% CCl_4, Q6 4% CCl_4, Q7 4% CCl_4, Q8 6% CCl_4, Q9 8% CCl_4 and Q10 12% CCl_4, and the samples recounted. A plot of efficiency (eff= CPM/AA) against the quench indicating parameter is shown. The QIP values for samples in these studies ranged from 550-650, an efficiency range of 35-45%.

an alkaline medium to give free Cu^{+} which then binds to a highly sensitive and selective detecting agent for Cu^{+}, bicinchoninic acid (BCA). The protein samples were mixed with the BCA reagent and incubated for two hours at room temperature, before the absorbance of the solution was read at 562nm wavelength in a Pye Unicam SP 1800 UV spectrophotometer. Membrane protein was determined against standard solutions of bovine serum albumin (BSA) containing 25-250μg protein.

Early protein determinations were made using a modification of the method of Lowry et al, (1951).

2.1.1g Tissue linearity of binding

Equilibrium binding was determined as described in section 2.1.1b except that the assay protein content was varied between 25-2000μg. In these experiments total and nsb was determined in triplicate

2.1.1h Kinetic experiments

1. Association

The membrane suspension (final assay concentration 200-500μg) and radioligand (final assay concentration $\leq$Kd) preincubated at 37^{o}C were combined in a total volume of 1ml. The tubes were rapidly mixed and incubated at 37^{o}C for varying time intervals (1-60min), before separating bound from free ligand by vacuum filtration using manifolds as described previously. Nsb was determined in parallel incubations. For each time point both total and nsb were determined in triplicate.

2. Dissociation induced by excess of cold competing ligand

The membrane preparation (final assay concentration 200-500μg) and radioligand (final assay concentration $\leq$Kd) were preincubated separately at 37^{o}C for 20min before being combined in a total volume of 50mls and incubated to equilibrium. Dissociation was initiated by adding and rapidly mixing 100μl of the non-specific ligand. Aliquots (1ml) were then removed at varying time periods and bound radioactivity separated from free as described previously. Nsb was determined in parallel incubations.

2.1.1i Saturation binding assays

Equilibrium binding was determined as described in section 2.1.1b except that the free ligand concentration was varied over a saturable range. Total binding, in triplicate, and nsb, in duplicate, were determined for each ligand concentration. Free ligand concentration was determined at each ligand concentration as described in section 2.1.1b.

2.1.1j Competition studies

Equilibrium binding of a fixed concentration of radioligand (≤Kd) was determined in duplicate as described in section 2.1.1b in the presence of a range (7-20) of concentrations of the competing ligand. Total and nsb were determined in duplicate before and after each competing ligand and the free ligand concentration was determined as in section 2.1.1b.

2.1.1k Data analysis

The theoretical basis of direct radioligand binding techniques is discussed in appendix 1.

1. Raw data manipulation

Raw data obtained from the liquid scintillation counter were transferred directly to a floppy disc data file on a Cromemco 64K or a BBC model B microcomputer. Transformation of the raw data into a format suitable for subsequent analysis was performed using a series of analysis programmes written for the Cromemco 64K or the BBC model B microcomputer.

2. Prefitting raw data

Experimental data were analysed using a combined graphical and analytical approach. A prefitting programme enabled a graphical output of the data, in an appropriate coordinate system. Isotherms describing the kinetic, saturation binding (Scatchard, 1949) or competition isotherms for single or multiple species of interacting or non-interacting sites could be superimposed upon the data points for visual inspection, and a series of graphical aids utilised to modify parameter estimates describing individual models. In this way it was possible to inspect visually the suitability of different models for describing

the experimental results. When appropriate, the parameter estimates obtained graphically were utilised as initial estimates for further determinations in programmes which allowed statistical analysis.

3. Analysis of saturation binding data

Fitting of data to the appropriate models was achieved using the Scafit non-linear regression analysis programme for LIGAND (Munson and Rodbard, 1980). Comparisons between different binding models were made using the extra sum of the squares principle as outlined by Munson and Rodbard, (1980), and shown in equation 1.

$$F = \frac{(SS_1 - SS_2) / (df_1 - df_2)}{(SS_2 / df_2)} \qquad \text{Equation 1}$$

SS and df refer to the residual sum of the squares and degrees of freedom associated with the two fits being compared. The subscript 1 is designated to the fit with highest number of degrees of freedom (i.e. the most complicated model of analysis). Statistical significance of the F value was determined from a table of the percentage of the F distribution using $df_1 - df_2$ and df_1 degrees of freedom as the numerator and denominator respectively.

4. Analysis of competition experiments

The inhibition of the radioligands by competing ligands was analysed graphically to estimate the IC_{50} (concentration of competitor displacing 50% of specifically bound radioligand), using a non-linear least squares programme specially designed for the interpretation of sigmoidal concentration curves in terms of total and non-specific binding as well as inhibition constants and curve steepness.

When Hill coefficients were not significantly different from unity the concentration of competitor displacing 50% of specific binding (IC_{50}) was converted to an affinity constant (Ki) using the expression derived by Cheng and Prusoff (1973) and shown in equation 2. In this expression

[L] and Kd represent the radioligand concentration and dissociation constant respectively.

$$Ki = \frac{IC_{50}}{1 + ([L]/Kd)} \qquad \text{Equation 2}$$

All data were initially analysed assuming a one site model of radioligand binding. The data, with Hill coefficients less than unity, were then analysed assuming a two site model, and the results of the curves fitting were statistically compared with those of the one site fit by an F test. The two site model was accepted if the observed fit was significantly better ($p<0.05$) than the one site fit. Some inhibitors of [^{3}H]-5HT binding to rat cerebrocortical membranes were analysed assuming a three site model.

5. Association experiments

The theoretical aspects underlying the methods of analysis of association kinetic binding data are discussed in appendix 1. The observed rate constant for association (K_{obs}) was obtained by fitting specific binding data, using non-linear regression analysis techniques, to models of binding which assume association with one or two species of non-interconverting sites. The association rate constant (K_1) was determined from K_{obs} as described in appendix 1.

6. Dissociation experiments

Theoretical aspects of the analysis are discussed in appendix 1. The dissociation rate constant K_{-1} was determined directly by fitting the specific binding data, using non linear regression analysis techniques, to models assuming dissociation of the radioligand from single or multiple species of interacting or non-interacting sites.

Statistical comparisons of the means of groups of data was made using the Students t-test for paired or unpaired data, where appropriate. A level of probability of $p<0.05$ was taken to indicate satistical significance.

2.1.2 Modifications to the basic technique

2.1.2a Effect of GTP

Saturation assays as described in section 2.1.1i and competition assays as described in section 2.1.1j were repeated in the presence of 0.1 or 1mM guanosine 5-triphosphate (GTP) (Tris salt) to assess nucleotide interactions with 5HT or α_2-adrenoceptor binding sites.

2.1.2b Effect of ascorbate

[^{3}H]-5HT is chemically unstable, and has been reported by numerous groups to be very sensitive to the incubation medium, resulting in a wide variation in the reported binding parameters; Kd values of 3-12nM have been reported. Initial experiments in this study were performed using the standard assay conditions, 5.7mM ascorbate and 4mM $CaCl_2$ described by Bennett and Snyder, (1976). Therefore the effects of these buffer constituents on specific [^{3}H]-5HT binding and the binding of other radioligands was investigated.

Washed rat cerebrocortical membranes were prepared as described in section 2.1.1a, and [^{3}H]-5HT binding assays were performed as described in section 2.1.1b, except for variations in the assay medium. Assays were performed in the presence or absence of ascorbate (5.7mM), $CaCl_2$ (4mM) and/or EDTA (0.5mM).

2.1.2c Effect of ions

The ionic composition of the assay buffer was varied and the effects of the monovalent ions Na^+ and K^+ (1-50mM) and divalent ions Ca^{2+} and Mg^{2+} (0.01-50mM) were examined.

2.1.2d Equilibrium temperature

The time to reach equilibrium and the amount of specific binding was determined at 22^{o}C and compared with the previous results for 37^{o}C.

2.1.2e Effect of pH

The effect of changing pH on the binding of the radioligands was also studied, to ensure the assays were performed at the optimum pH. All assay additions, membranes, radioligand and drugs, were prepared in the appropriate pH assay buffer. Experiments were subsequently carried out as described previously with the only

modification being the difference in the pH of the buffer used.

2.1.3 Specific 5HT binding assays

The above methodology describes in general terms the principle techniques used in a receptor binding assay. However, modifications are required to optimise the requirements for different receptors and radioligands. Specific assays for 5HT subtypes are described below:-

2.1.3a $5HT_{1A}$ binding sites labelled by [^{3}H]-5HT

$5HT_{1A}$ assays were performed by incubating washed rat cerebral membranes (2.1.1a) with [^{3}H]-5HT (2nM) for 10min at 37^{o}C, in the presence or absence of 12-20 concentrations of the competing drug in a final volume of 1ml Tris assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4 at 37^{o}C). Nsb was defined as the concentration bound in the presence of 1μM spiperone (Middlemiss, 1984). Saturation experiments were performed under similar conditions using a range of concentrations of [^{3}H]-5HT (0.2-12nM).

2.1.3b $5HT_{1A}$ binding sites labelled by [^{3}H]-WB4101

Assays were performed using a modification of the method described by Norman et al, (1985). Briefly, washed rat membranes (0.5mg protein) were incubated with [^{3}H]-WB4101 (2nM) for 40min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4 at 37^{o}C). Nsb was defined using 10μm 5HT or 3μm buspirone. [^{3}H]-WB4101 assays were all performed in the presence of 30nM prazosin to mask α_1-adrenoceptors. Saturation experiments were performed under identical conditions using a range of concentrations of [^{3}H]-WB4101 (0.5-8nM).

2.1.3c $5HT_{1A}$ binding sites labelled by [^{3}H]-8-OH-DPAT

The $5HT_{1A}$ assays using [^{3}H]-8-OH-DPAT were performed using a modification of the method described by Gozlan et al, (1983). Briefly, aliquots of the final membrane suspension (0.5mg protein) were incubated for 10min at 37^{o}C with [^{3}H]-8-OH-DPAT (1nM) in the presence or absence of 12

concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4). Specific binding was determined using 10μm 5HT or 3μm buspirone. Saturation experiments were performed in a similar manner using a range of concentrations of [^{3}H]-8-OH-DPAT (0.2-12 nM).

Filter binding of [^{3}H]-8-OH-DPAT was assessed as described in section 2.1.1d.

2.1.3d $5HT_{1B}$ binding sites labelled by [^{3}H]-5HT

The binding of [^{3}H]-5HT to the $5HT_{1B}$ subtype was measured under the same conditions as described above for the binding to the $5HT_{1A}$ subtype (2.1.3a) except that 1μM spiperone was included in the assay mixture (Middlemiss, 1984). Specific binding was determined using 10μm 5HT.

2.1.3e $5HT_{1B}$ binding sites labelled by [^{125}I]-CYP

Assays were performed using a modification of the method described by Hoyer et al, (1985a). In the presence of 30μM isoprenaline (in order to supress binding to β-adrenoceptors) [^{125}I]-CYP (150pM) was incubated to equilibrium, 90min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 250μl buffer (50mM Tris HCl; pH 7.4). Specific binding was determined in the presence of 10μm 5HT.

Saturation assays were performed under identical conditions using a range of concentrations of [^{125}I]-CYP (5-500pM).

2.1.3f [^{3}H]-ketanserin binding to rat cerebral membranes

Washed rat cerebral membranes were incubated with [^{3}H]-ketanserin (1nM) for 15min at 37^{o}C in a final volume of 1ml Tris assay buffer (50mM Tris HCl containing 4mM $CaCl_2$, 5.7mM ascorbate, 10μm pargyline; pH 7.4 at 37^{o}C), in the presence or absence of a range of concentrations (7-22) of the competing drug. Nsb was determined in parallel experiments in the presence of 2μm methysergide. Saturation assays were performed under identical conditions using a range of concentrations of [^{3}H]-ketanserin (0.05-5nM).

2.1.3g [^{3}H]-Mianserin binding to rat frontal cortex membranes

Assays were performed using the method of Peroutka and Snyder, (1981) with some modifications. In brief, washed rat frontal cortex membranes, as described in section 2.1.1a, were incubated with [^{3}H]-mianserin for 45min at 25^{o}C in a final volume of 1ml. Saturation assays were performed using a concentration range of 0.5-10nM [^{3}H]-mianserin whereas competition experiments employed a fixed concentration of 1nM [^{3}H]-mianserin. Specific binding was determined using 2μm methysergide and represented approximately 50% of total binding at 1nM [^{3}H]-mianserin. Assays were performed in the presence of the H_1 antagonist triprolidine (0.3μm).

2.1.3h [^{3}H]-LSD binding to human platelet membranes

Experiments were carried out using a modification of the assay described by Geaney et al, (1984). Assay tubes containing 0.5ml of the platelet membrane suspension (approximately 1ml whole blood) were incubated with 100μl of [^{3}H]-LSD in the absence or presence of a displacing agent in a final volume of 1ml for 4h at 37^{o}C. Saturation curves were performed using a concentration range of 0.25-2.5nM [^{3}H]-LSD whereas competition curves employed a fixed concentration of 0.5nM. Specific binding was determined using 2μm methysergide or 0.5μM spiperone and represented approximately 65% of total binding at 0.5nM [^{3}H]-LSD.

2.1.3i [^{125}I]-LSD binding to vascular smooth muscle

Aliquots of the membrane preparation were incubated with [^{125}I]-LSD (0.01-4nM) in a final volume of 250μl for 60min at 37^{o}C. Specific binding was determined in the presence of 2μm methysergide. Competition experiments were performed using a fixed concentration of [^{125}I]-LSD (0.2nM). The reaction was terminated by immediate filtration over Whatman GF/B filters that had been prewashed with buffer. The filters were washed twice with 5ml ice-cold buffer and counted in a LKB 1282 gamma counter.

2.1.3j [^{125}I]-LSD binding to rat frontal cortex membranes

[^{125}I]-LSD binding to rat frontal cortex membranes was performed esentially as described above. A protein concentration of 100μg was found to be adequate.

2.1.4 <u>5HT binding sites in the gerbil brain</u>

An investigation of the characterisation and distribution of 5HT binding sites in the gerbil brain was undertaken. Gerbil brain membranes were prepared as described in section 2.1.1a and the assays performed as described in section 2.1.1b. Initial studies established the normal binding characteristics of the gerbil 5HT binding sites before assessing the effects of ischaemia.

1. Non-recovery model

The effect of 3h unilateral carotid ligation on the affinity and number of $5HT_2$ binding sites in the gerbil frontal cortex was determined. The surgery was carried out as described in section 2.3.1a. After 3h the animals were killed, the brains rapidly removed and the frontal cortex separated into left and right hemispheres and stored under liquid nitrogen until required. Tissues were pooled, 2 right or left frontal cortices, for each membrane preparation and $5HT_2$ binding assays were performed using [^{3}H]-ketanserin as described in sections 2.1.1 and 2.1.3f.

Striatal tissue from the above study was retained so that the effect on $5HT_{1B}$ binding sites could also be assessed. The sites were labelled by [^{125}I]-CYP and the assay conditions were as described in section 2.1.1 and 2.1.3e.

The effect of 3h death on $5HT_2$ binding sites

3h unilateral ischaemia is a very severe model, it was therefore necessary to investigate if the changes seen were in any way similar to 3h postmortem changes in $5HT_2$ binding sites. Death was induced by cervical dislocation, air embolism or decapitation. Animals were maintained at 37^{o}C for 3h before dissecting out the frontal cortex and assaying the tissue as described in sections 2.1.1 and 2.1.3f.

2. Recovery model

The recovery 5min bilateral ischaemia model was carried out as described in section 2.3.1b. After 72h the animals were again anaesthetised with 5% halothane in a 70% nitrous oxide / 30% oxygen mix, decapitated and the brains rapidly removed and the frontal cortex and hippocampus dissected out. Frontal cortices from sham-operated or ligated animals were used to determine the effect on $5HT_2$ binding sites.

Hippocampal tissue (3/membrane preparation) was used to determine the effect on the affinity and density of $5HT_{1A}$ binding sites. Assays were carried out using [^{3}H]-8-OH-DPAT as described in section 2.1.1 and 2.1.3c. Additional studies were performed using [^{3}H]-WB4101 and [^{3}H]-5HT, the later ligand was also used to look at the effect of 10min bilateral ligation followed by 1 or 2h reperfusion.

2.1.5 <u>5HT binding sites in peripheral tissues</u>

2.1.5a [^{3}H]-5HT binding to rat lung membranes

Membranes from rat parenchymal lung tissue were prepared essentially as described in section 2.1.1a with the exception that it proved necessary to filter the membranes through cheesecloth after every wash to remove connective tissue. Saturation binding assays were performed as described in sections 2.1.1 and 2.1.3a using a range of concentrations of [^{3}H]-5HT (0.2-12nM).

2.1.5b $5HT_1$ receptor on guinea-pig and rabbit atria

Saxena et al (1985), demonstrated the existence of $5HT_1$ receptors in cat heart tissue. A study was undertaken to assess $5HT_1$ binding in guinea-pig and rabbit heart tissue.

Guinea-pig and rabbit atrial membranes were prepared as described in section 2.1.1a for the rat cerebral membranes, except that as for the rat lung membranes, the homogenate was filtered through cheesecloth before each centrifugation. Saturation assays were performed using a concentration range of 0.5-20nM [^{3}H]-5HT.

2.1.5c $5HT_2$ receptors on human platelets

Human platelet membranes were prepared as described in section 2.1.1a, and assays performed using [^{3}H]-LSD as described in section 2.1.3h.

2.1.5d Pig Coronary Artery

Membranes were prepared as described in section 2.1.1a. A modification of the assay described by Engel et al, (1984a), for the binding of [^{125}I]-LSD to guinea-pig ileum was used in these experiments to study the 5HT receptor subtypes on PCA membranes. Assays were performed as described in section 2.1.3i.

2.1.5e Human Umbilical Artery

The human umbilical artery smooth muscle preparation has proved a useful method for studing $5HT_2$ receptors (McGrath et al, 1985) with the advantage of using a human tissue. These experiments describe direct binding studies to HUA. Assays were performed essentially as described previously using [^{125}I]-LSD as radioligand and 2μm methysergide to define non-specific binding.

2.1.5f [^{3}H]-Ketanserin binding to rat lung membranes

Membranes from rat parenchymal lung tissue were prepared as described in section 2.1.5a. Saturation assays were performed as described in sections 2.1.1 and 2.1.3f using a range of concentrations of [^{3}H]-ketanserin (0.05-5nM).

2.1.6 <u>Alpha-adrenoceptor binding studies</u>

2.1.6a Membrane preparation

Male Sprague-Dawley rats (150-200g) were killed by cervical dislocation, the brains rapidly removed and dissected on ice. Cerebral cortices or hippocampi (5/membrane) were homogenised in 25 volumes of Tris buffer (50mM Tris HCl, 5mM EDTA: pH 7.4 at 25^{o}C) using a polytron PT 10 tissue disrupter (setting 10; 2 x 10s bursts). The homogenate was then centrifuged at 38000xg for 15min. After a second wash, the pellet obtained was resuspension Tris assay buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4 at 25^{o}C) before undergoing two further washes. The final pellet was resuspended in assay buffer for direct use in binding assays or stored under liquid nitrogen.

2.1.6b α-adrenoceptor binding assays

1. [^{3}H]-Prazosin binding to rat cerebrocortical membranes

α_1-adrenoceptor competition binding assays were performed by incubating washed rat cortex membranes (300μg

protein) with [^{3}H]-prazosin (0.5nM) in the presence or absence of the competing ligand in a total volume of 1ml Tris assay buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was defined as the concentration of bound ligand in the presence of 10μm phentolamine. Following equilibrium (30min at 25^{o}C) bound ligand was separated from free by vacuum filtration over Whatman GF/B glass fibre filters, which were then washed with 4 x 5ml ice cold buffer. Radioactivity was determined as described in section 2.1.1e and data analysed as described in section 2.1.1k.

Saturation assays were performed under identical conditions using a range of concentrations of [^{3}H]-prazosin (0.01-8nM).

2. [^{3}H]-Yohimbine binding to rat cerebral membranes

α_2-adrenoceptor competition binding assays were performed by incubating washed rat cerebral membranes (500μg protein) with [^{3}H]-yohimbine (1.5nM) in the presence or absence of a range of 12 concentrations of the competing ligand in a total volume of 500μl Tris assay buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4 at 25^{o}C). Non-specific binding was defined as the concentration of bound ligand in the presence of 10μm phentolamine. Specific binding represented 60-70% of total binding at 1.5nM [^{3}H]-yohimbine. Following equilibrium (30min at 25^{o}C), bound radioactivity was separated and determined as described above, or using the Brandel cell harvester as described in section 2.1.1c. Saturation assays were performed under similar conditions using a range of concentrations of [^{3}H]-yohimbine (0.1-15nM).

3. [^{3}H]-Idazoxan binding to rat cerebral membranes

Initial experiments were performed under identical conditions to [^{3}H]-yohimbine binding assays, using a Tris HCl buffer system (50mM Tris HCl, 0.5mM EDTA; pH 7.4 at 25^{o}C). Membrane were incubated with [^{3}H]-idazoxan (1nM) to equilibrium (15min) at 25^{o}C in a final volume of 1ml. Specific binding was determined using 3μm phentolamine and represented 55-65% of total binding at 1nM [^{3}H]-idazoxan. Saturation experiments were carried out under identical

conditions using a range of concentrations of [^{3}H]-idazoxan (0.1-12nM). Membranes were collected through Whatman GF/B filters, which were washed with 2 x 5ml Tris buffer (50mM Tris HCl, pH 7.4 at 25^{o}C), and the radioactivity determined as described above.

3. [^{3}H]-Yohimbine binding to human platelet membranes

Membranes were prepared as described in section 2.1.1a. The binding assay used was a modification of the assay described by Cheung et al, (1982) and very similar to the assay described above for [^{3}H]-yohimbine binding to rat cerebral membranes. Briefly platelet membranes (1ml whole blood/ assay tube) were incubated with [^{3}H]-yohimbine (1.5nM) in the presence or absence of the competing drug for 30min at 25^{o}C in a Tris HCl assay buffer (50mM Tris HCl, 0.5mM EDTA, pH 7.4) in a final volume of 500μl. Specific binding was determined using 10μm phentolamine and represented 75-85% of total binding at 1.5nM [^{3}H]-yohimbine. Bound radioactivity was separated and determined as described above. Saturation assays were performed under identical conditions using a range of concentrations of [^{3}H]-yohimbine (0.1-15nM).

4. [^{3}H]-Idazoxan binding to human platelets

Platelet membranes were prepared as described in section 2.1.1a and incubated with [^{3}H]-idazoxan in a Tris HCl or physiological buffer using the conditions for the assay described above for [^{3}H]-idazoxan binding to rat cerebral cortex.

2.1.6c The effect of assay buffer constituents on [^{3}H]-yohimbine and [^{3}H]-idazoxan binding

Preliminary studies were undertaken to assess the binding characteristics of the two radioligands using the following incubation mediums:-

1. Tris HCl buffer pH 7.4 at 25^{o}C.
2. Physiological salt solution pH 7.4 at 25^{o}C containing (mM): NaCL, 118; KCL, 4.8; $CaCL_2$, 1.3; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; equilibrated at 25^{o}C with 95% O_2 / 5% CO_2 before use; pH 7.4.
3. Tris HCl buffer pH 7.4 at 25^{o}C containing 2.6mM Ca^{2+}
4. Tris HCl buffer pH 7.4 at 25^{o}C containing 5mM Mg^{2+}

2.1.6d The effect of chemical modifying agents on α_2-adrenoceptor binding sites

Membrane pretreatments: Membranes (1-4mg/ml protein) were preincubated with 1 or 10mM N-ethylmaleimide (NEM) or phenylglyoxal (PGL) in 50mM Tris buffer (pH 7.4) in a final volume of 0.5ml. Preincubations were performed at 37°C for 20min and terminated by addition of 0.5ml ice cold buffer containing 8% (w/v) polyethyleneglycol and centrifuged for 1min at 30000xg. The membranes were washed twice more and finally resuspended in buffer only for use in the binding assays. This method was adapted from Mattens et al, (1984).

Saturation binding assays and competition assays were performed with control and pretreated rat cortex and hippocampus membranes using [^{3}H]-yohimbine to label the α_2-adrenoceptors as described in section 2.1.6b.

2.2 5HT functional assays

2.2.1a 5HT autoreceptor

Presynaptic receptors regulating 5HT, can be studied using K^+ - evoked release of [^{3}H]-5HT from preloaded rat frontal cortex slices.

1. Preparation of slices

Rat frontal cortices were rapidly dissected and chopped at 300μm intervals in two directions using a McIlwain tissue chopper. The slices were transferred to 5ml of gassed Krebs buffer (mM): 118 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 1.3 $CaCl_2$, 10 glucose, 0.06 ascorbic acid, 0.03 disodium EDTA; gassed with 95%/5% O_2/CO_2 at pH 7.4) containing 1μm pargyline. After a 15min preincubation period at 37°C, the slices were washed three times resuspended in 5ml Krebs buffer containing 0.1μm [^{3}H]-5HT and incubated for 30min. The slices were then washed three times with cold Krebs buffer and incubated for a further 30min to allow efflux of [^{3}H]-5HT to reach equilibrium.

2. Assay

Drugs were added to the slices at 2min (agonists) or

12min (antagonists) before exposure to elevated levels of K^+ (60K). All experiments using agonists were performed in the presence of 5μM of the uptake blocker imipramine. After 5min incubation at 37^{o}C, the slices were pelleted by a 2min centrifugation at 1000xg (or by rapid filtration over Whatman GF/B filters supported on a 12 port manifold system). The samples were then stored on ice where appropriate to prevent further release. Aliquots (250μl) were prepared for scintillation counting. The remaining medium was aspirated and the tissue pellet dissolved in 500μl Soluene before counting.

2.2.1b Platelet aggregration

Human blood from male and female volunteers was collected in 3.9% sodium tricitrate (ratio 9:1). Platelet rich plasma (PRP) was obtained by spinning the blood at 200xg for 10min. The PRP was adjusted to $2.5x10^5$ - $5x10^4$ platelets mm^3, with platelet poor plasma (PPP) on a platelet coulter counter. A sample of PPP was obtained by a second spin of PRP at 1440xg for 10min.

The measurement of platelet aggregation was performed using a PAP4 aggregometer. The rate and extent of aggregation were assessed by measuring the slopes of percentage transmission, 100% transmission set using PPP.

Platelet aggregations were carried out in response to 5HT (10μM) as the amplification effects of 5HT were found to be greatest on responses induced by threshold concentration of collagen. The ability of various antagonists to inhibit the 5HT amplification of platelet aggregation induced by collagen was examined.

Aliquots of PRP were pre-incubated at 37^{o}C with the test compound or distilled water for 3min before the addition of collagen which again was allowed to equilibrate for 3min before 5HT was added. The reaction was followed for a further 3min.

2.2.1c 5HT mediated contraction of PCA

Pig hearts were obtained from the abattoir. The lateral descending coronary arteries were removed and cut into helical strips (2x15mm). Four preparations were obtained from each coronary artery. The strips were suspended in

30ml organ baths containing Krebs salt solution (NaCL 118.93mM, KCL 4.69mM, $MgSO_4$ 1.01mM, $KHPO_4$ 1.18mM, $NaHCO_3$ 25mM, $CaCL_2$ 2.5mM, 11.1mM glucose; pH 7.4, gassed with 5% CO_2 in 95% oxygen) at 37°C. The tension of the strips was recorded isometrically, with the strips stretched to an initial tension of 500mg. The strips were allowed to equilibrate for 2h, during which time the bathing medium was changed every 15min. During this time the resting tension of the strips declined to about 100 to 200mg which then remained constant throughout the experiment. Pargyline (10μM) was added to the baths 30min before the agonist to prevent inactivation by monoamine oxidase. In each experiment two cumulative concentration response curves (CRC's) for 5HT were established. Graded concentrations of antagonist were added to 3 of the strips, whilst the 4th strip acted as control, and were allowed to act for 30min before a third 5HT CRC was determined. The increase in tension was plotted against agonist concentration on a log scale. The degree of antagonism was qualified in terms of the dose-ratio, which was determined by the ratio of the EC_{50} values before and after the additions of antagonist. Correction was made for any spontaneous change in sensitivity by dividing the dose-ratio obtained following the antagonist by the dose-ratio obtained in the control strip.

The results were plotted graphically in the form of a Schild plot (Arunlakshana and Schild, 1959) for the estimation of pA_2 values. The slope and intercept were also assessed using linear regression.

2.3 Gerbil stroke models

2.3.1a Non-recovery unilateral model

The availability of a suitable animal model facilitates greatly experimental study of the disease and ultimately the pharmacological intervention necessary to arrest or cure the disease in man. The finding that the blood supply to the brain of the Mongolian gerbil is unique in lacking

communicating arteries which make up the circulus arteriosus has made it a suitable model for the study of stroke (Kahn, 1972). Ligation of the one common carotid artery has been reported to cause a unilateral hemispheric infarction (Levine & Payan, 1966) with an alteration in the level of neurotransmitters. Unfortunately only 30-50% of gerbils are susceptible to developing ipsilateral hemispheric ischaemia following unilateral carotid ligation. Therefore separation of stroke-prone from stroke-resistant animals must be achieved before any assessment of biochemical changes can be made.

Adult gerbils, weight range 60-80g, were anaesthetised with 6mg pentobarbital (i.p.) and the right common carotid artery exposed in the paratracheal region, dissected free of the accompanying vagosympathetic nerve trunk and ligated. In the sham-operated animals, the carotid artery was exposed, the ligature put in place but not tied. The animals were maintained under anaesthetic and given a top up dose of 1mg pentobarbital (i.p.) if required. After 3h the animals were decapitated, the brain rapidly removed and a microscopic (x40) examination of the anterior blood supply made on ice before separating the brain into left and right hemispheres and dissecting out the appropriate regions.

2.3.1b Recovery bilateral model

A second model, 5min global ischaemia and 72h recovery, involves the bilateral occlusion of both common carotid arteries. Global models of ischaemia such as the rat 4 vessel occlusion (4VO) model (Pulsinelli and Brierley, 1979) are difficult to produce because in addition to ligating the carotid arteries, the vertebral arteries have to be cauterised which is both surgically demanding and time consuming. However, ligation of both common carotid arteries in the gerbil produces a global cerebral ischaemia in all animals with an inadequate anastomosis between the anterior and posterior circulatory systems; this is suggested to be 60% by Levy and Brierley, (1974) and estimated to be closer to 80% in these studies.

Adult gerbils weight range 60-80g, were anaesthetised

with 5% halothane in a 70% nitrous oxide / 30% oxygen gas mixture. Halothane was then reduced to 1.5%, to minimise the effect of the anaesthetic, and maintained at this level for the remainder of the surgical procedure. The left and right common carotid arteries were exposed through a paratracheal incision and simultaneously occluded with microvascular clamps. The clamps were removed after 5min, the wound dusted with antibiotic powder and surgically closed. Animals regained consciousness within 2min. Seventy two hours following induction of the 5min period of ischaemia, the animals were killed, the brains rapidly removed and dissected on ice. Identical surgical procedures were carried out on the sham-operated animals with the exception of the occlusion of the carotid arteries.

2.3.2 The effect of unilateral occlusion on the electroencephalogram of the Mongolian gerbil

This study was to determine if a correlation exists between inhibition of the electroencephalogram (EEG) activity and cerebrovascular deficit. Gerbils weighing 60-80g, were anaesthetised with 5% halothane in a 70% nitrous oxide / 30% oxygen mixture. After induction the halothane concentration was reduced to 1.5% and maintained at this level throughout the remainder of the experiment. The EEG was recorded by subcutaneous needle electrodes between the left and right temporal region. Following recording of a control trace the right or both common carotid arterys were ligated as described above and recording resumed. At termination of the experiment, the brain was perfuse fixed with 10% formal saline with added Indian ink by left ventricular injection. The circulus arteriosus was examined microscopically for anatomical classification.

2.3.3 The effect of cerebral ischaemia on 5HT levels

These studies describe a simple subjective system for assessing the possibility of stroke in an animal by analysis of 5HT changes and where necessary by an anatomical investigation of the connections that make up

the circulus arteriosus.

Surgical procedures were carried out as described for the non-recovery gerbil stroke model. After a 30min, 1h or 3h unilateral ligation, the anterior vascular anatomy of the animals was carefully graded before dissecting out the the left and right frontal cortex or corpus striatum, which were then weighed before being homogenised in 2ml 0.1M perchloric acid and stored under liquid nitrogen until required. The effect of a 5min bilateral occlusion of both common carotid arteries on the 5HT and 5HIAA levels in the frontal cortex was also determined for direct comparison with binding data.

Determination of 5HT levels

To 1ml of sample, 3ng dihydroxybenzylamine (DHB) was added as internal standard. To measure 5HT, samples were centrifuged at 9000xg for 5min and 100µl of the supernatant was injected onto a high performance liquid chromatography system (HPLC) coupled to a coulometric electrochemical detector (ECD) maintained at a carbon electrode voltage of +0.4 V. Indolamine separation was achieved by reverse-phase chromatography using an octadecyl polymer coated silica column (Ultrasphere i.p., 250 x 4.6mm) and a short pre-column (60 x 5mm i.d.) packed with an octadecyl coated pellicular support (CO: Pell ODS, Whatman). The mobile phase was composed of a water:methanol mixture (90:10 v/v) containing 0.1mM KH_2PO_4, 0.3mM sodium octyl sulphonate and 0.1mM EDTA, and the flow rate was maintained at 1ml/min. All chemicals used were HPLC grade. The mobile phase was gassed with helium and filtered before use.

5HT and 5-hydroxyindole acetic acid (5HIAA) standards were assayed under the same conditions at concentrations of 5, 2, 1, and 0.5ng/100µl. New standards were made up every day and remained stable for up to 8h if kept on ice and in the dark.

Breakdown products of [^{3}H]-5HT, after passing through the column, were collected at 30s intervals and the radioactivity determined by liquid scintillation spectrometry.

2.3.4 Functional correlates for changes in 5HT binding

2.3.4a The effect of the 5min bilateral ligation followed by 72h recovery on the number of quipazine induced head shakes

Gerbils were assessed for head twitch behaviour pre- and post-ischaemia. The animals were divided into two groups, sham-operated and ligated, and control numbers of head shakes were determined. The head twitch behaviour was provoked by subcutaneous administration of quipazine (25mg/kg) in the back of the neck. 30min later the number of head twitches were counted over a 15min period. The animals were then rendered ischaemic, by 5min bilateral ligation, and recovered for 72h. They were retested 24 and 72h post-ischaemia. Identical surgical procedures were carried out on the sham-operated animals with the exception of the occlusion of the carotid arteries. Head twitches were counted without prior knowledge of the treatment given.

2.3.4b The effect of 3h unilateral ligation on the 5HT binding sites in the gerbil as assessed by autoradiography

Animals were subjected to a 3h unilateral ligation as described in section 2.3.1b. The animals were then killed, the brains rapidly removed and an assessment of the cerebral vasculature made. The brains were then prepared for autoradiographic visualisation of $5HT_2$ binding sites. 10 micron sections of frozen gerbil brain were prepared on a microtome cryostat and thaw-mounted onto gelatin-coated coverslips. Sections of frontal cortex were dried and incubated in Tris HCl buffer containing 1nM [^{125}I]-LSD for 60min at room temperature to label $5HT_2$ binding sites. Parallel sections were prepared containing 2μM methysergide to determine non-specific binding. Following the incubation the sections were washed for 2x10min in fresh ice cold buffer and dried under a cold air stream. The sections were then exposed to Hyperfilm ^{3}H for 4.5 days, in a Harvard cassette with rare earth intensifying screens. The resultant autoradiograms were developed and printed

onto photographic paper.

2.3.4c Behavioural observations

Animals were subjected to a 5min bilateral or unilateral ligation as described in section 2.3.1b and allowed to recover. The animals were then carefully observed for circling behaviour towards the lesioned hemisphere, which is associated with the development of cerebral infarction (Kahn, 1972) and for any evidence of "5HT behavioural syndrome" (Lucki et al, 1984; Tricklebank, 1985).

2.4 Drugs

Reagents used were of the highest analytical grade. Drugs were kindly donated by their manufacturers, synthesised at Syntex (Palo Alto) or purchased. The following drugs were used:-

(-)Adrenaline bitartrate (Sigma), BDF-6143 hydrochloride*, Bufotenine oxylate (Sigma), Buspirone hydrochloride@ (Bristol-Meyers), 5CT (Glaxo), Compound X*, Cyanopindolol*, Cyproheptadine hydrochloride (Merck, Sharp and Dohme), Desipramine hydrochloride (Geigy), 5,6-dihydroxytryptamine (Sigma), DP-5CT*, Domperidone (Janssen), Dopamine hydrochloride (Sigma), Histamine dihydrochloride (Sigma), 8-OH-DPAT bromide (Research Biochamicals), 5HT creatinine sulphate (Sigma), Idazoxan*, Imiloxan hydrochloride (Syntex), Imipramine hydrochloride (Sigma), Indoramin hydrochloride (Wyeth), Isoprenaline bitartrate (Sigma), Ketanserin tartrate (Janssen), d-Lysergic acid diethylamine (Sandoz), MDL72222 (Merrell Dow), Mianserin (Organon), Mesulergine (Sandoz)*, Methysergide bimaleate (Sandoz), MJ 13653*, N-ethylmaleimide (Sigma), (-)Noradrenaline bitartrate* (Sigma), Oxymetazoline hydrochloride*, Panuramine (Wyeth), Phentolamine mesylate (Ciba), Pindolol (Sandoz), Phenylglyoxal (Sigma), Prazosin hydrochloride (Pzifer), Propranolol hydrochloride (ICI), Quipazine maleate (Miles), Rauwolscine hydrochloride (Carl Roth), Ritanserin

(Janssen), RU24969 (Roussel), Spiperone (Janssen), TFMPP hydrochloride (Research Biochemicals), Triprolidine hydrochloride (Wellcome), UK 14304 tartrate (Pzifer), WB4101 (Ward Blenkinsop), WY 26703 hydrochloride (Wyeth), Yohimbine hydrochloride, (Sigma), Zimeldine hydrochloride (Astra).

Radioligands:-

Amersham

[^{3}H]-5HT (20Ci/mmol), [^{3}H]-8-OH-DPAT (206Ci/mmol),
[^{3}H]-WB4101 (21Ci/mmol), [^{125}I]-CYP (1890Ci/mmol),
[^{3}H]-lSD (11.8Ci/mmol), [^{3}H]-Idazoxan (60Ci/mmol),
[^{125}I]-LSD (2000Ci/mmol).

Dupont

[^{3}H]-Ketanserin (61.8Ci/mmol),
[^{3}H]-Mianserin (10.2Ci/mmol),
[^{3}H]-Yohimbine (87Ci/mmol),
[^{3}H]-Prazosin (82Ci/mmol).

* Compounds synthesised by Syntex (Palo Alto).

@ This compound is a novel drug still undergoing research within the company. At such time when the drug comes under a patent this thesis shall be updated to include the available data. It is the same compound referred to by S.J. MacLennan (1986).

AIMS OF THESIS

During the course of this thesis an enormous amount of work on 5HT receptors has been published. When appropriate, experiments have been designed to take advantage of as well as add to current knowledge.

The initial objective of this thesis was the characterisation of 5HT subtypes using ligand binding techniques. Methods were devised for the study of :-

1. $5HT_1$ binding sites
2. $5HT_2$ binding sites

Once specific and selective assays had been established, the aims of the study were then to evaluate:-

3. functional correlates for 5HT binding sites
4. the effect of cerebral ischaemia on 5HT neuropharmacology
5. possible 5HT/NA interactions, including the possibility of α_2-adrenoceptor subtypes

Chapter 3: STUDIES ON $5HT_1$ RECEPTOR SUBTYPES

3. STUDIES ON $5HT_1$ RECEPTOR SUBTYPES

3.1 Introduction

Ligand binding studies have shown that there are at least four $5HT_1$ binding sites, which have been designated $5HT_{1A}$, $5HT_{1B}$, $5HT_{1C}$ and $5HT_{1D}$. Although Bradley et al, (1986) hesitated to include the $5HT_1$ subtypes in their functional classification, referring to all such receptors as "$5HT_1$-like", it is now clear that functionally distinct $5HT_1$ subtypes occur and that these show a similar pharmacological profile to the $5HT_1$ binding sites.

$5HT_1$ Binding sites

$5HT_1$ binding sites all show nanomolar affinity for 5HT and can be labelled using [^{3}H]-5HT, unlike $5HT_2$ and $5HT_3$ binding sites which show approximately 400 and 25 fold lower affinity respectively.

$5HT_{1A}$ binding sites are found in peripheral and central tissues in many species. They are widely distributed in the brain, particularly in the hippocampus, and are located both pre- and post-synaptically (Hall et al, 1985). Several compounds show selectivity for this site including N,N Dipropyl 5-carboxamidotryptamine (DP-5CT) (Hagenbach et al, 1986), 8-OH-DPAT (Hjorth et al, 1982), buspirone (Hjorth and Carlsson, 1982) and ipsapirone (Glaser et al, 1985). Activation of central $5HT_{1A}$ receptors results in neuronal inhibition, hypotension and bradycardia, aspects of the 5HT behavioural syndrome, hyperalgesia, hyperphagia and induction of a discriminative stimulus. $5HT_{1A}$ peripheral receptors mediate enteric neuronal inhibition and contraction of certain blood vessels, such as the basilar artery in the dog (Peroutka et al, 1983).

The $5HT_{1A}$ site is usually studied in the rat cortex or hippocampus using [^{3}H]-8-OH-DPAT. It is controversial as to whether $5HT_{1A}$ compounds such as 8-OH-DPAT, buspirone and ipsapirone act as agonists, partial agonists or antagonists. On [^{3}H]-acetylcholine release from

electrically stimulated guinea-pig ileum, buspirone and ipsapirone act as antagonists whereas 8-OH-DPAT acts as a partial agonist (Fozard and Kilbinger, 1985). All three compounds act as agonists at the $5HT_{1A}$ presynaptic receptor located on serotonergic cell bodies in midbrain raphe nuclei (Hutson et al, 1986). However, an antagonist/ partial agonist action has been described for buspirone for the reversal of 8-OH-DPAT-induced behavioural syndrome (Clague and Spedding, 1987). Hypothermia following 8-OH-DPAT is suggested as a possible index of pre-synaptic $5HT_{1A}$ function (Goodwin et al, 1987) although disputed by Hutson et al, (1986). The '5HT behavioural syndrome' following 8-OH-DPAT is suggested as an index of post-synaptic $5HT_{1A}$ receptor sensitivity (Goodwin et al, 1987).

$5HT_{1B}$ binding sites are found in rodents (Pedigo et al, 1981), although controversy exists as to whether they are also found in man (Middlemiss et al, 1986). Several compounds show high affinity for this site such as 5CT (Saxena and Verdouw, 1985), RU24969 (Euvrard and Boissier, 1980) and some β-blockers e.g. propranolol, pindolol (Middlemiss et al, 1977; Nahorski and Willcocks, 1983), although none of these compounds are selective. Recently a selective $5HT_{1B}$ ligand: CGS 12066B, has been introduced (Neale et al, 1987). Some evidence suggests that the $5HT_{1B}$ subtype may be the inhibitory autoreceptor (Engel et al, 1986). However, a postsynaptic binding site has been identified in the rodent brain which is inhibited by $5HT_{1B}$ compounds RU24969 and 5CT but not by the β-adrenoceptor antagonists. The $5HT_{1B}$ site is generally studied in rat cortex or striatum using [^{125}I]-iodocyanopindolol ([^{125}I]-CYP) in the presence of 30μM isoprenaline (Hoyer et al, 1985a). The $5HT_{1B}$ binding site is widespread in rodent brain.

$5HT_{1C}$ binding sites are found in the brain of a number of species particularly higher mammals such as man and pig (Pazos et al, 1984a and b). Large numbers are found in the

choroidal plexus suggesting a possible link with CSF production although this has still to be established. A few compounds show high affinity for this site although, as yet, no selective compound has been identified. The $5HT_{1C}$ site is generally studied in the rat or pig choroidal plexus using [^{3}H]-mesulergine as the radioligand (Pazos et al, 1984b). A recent interesting report describes the successful cloning of the $5HT_{1C}$ receptor cDNA and the expression of functional $5HT_{1C}$ receptors in Xenopus oocytes injected with rat brain mRNA (Lubbert et al, 1987).

A $5HT_{1D}$ subtype has been suggested to exist because 50-70% of specific, high affinity $5HT_1$ binding of [^{3}H]-5HT in the bovine brain cannot be described as 1A, 1B or 1C (Heuring and Peroutka, 1987). The site is characterised by high affinity for 5CT, intermediate affinity for RU24969 whereas 8-OH-DPAT is weak and the β-blockers are inactive. The site is widespread in bovine and human brain particularly in the globus pallidum and the substantia nigra. At present there is no known functional correlate but there are many "$5HT_1$-like" responses which show some pharmacological similarities (Cohen and Fludzinski, 1987; Charlton et al, 1986).

As described in the introduction, 5HT binding studies were first carried out by Bennett and Snyder, (1976) using [^{3}H]-5HT and [^{3}H]-LSD. These were the only radioligands available for the study of $5HT_1$ binding sites at the start of this thesis. [^{3}H]-5HT was used in early studies to investigate further $5HT_1$ binding sites.

An extensive study of the binding of [^{3}H]-5HT to rat cerebral membranes was undertaken and included:-

1. a careful assessment of ligand binding assays.
2. a detailed assay development; equilibrium binding assays, binding kinetics, the influence of assay conditions including pH, temperature, buffer ions and other modulators.
3. a large number of competition curves were generated to determine if multiple "states" or multiple binding

sites were present.

Binding assays were established for the $5HT_{1A}$ and $5HT_{1B}$ binding sites in the rat cortex, hippocampus and striatum. [^{3}H]-5HT proved to be unstable, therefore more stable and selective radioligands were used as they became available. A comparison of the binding characteristics of $5HT_1$ subtypes was made using:- [^{3}H]-5HT, [^{3}H]-WB4101 and [^{3}H]-8-OH-DPAT to study the $5HT_{1A}$ binding site and [^{3}H]-5HT and [^{125}I]-CYP to study the $5HT_{1B}$ binding site.

The characterisation and distribution of $5HT_1$ binding sites in the gerbil brain was undertaken. The gerbil was chosen as it is a useful model for examining the effects of cerebral ischaemia on receptors and receptor mediated processes, (see chapter 5).

A number of attempts were made to establish $5HT_1$ binding assays using peripheral tissues in the rat, namely heart and lung.

Finally, where possible, functional correlates for the $5HT_1$ binding sites were undertaken.

The figures and tables are included at the end of the results section.

3.2 Results

3.2.1a Equilibrium binding studies

Preliminary results

Preliminary studies indicated that [^{3}H]-5HT bound to a saturable population of binding sites on rat cerebrocortical membranes with nanomolar affinity. However, the binding was complex and required careful resolution. The first problem encountered was the chemical instability of the [^{3}H]-5HT ligand available. Fig. 3.1 shows the composition of [^{3}H]-5HT stored for two months in a sealed vial at 4^{o}C. Separation of [^{3}H]-5HT by HPLC indicated that some of the breakdown products were electrically active i.e. gave a signal on the electro-chemical detector. Comparison of the HPLC trace and the radioactivity determined from the collected eluates indicated that almost all (90%) of the accumulated electroactive peaks were radioactive, although only 33% of the radioactivity was 5HT. Therefore care had to be taken at all times to store the [^{3}H]-5HT as recommended, to use only at > 96% purity and to prevent breakdown of the ligand during the assay. Inclusion of ascorbic acid in the assay was found to prevent the decomposition of [^{3}H]-5HT, however, ascorbate itself has effects on the $5HT_1$ binding assay (see below).

3.2.1b Separation of bound from free ligand

Preliminary kinetic experiments indicated a relatively slow rate of dissociation ($t_{1/2}$ > 1min) of [^{3}H]-5HT from washed cerebral membranes indicating the suitability of using vacuum filtration procedures for separating bound from free ligand. Specific binding of [^{3}H]-5HT was not markedly different when filtration was performed using 2-5 x 5ml washes with buffer at room temperature (fig. 3.2). Washing with room temperature or ice cold buffer made no difference to the recoverable amount of specific binding. However, the optimum conditions was the use of 3 x 5 ml washes with buffer at room temperature.

Care was taken to close the vacuum after filtering each

manifold and to remove the lid and dry the filters. This was carried out in order to prevent any further dissociation of the ligand-receptor complex due to residual moisture left on the filter. The vacuum was applied at a constant 22mm Hg. Higher vacuum pressure resulted in a significant decrease in recoverable specific binding probably due to protein loss through the glass fibre filter. The vacuum was applied for 1min before filtering and a 5ml wash used to wet the filter. Any port filtering too quickly or too slowly was discarded from the assay.

Later assays were developed using a 24 place Brandel cell harvester (M-24R) to separate bound from free. In this method 2 x 10s washes (flow rate 1.5L/min) with room temperature buffer was found to be the optimum conditions for filtration. The vacuum was applied at a constant 22mm Hg and was opened 30s before filtering and a 10s wash used to wet the filter. After filtering the filters were dried for 5s under vacuum. The Brandel proved to be advantageous allowing 24 samples to be filtered and dried under identical conditions with a higher recovery of counts. In addition the Brandel gave rise to greater reproducibility than that obtained through millipore manifolds.

3.2.1c Filter binding of ligands

Filter binding to Whatman GF/B filters of [^{3}H]-5HT was negligible through the saturation range of 0.2-12nM. Antiabsorbants applied to the filter such as bovin serum albumin (2%) had no significant effect on the amount of recoverable specific binding and therefore were not used in these studies. Competing ligands at the maximal concentrations used in displacement studies had no significant effect on filter binding.

3.2.1d Tissue linearity of binding

Specific binding of [^{3}H]-5HT (2nM) was linear over a protein level of 50-1200μg. In all studies a protein level well within this range was used.

3.2.1e Kinetic experiments

Association

[^{3}H]-5HT (2nM) bound rapidly to rat cerebrocortical membranes (500μg) at 37^{o}C reaching half maximal binding in 1.2min and attaining equilibrium in 3min (fig. 3.3). All subsequent experiments were performed using a 10min incubation period.

Dissociation

The binding was found to be completely reversible since, when excess unlabelled 5HT (10μM) was added, the bound [^{3}H]-5HT was completely dissociated in 3min at 37^{o}C (fig. 3.3). The half life for dissociation of bound [^{3}H]-5HT was difficult to determine at 37^{o}C. However, at 22^{o}C a dissociation rate constant (K_{-1}) of 0.0025/s was calculated. The association rate constant at 22^{o}C was $3x10^5$/M/s. Therefore the dissociation constant (Kd) determined from the ratio of K_{-1}/K_1 was found to be 8.3nM.

3.2.1f Definition of non-specific binding

5HT itself was found to displace [^{3}H]-5HT from rat cerebral membranes in a monophasic manner with a Hill slope not significantly different from unity (see table 3.5). Displacememt of specific binding reached a plateau at 2μM, (fig. 3.4), and remained constant up to 200μM. For this reason 5HT at 10μM (>1000 fold excess) was used to define non-specific binding in all subsequent studies. Under these conditions 75% specific binding was available at 2nM [^{3}H]-5HT.

3.2.1g Saturation binding assays

[^{3}H]-5HT was found to bind with high affinity to rat cerebral cortex (Kd 4.8±0.4nM; Bmax 290±6fmol/mg protein) (fig. 3.5) and rat striatum (Kd 4.55±0.5nM; Bmax 395±12fmol/mg protein) when assayed at 37^{o}C using a concentration range of 0.2-12nM. The nsb, defined by 10μM 5HT, was approximately linear over this concentration range. Scatchard transformations of the specific binding isotherm showed a very slight upward curvature. This could not be statistically resolved and therefore no multiple [^{3}H]-5HT binding sites could be assumed from saturation binding data, although the possibility of two or more

binding sites with approximately the same affinity for [^{3}H]-5HT could not be ruled out.

Fillion et al, (1978) suggested a second low affinity binding site for [^{3}H]-5HT (Kd 10-30nM). Under the conditions described in this study no binding of [^{3}H]-5HT to a low affinity site was observed.

3.2.2 In vitro modulation of specific [^{3}H]-5HT binding

3.2.2a Effect of GTP

Fig. 3.6 shows the Scatchard transformation of the saturation data for the binding of [^{3}H]-5HT to rat cerebral membranes in the presence or absence of 1mM GTP. GTP reduces significantly the affinity of [^{3}H]-5HT for the $5HT_1$ binding sites (Kd 4.8 to 8.2nM). In competition experiments in the presence of 1mM GTP all agonists/partial agonists tested (5HT, 5CT and 8-OH-DPAT) and $5HT_2$ antagonists showed a significant decrease in affinity (table 3.1). The results are presented as apparent IC_{50} values (i.e. IC_{50} values corrected for the free ligand concentration) because even in the presence of GTP all the compounds except 5HT itself showed Hill slopes of less than unity.

3.2.2b Effect of ascorbate

Specific [^{3}H]-5HT binding to rat cerebral cortex is decreased in the presence of 5.7mM ascorbate (table 3.2). However, if 4mM $CaCl_2$ is included in the incubation medium specific binding is maintained. The inclusion of EDTA (0.5mM) also protects against the deleterious effects of ascorbate (table 3.2).

3.2.2c Effect of ions

Monovalent cations, 1-50mM NaCl and 1-50mM KCl, were found to inhibit specific [^{3}H]-5HT binding and therefore were not used. However, divalent cations, Ca^{2+} and Mg^{2+}, over a concentation range of 1-10mM enhanced specific [^{3}H]-5HT binding, above this concentration the binding is inhibited (fig. 3.7). A concentration of 4mM divalent ions was used in all subsequent assays.

3.2.2d Equilibrium temperature

Specific binding of [^{3}H]-5HT to rat cerebrocortical membranes at 37^{o}C reached equilibrium within 10min, even

at the lowest ligand concentration. At 22°C equilibrium was reached within 30min with no significant difference in the Kd or Bmax (table 3.3).

3.2.2e Effect of pH

The effect of pH on specific [^{3}H]-5HT binding is shown in fig. 3.8. The binding was stable over the pH range 7.2-7.8 and then declined at higher pH values. A pH of 7.4 was chosen for all subsequent assays unless otherwise stated.

3.2.3 Specific 5HT$_1$ binding assays

3.2.3a 5HT$_1$ binding sites labelled by [^{3}H]-5HT

The earliest studies in this thesis used [^{3}H]-5HT to label 5HT binding sites in the rat cerebral cortex and corpus striatum. Spiperone was found to inhibit the binding of [^{3}H]-5HT in a biphasic manner (fig. 3.9) as first shown by Pedigo et al, (1981). Therefore a further investigation of the 5HT subtypes was undertaken.

1. Rat striatal membranes

Studies of the high affinity, spiperone sensitive [^{3}H]-5HT binding site were performed using 1μM spiperone to determine nsb. The binding site could be termed 5HT$_{1A}$ by the definition of Middlemiss, (1984). This work was carried out before the availability of selective 5HT$_{1A}$ ligands. Table 3.4 shows the affinity values of a wide range of compounds determined for this site. 5HT itself showed nanomolar affinity whereas other agonists NA, DA and histamine were inactive. α_2-Adrenoceptor antagonists, yohimbine and phentolamine showed low affinity. However, the β-adrenoceptor antagonists propranolol and pindolol showed moderate affinity. The 5HT$_2$ antagonists ketanserin, cyproheptadine and mianserin showed low affinity whereas D-LSD showed high affinity, and methysergide and spiperone showed moderate affinity. It was concluded that [^{3}H]-5HT under these conditions labels a site in the rat corpus striatum which is a 5HT$_1$ subtype, i.e. having high affinity for 5HT itself, and not a 5HT$_2$ subtype since it has moderately low affinity for classical 5HT$_2$ antagonists.

The affinity values of the same series of compounds were determined using [^{3}H]-5HT binding to rat corpus striatum in the presence of 1μM spiperone, to block the high affinity spiperone sensitive part of the binding. Nsb was defined using 10μM 5HT. The remaining portion of binding (approximately 55%) is to a $5HT_{1B}$ subtype (Middlemiss, 1984). The results are presented in table 3.4. 5HT was the only active agonist showing nanamolar affinity. D-LSD again showed high affinity and methysergide moderate affinity although both were lower than that obtained at the $5HT_{1A}$ site. Pindolol and propranolol showed higher affinity for the $5HT_{1B}$ binding site. From this data it was concluded that in the presence of 1μM spiperone [^{3}H]-5HT labels a $5HT_{1B}$ subtype.

2. Rat cerebrocortical membranes

Later studies were performed in the rat cortex using computer modelling to determine the $5HT_1$ subtypes.

5HT was found to displace [^{3}H]-5HT from rat frontal cortex membranes in a monophasic manner (table 3.5). However, all other compounds tested showed biphasic or triphasic displacement curves, as determined by computer-assisted analysis. The affinity values are presented in table 3.5. The greatest selectivity between the two sites was obtained with 8-OH-DPAT (>1,000 fold). Triphasic competition curves were obtained for 5CT (fig 3.10) and RU24969 against [^{3}H]-5HT binding to rat cortex membranes providing evidence that [^{3}H]-5HT labels 3 distinct $5HT_1$ sites in these membranes.

Therefore [^{3}H]-5HT was found to label clearly two distinct binding sites, $5HT_{1A}$ and $5HT_{1B}$. The subtypes could be distinguished by pharmacolgical manipulation of the assay or using computer assisted analysis. The $5HT_{1A}$ binding site was characterised by high affinity for 8-OH-DPAT, spiperone and methysergide whereas RU24969 and pindolol showed higher affinity for the $5HT_{1B}$ subtype (see also Table 3.12).

Other [^{3}H]-5HT high affinity binding sites?

The triphasic displacement observed for 5CT and RU24969 inhibition of [^{3}H]-5HT binding in the rat cerebral cortex provided evidence for a third high affinity binding site. However, because of the small percentage of binding it was difficult to fully characterise this site. An attempt to study this site was made using [^{3}H]-5HT in the presence of 100nM 8-OH-DPAT to block $5HT_{1A}$ and 100nM cyanopindolol to block $5HT_{1B}$ binding. Specific binding was defined using 10μM 5HT. Under these limited conditions, only 20% specific binding, 5HT still showed high affinity (IC_{50} 55nM) and the $5HT_2$ antagonists methysergide, mianserin and ketanserin showed moderate affinity, whereas the β-blockers pindolol and propranolol were inactive. The α-adrenoceptor antagonist yohimbine was also inactive.

Experiments to be discussed in chapter 6 showed [^{3}H]-yohimbine to label two distinct binding sites with nanomolar affinity. Yohimbine has also been reported to have moderately high affinity for the 5HT response in the rat fundus. Therefore can a high affinity 5HT binding site, which has nanomolar affinity for yohimbine, be detected in the rat brain. Yohimbine showed only low affinity for the three [^{3}H]-5HT binding sites described above, $5HT_{1A}$ (IC_{50} 1250nM), $5HT_{1B}$ (IC_{50} >10,000nM) and $5HT_{1C}$ (IC_{50} >10,000nM). However, no other high affinity [^{3}H]-5HT binding site could be detected in the rat cerebrum in the presence of known $5HT_{1A}$, $5HT_{1B}$ and $5HT_{1C}$ selective compounds (fig. 3.11).

Therefore the investigation for a yohimbine sensitive [^{3}H]-5HT binding was continued in a higher species. A fourth [^{3}H]-5HT binding site was reported to exist in pig and human brain (Hoyer et al, 1987a) and bovine brain (Heuring and Peroutka, 1987). Unlike the rat, in the presence of 100nM 8-OH-DPAT (to block $5HT_{1A}$ binding) and 100nM mesulergine (to block $5HT_{1C}$ binding) 30% specific [^{3}H]-5HT binding remains in the bovine frontal cortex. This binding was not blocked by 100nM cyanopidolol but was displaced by 5HT (7nM) and yohimbine (52nM).

Are any of the high affinity 5HT binding sites related to the high affinity 5HT uptake site?

Table 3.6 shows the affinity values of 5HT uptake blockers for the $5HT_{1A}$ and $5HT_{1B}$ binding sites labelled by 2nM [^{3}H]-5HT. The 5HT uptake blockers, although potent at inhibiting 5HT uptake are devoid of affinity at the $5HT_{1A}$ and $5HT_{1B}$ binding sites.

Further studies on subtypes of $5HT_1$ binding sites on rat cerebral membranes were performed using more selective radioligands.

3.2.3b $5HT_{1A}$ binding sites labelled by [^{3}H]-WB4101

In the presence of 30nM prazosin, [^{3}H]-WB4101 was found to label a single population of sites in the rat cortex (Kd 1.9±0.6nM; Bmax 121±20fmol/mg protein) and the rat hippocampus (Kd 1.25±0.3nM; 420±30fmol/mg protein). The site is characteristic of the $5HT_{1A}$ binding site as shown by its high affinity for 5HT and $5HT_{1A}$ selective compounds 8-OH-DPAT and buspirone (table 3.7). All inhibition curves were described adequately by a model assuming one binding site. The Hill coefficents determined were not significantly different from unity. It was found that a number of compounds showed higher affinity for the hippocampal $5HT_{1A}$ binding site, including 5HT and the $5HT_{1A}$ selective ligands 8-OH-DPAT, buspirone and WB4101 and the α_2-adrenoceptor antagonists yohimbine and rauwolscine. 5HT and yohimbine showed statistically significantly higher affinity for the hippocampus $5HT_{1A}$ binding site, $p<0.02$ and $p<0.01$ respectively.

3.2.3c $5HT_{1A}$ binding sites labelled by [^{3}H]-8-OH-DPAT

[^{3}H]-8-OH-DPAT was shown to label a saturable population of high affinity sites in the rat cortex (Kd 1.3±0.8nM; Bmax 102±10fmol/mg protein) and hippocampus (Kd 1.1±0.7nM; Bmax 534±18fmol/mg protein). 5HT was found to have high affinity (nM) for the [^{3}H]-8-OH-DPAT binding site as did $5HT_{1A}$ selective compounds 8-OH-DPAT, buspirone and WB4101 whereas the $5HT_2$ selective compounds ketanserin and the

$5HT_{1C}$/$5HT_{2}$ selective compound mesulergine were inactive (table 3.8). Compounds with high $5HT_{1B}$/$5HT_{1A}$ selectivity ratios were not available for this study. The Ki value determined for RU24969 is a log order less than its reported affinity for $5HT_{1B}$ sites. 8-OH-DPAT has low affinity for the $5HT_{1B}$ site labelled by [^{125}I]-CYP in the presence of 30μM isoprenaline (pKi 4.20) or [^{3}H]-5HT in the presence of 1μM spiperone (pKi<5). Therefore [^{3}H]-8-OH-DPAT is unlikely to label a $5HT_{1B}$ binding site.

In contrast to the inhibition curves for [^{3}H]-5HT binding to rat cerebral cortex, all the compounds tested displaced [^{3}H]-8-OH-DPAT with Hill slopes not significantly different from unity.

The effect of GTP on [^{3}H]-8-OH-DPAT binding

The effect of GTP on the affinity of compounds for the $5HT_{1A}$ site labelled by [^{3}H]-8-OH-DPAT was examined. The inclusion of 1mM GTP in saturation assays resulted in a significant increase in the Kd of [^{3}H]-8-OH-DPAT for $5HT_{1A}$ binding sites (1.3±0.8nM to 4.1±0.6nM; n=3; $p<0.05$) with no change in the Bmax (102±10 to 105±15fmol/mg protein). Similarly a two to three fold increase in the Ki values for inhibition of [^{3}H]-8-OH-DPAT binding was found for 5HT and buspirone. Fig. 3.12 shows the inhibition of [^{3}H]-8-OH-DPAT binding to rat cerebrocortical membranes by the putative $5HT_{1A}$ agonist/antagonist buspirone in the presence or absence of 1mM GTP. The results would suggest that buspirone is at least a partial agonist at this site.

[^{3}H]-8-OH-DPAT filter binding

Peroutka and Demopulos, (1986) suggested that [^{3}H]-8-OH-DPAT binds specifically to glass fibre filters when the assay is performed in a 50mM Tris HCl buffer. However, they reported that if 0.1% ascorbate is added to the buffer there is no apparent specific labelling of Whatman GF/B filters. Since the present studies were carried out using Whatman GF/B filters specific filter binding of [^{3}H]-8-OH-DPAT was investigated. The assay conditions for [^{3}H]-8-OH-DPAT binding are described in section 2.1.3d. The incubation medium consisted of 50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4 at 37^{o}C, and under

these conditions no specific filter binding was found (fig. 3.13).

3.2.3d $5HT_{1B}$ binding sites labelled by [^{125}I]-CYP

[^{125}I]-CYP was found to bind to a single class of high affinity binding sites (Kd 0.1±0.05; Bmax 190±12fmol/mg protein) on rat corpus striatum. The binding site showed the characteristics of a $5HT_{1B}$ site, ie high affinity for 5HT, 5CT and RU24969 but only low affinity for the $5HT_{1A}$ selective compound 8-OH-DPAT and the $5HT_{1C}$/$5HT_2$ selective compound mesulergine. It also showed moderate affinity for the β-blockers propranolol and pindolol (table 3.9).

3.2.4 A comparison of the binding of different radioligands

The density of binding sites labelled by [^{3}H]-8-OH-DPAT (102fmol/mg protein) and [^{3}H]-WB4101 (112fmol/mg protein) in the rat cerebral cortex are similar. The characterisation of the two sites by the 5HT ligands is shown in table 3.10 and the correlation drawn in fig. 3.14 ($r=0.971; p<0.001$). A good correlation was found indicating that both ligands label a similar population of binding sites. Both radioligands unlike [^{3}H]-5HT appeared to label a single population of binding sites. However, as with [^{3}H]-5HT, the binding of both ligands is regulated differentially by divalent cations and guanine nucleotides. This is consistent with the view that both ligands act as agonists at the $5HT_{1A}$ subtype. As [^{3}H]-8-OH-DPAT was the most selective ligand identified, and since [^{3}H]-WB4101 assays have to be performed in the presence of an α_1-adrenoceptor antagonist, [^{3}H]-8-OH-DPAT was used in further studies of $5HT_{1A}$ binding sites.

A similar comparison was made of the binding of [^{3}H]-5HT in the presence of 1μM spiperone in the rat cortex with [^{125}I]-CYP to rat striatal membranes. The affinity values for inhibition of [^{3}H]-5HT and [^{125}I]-CYP binding are presented in tables 3.12 and 3.9 respectively. A similar affinity was determined for 5CT, RU24969, pindolol and propranalol, suggesting that both radioligands probably bind to the same population of sites, the $5HT_{1B}$ subtype.

3.2.5 A comparison of the binding in different rat brain regions

The distribution of $5HT_1$ subtypes in the rat brain is shown in table 3.11. The pharmacological characterisation of each subtype in the rat cerebral cortex is shown in table 3.12.

3.2.6 $5HT_1$ binding sites in the gerbil brain

In the gerbil, as in the rat, regional differences in the binding of [^{3}H]-5HT were found. The highest density was found in the hippocampus, followed by striatum and cortex. The use of selective ligands showed that both $5HT_{1A}$ and $5HT_{1B}$ binding sites were present in the gerbil. The largest number of $5HT_{1A}$ sites occured in the hippocampus whereas $5HT_{1B}$ binding sites were prominent in the cortex and striatum. The data from saturation experiments is presented in table 3.13. A typical saturation curve of [^{3}H]-8-OH-DPAT binding to gerbil hippocampus is shown in fig. 3.15.

Inhibition of [^{3}H]-5HT binding in the gerbil cortex was carried out using a wide range of 5HT ligands. 5HT itself showed monophasic displacement curves and, as for the rat, 10μM 5HT was used to define nsb. However, all the other compounds tested showed biphasic displacement curves. Affinity values are presented in table 3.14. 5CT and RU24969 both produced biphasic displacement curves unlike in the rat cortex. No evidence of a third site could be found.

The affinity value for 8-OH-DPAT (7.95) at the $5HT_{1A}$ site was three log orders higher than its affinity for the $5HT_{1B}$ subtype (<5). RU24969 shows higher affinity for the $5HT_{1B}$ site (8.74) compared with its affinity for the $5HT_{1B}$ site (7.31). 8-OH-DPAT and RU24969 inhibit total [^{3}H]-$5HT_{1A}$ binding, using 10μM 5HT to define nsb, in a biphasic manner as shown in fig. 3.16, 40% of the binding is to the $5HT_{1A}$ subtype whereas 60% is to the $5HT_{1B}$ subtype. D-LSD showed high affinity for [^{3}H]-5HT binding (8.9) and a monophasic inhibition curve (fig. 3.16).

These results suggest that [^{3}H]-5HT labels only two high

affinity sites in the gerbil frontal cortex. A good correlation can be made between the affinity values for inhibition of [^{3}H]-5HT binding to putative $5HT_{1A}$ binding sites in the rat and gerbil ($r<0.952$; $p<0.001$) (fig. 3.17). Interestingly yohimbine shows 10 fold higher affinity for the rat $5HT_{1A}$ binding site. A similar correlation can be drawn between the $5HT_{1B}$ binding site in the rat and gerbil cortex ($r<0.988$; $p<0.001$)

3.2.7 $5HT_1$ binding sites in peripheral tissues

3.2.7a [^{3}H]-5HT binding to rat lung membranes

Saturation experiments of [^{3}H]-5HT binding to rat lung parenchyma membranes showed only a small percentage of specific binding: approximately 20% at 2nM [^{3}H]-5HT compared with 75% in the rat cortex. Scatchard transformations of the specific binding data showed [^{3}H]-5HT binding to a low number (Bmax 30fmol/mg protein) of high affinity sites (Kd 4nM) (fig. 3.18). The binding was specifically inhibited by cold 5HT (IC_{50} 22nM). The only other compound found to displace the binding was 5CT (IC_{50} 200nM), 5HT uptake blockers imipramine and desimipramine (DMI) were inactive.

3.2.7b $5HT_1$ receptor on guinea-pig and rabbit atria

No detectable specific binding was found for [^{3}H]-5HT binding to guinea-pig or rabbit atrial membranes at ligand concentrations up to 20nM. More selective ligands with higher specific activity are needed if specific binding to guinea-pig and rabbit atria is to be demonstrated.

3.2.8 Functional $5HT_1$ binding sites

3.2.8a $5HT_1$ autoreceptor

Release of [^{3}H]-5HT from rat frontal cortex slices was found to be [K^+]- and [Ca^{2+}]-dependent. Release of [^{3}H]-5HT was modulated by a presynaptic 5HT receptor as indicated by the concentration-dependent inhibition of the $5HT_1$ agonists 5HT and 5CT. The effect of [K^+] on the release of [^{3}H]-5HT is shown in fig. 3.19. From a [K^+] concentration

curve a concentration of 60 mM K^+ was chosen for all subsequent studies. Fig. 3.20 shows the dose-dependent inhibition of [^{3}H]-5HT release by 5HT (IC_{50} $6x10^{-7}$ M). 5CT (IC_{50} $3x10^{-7}$ M) was also shown to inhibit the release whereas the $5HT_2$/$5HT_{1C}$ antagonist mianserin and the $5HT_3$ antagonist MDL 72222 did not affect the 5HT-induced inhibition.

3.2.8b Effect of chronic dosing of an antidepressant on $5HT_{1A}$ binding sites in the rat hippocampus

Using binding techniques, a receptor function can be examined indirectly. A drug of known action can be given to an experimental animal and then the receptors assessed by ligand binding assays.

Considerable evidence exists to show chronic antidepressant treatment causes a decrease in the density of β-adrenoceptors in rat brain. However, less is known about the effect that antidepressant treatment has on other receptor systems. A decrease in the density of $5HT_2$ binding sites has been reported to occur following antidepressant treatment and this has lead to the suggestion the $5HT_2$ sites play a role in depression. This study was carried out to determine if chronic administration of DMI, a clinically effective antidepressant, affected the $5HT_{1A}$ binding site characterised in the hippocampus.

Forty male sprague dawley rats (150-200g) were allocated into two groups and dosed with either distilled water or desmethylimipramine (7.5mg/kg i.p. o.d.) for 14 days. Twenty four hours after the last dose the animals were sacrificed. The brains were dissected on ice and the hippocampal tissue removed and stored under liquid nitrogen. Membranes were prepared from pooled tissue (3-4/preparation) as described in section 2.1.1a and [^{3}H]-8-OH-DPAT saturation assays performed as described in section 2.1.3c. A significant decrease in the number of $5HT_{1A}$ binding sites was seen in the DMI treated animals compared with control (table 3.15). A slight but not significant increase in affinity was also seen.

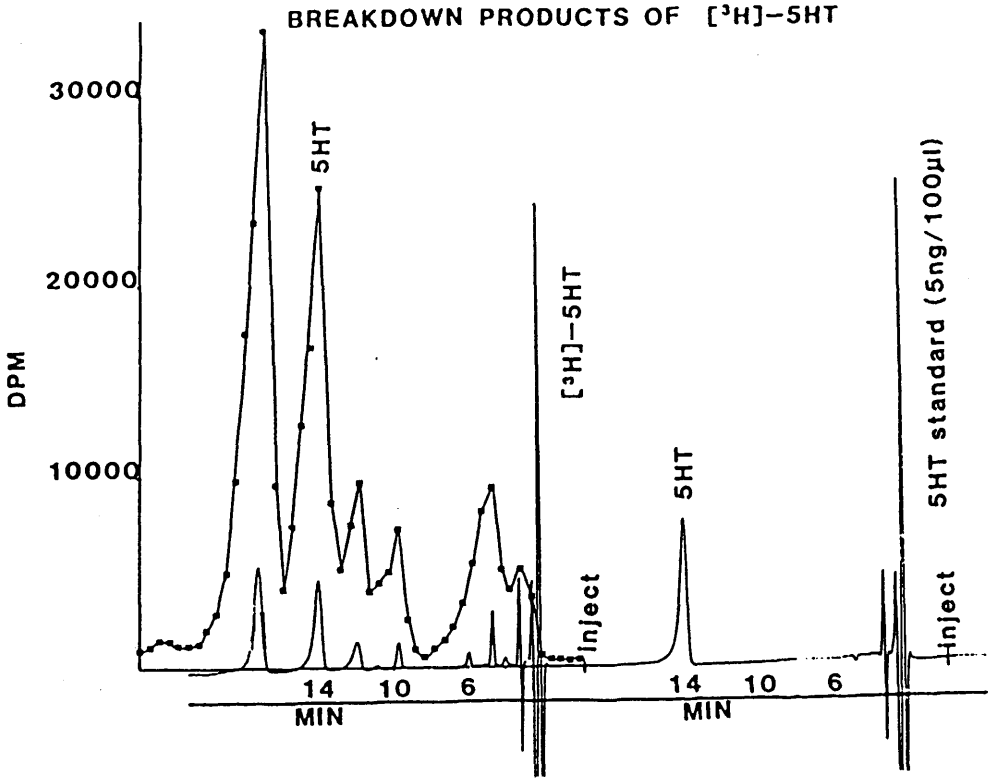


Fig. 3.1 shows the composition of [^{3}H]-5HT stored for two months in a sealed vial at 4^{o}C. Separation of [^{3}H]-5HT by HPLC and collection of the fractions for scintillation counting, indicated that some of the breakdown products were both electrically active and radioactive. Almost all (90%) of the accumulated electroactive peaks were radioactive, although only 33% of the radioactivity was 5HT.

EFFECT OF WASHING ON RECOVERABLE SPECIFIC [^{3}H]-5HT BINDING

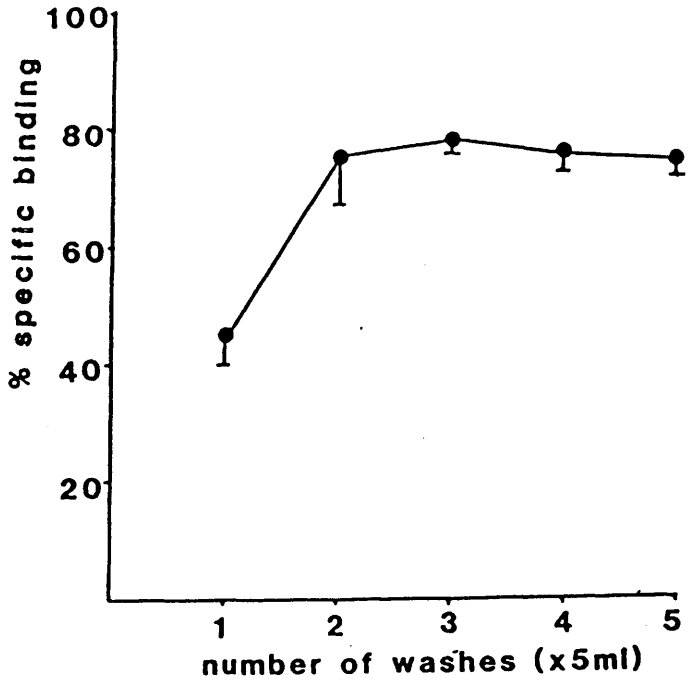


Fig. 3.2 shows the effect of washing the filters, to seperate bound from free radioactivity, on the recoverable percentage of specific binding. Specific binding was determined by subtracting nsb from total binding.

ASSOCIATION & DISSOCIATION OF [^{3}H]-5HT BINDING TO RAT CEREBRAL CORTEX

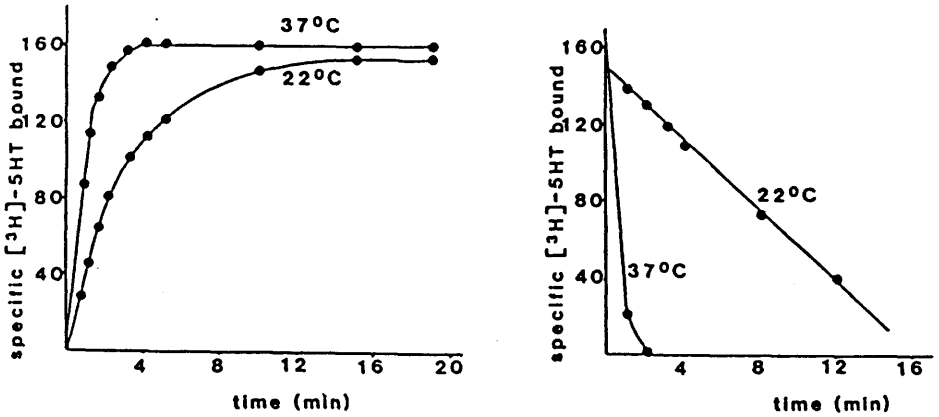


Fig. 3.3 Association and dissociation of [^{3}H]-5HT binding.
Association; membranes were incubated with [^{3}H]-5HT (2nM) at 37°C or 22°C for varying time intervals, before separating bound from free ligand as described in methods.
Dissociation; membranes were incubated with [^{3}H]-5HT (2nM) at 37°C or 22°C to equilibrium before dissociation was initiated by adding 10μm cold 5HT. Aliquots were then removed at varying time intervals and the bound radioactivity separated from free.
Specific binding was determined by subtracting nsb from total and is expressed in fmol/mg protein.

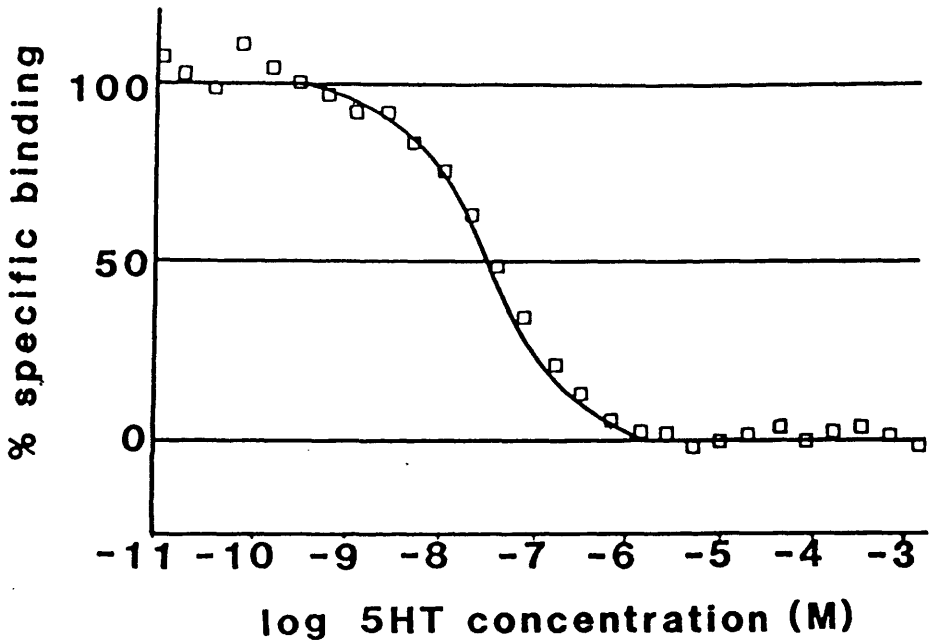


Fig. 3.4 Inhibition studies of [^{3}H]-5HT to rat cerebrocortical membranes were conducted over a concentration range of $1x10^{-11}$ -$1.34x10^{-3}$ M 5HT. Incubations were performed for 10min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4). The data describes a single experiment performed in duplicate.

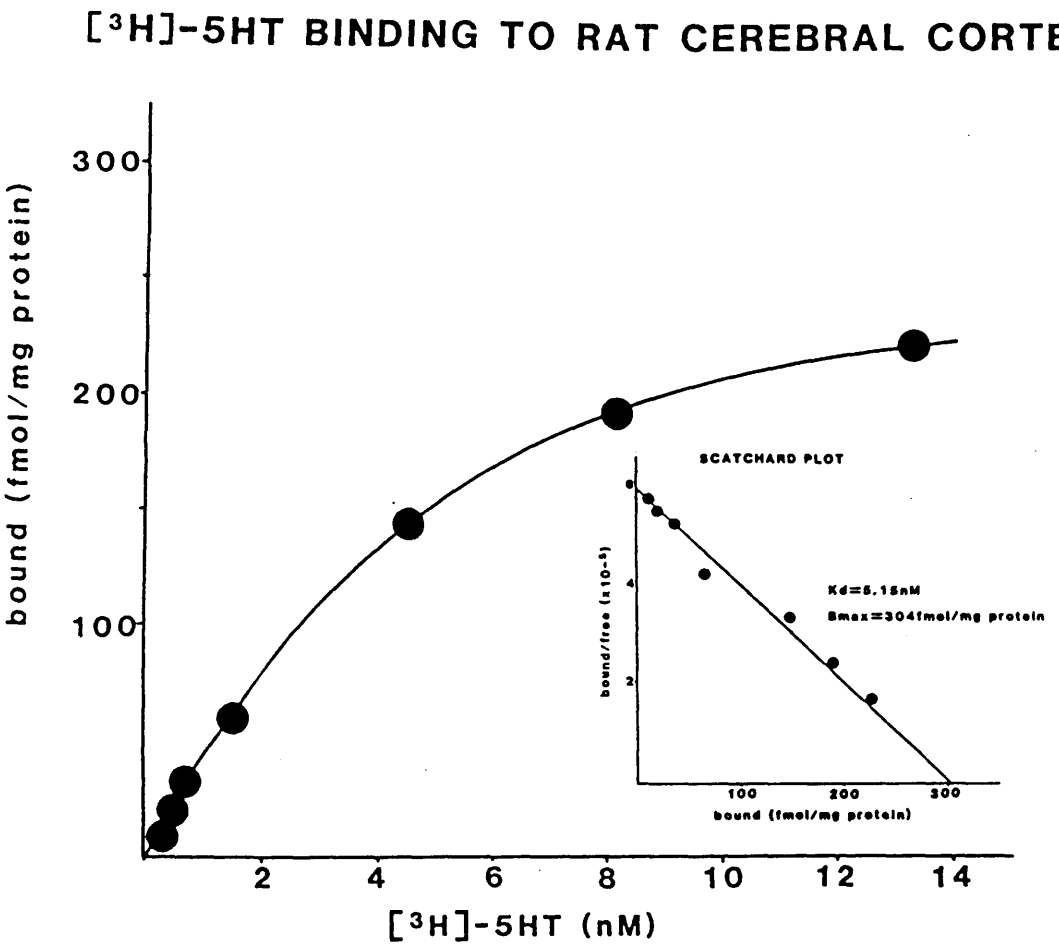


Fig. 3.5 Specific binding of [^{3}H]-5HT (1-14nM) to rat cerebrocortical membranes. Specific binding is defined as that binding displaced by 10μm 5HT, and the data describes a single experiment performed in duplicate.

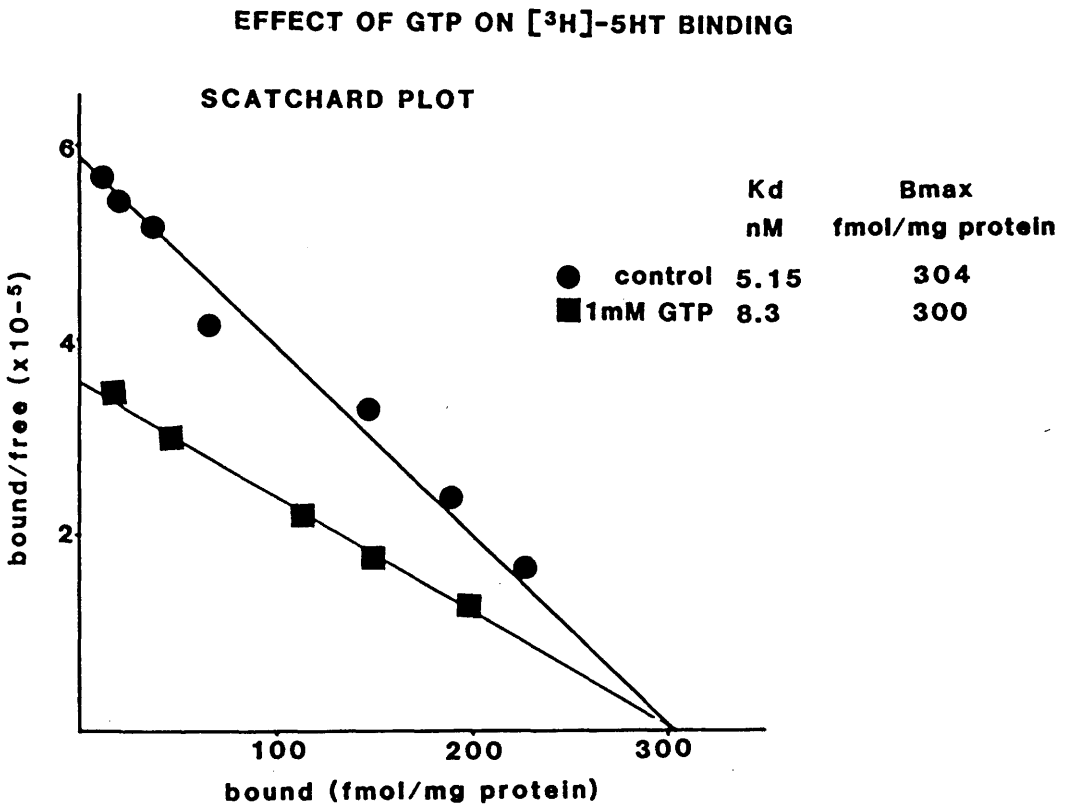


Fig. 3.6 Scatchard tranformations of the specific binding of [^{3}H]-5HT (1-12nM) to rat cerebrocortical membranes in the absence or presence of 1mM GTP.

EFFECT OF CATIONS ON THE SPECIFIC BINDING OF [^{3}H]-5HT TO RAT CEREBROCORTICAL MEMBRANES

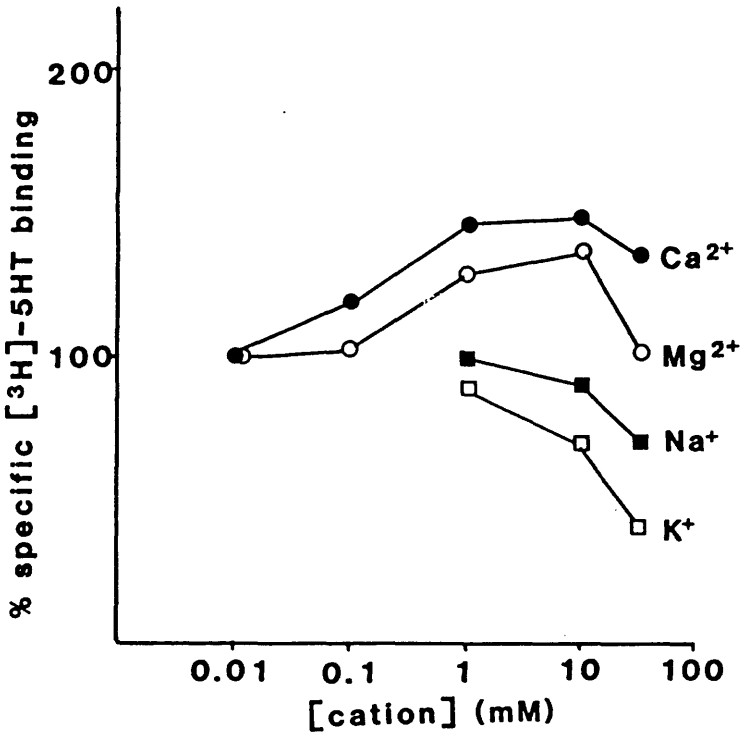


Fig. 3.7 Membranes were incubated with [^{3}H]-5HT (2nM) for 10min at 37^{o}C in 50mM Tris HCl buffer (pH 7.4) containing $CaCl_2$ (0.01-50mM), $MgSO_4$ (0.01-50mM), NaCl (1-50mM) or KCl (1-50mM). The specific binding of [^{3}H]-5HT is expressed as a percentage of that found in the absence of cations. Each point represents the mean of three experiments performed in duplicate.

EFFECT OF INCUBATION pH ON SPECIFIC BINDING OF [^{3}H]-5HT TO RAT CEREBROCORTICAL MEMBRANES

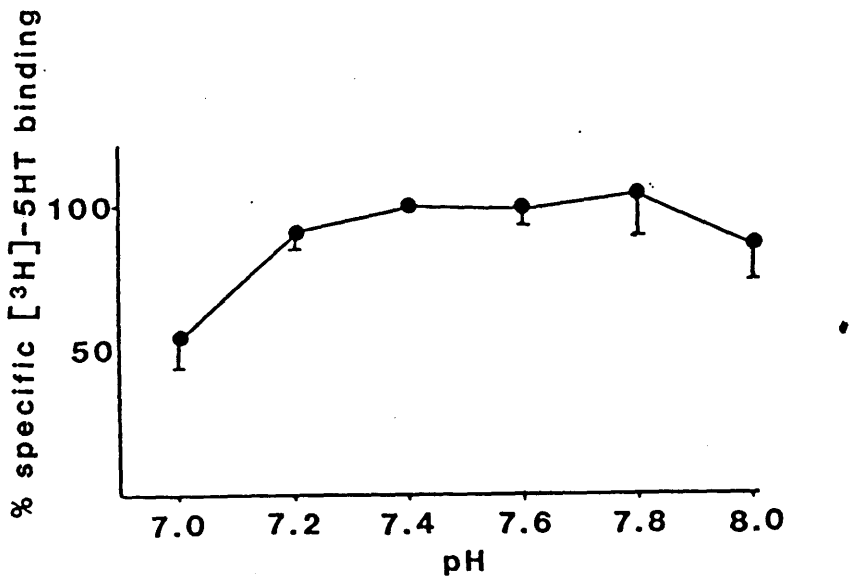


Fig. 3.8 Membranes were incubated with [^{3}H]-5HT (2nM) for 10min at 37^{o}C in 50mM Tris HCl assay buffer, pH 7.0-8.0. The specific binding of [^{3}H]-5HT is expressed as a percentage of that determined at pH 7.4. Data represents the mean of two experiments performed in duplicate.

SPIPERONE INHIBITION OF [^{3}H]-5HT BINDING TO RAT STRIATAL MEMBRANES

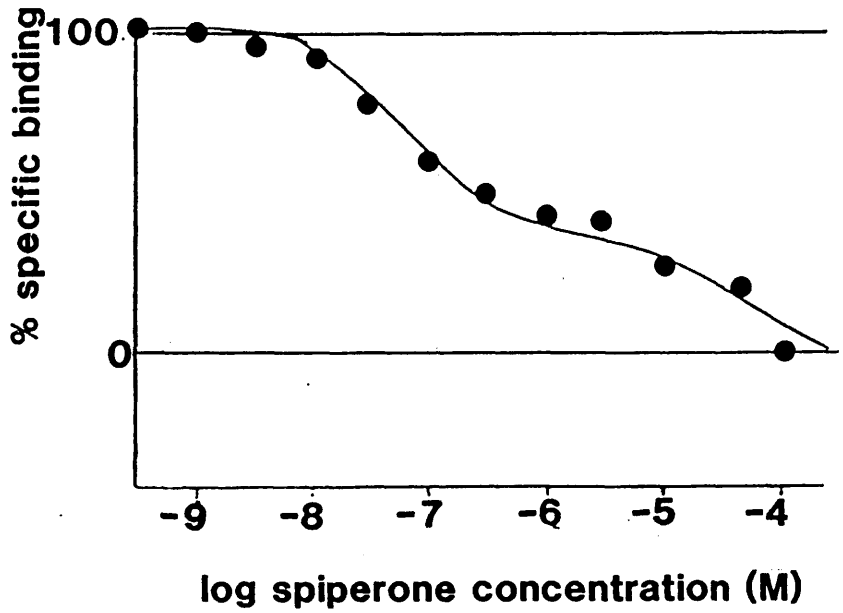


Fig. 3.9 Inhibition studies of [^{3}H]-5HT binding to rat striatal membranes were conducted over a concentration range of $3x10^{-10}$ -$1x10^{-4}$ M spiperone. Incubations were performed for 10min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline, pH 7.4). Non-specific binding was determined in the presence of 10μm 5HT. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.01$.

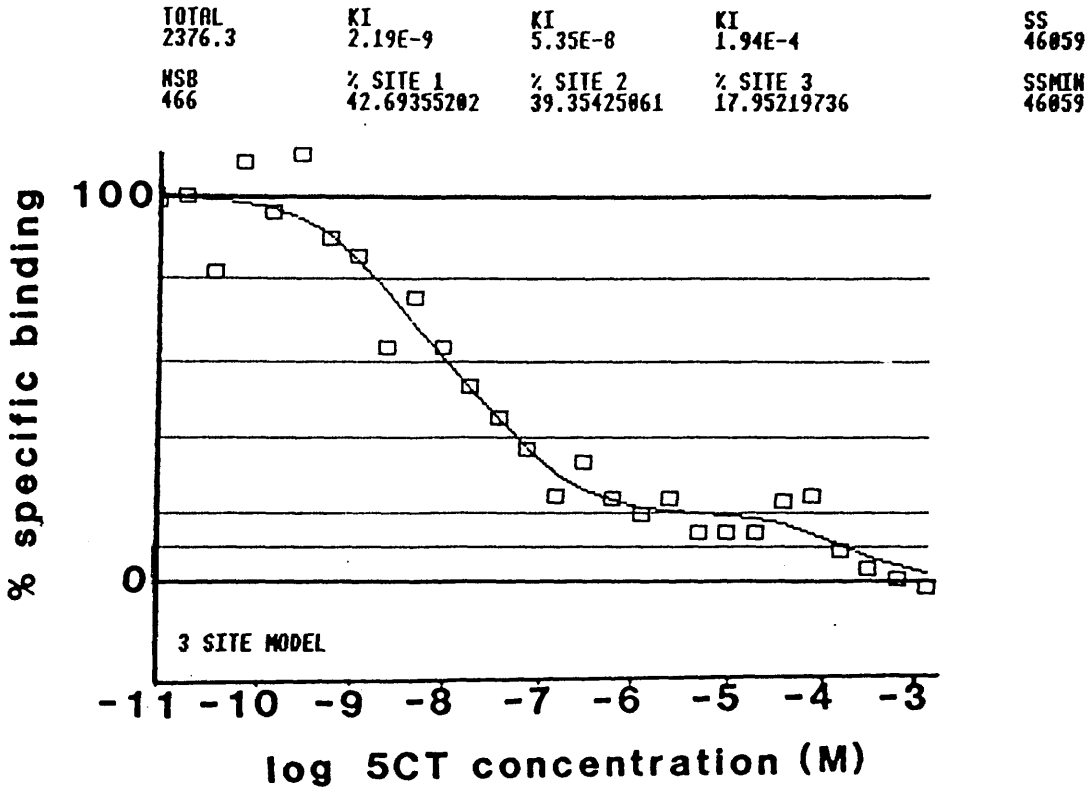


Fig. 3.10 Inhibition studies of [^{3}H]-5HT to rat cerebrocortical membranes were conducted over a concentration range of $1x10^{-11}$ -$1.34x10^{-3}$ M 5CT. Incubations were performed for 10min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4). Non-specific binding was determined in the presence of 10μm 5HT. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a three site fit was significantly better than a one or two site fit, $p<0.001$.

REMAINING SPECIFIC [^{3}H]-5HT BINDING IN THE RAT CEREBROCORTICAL MEMBRANES

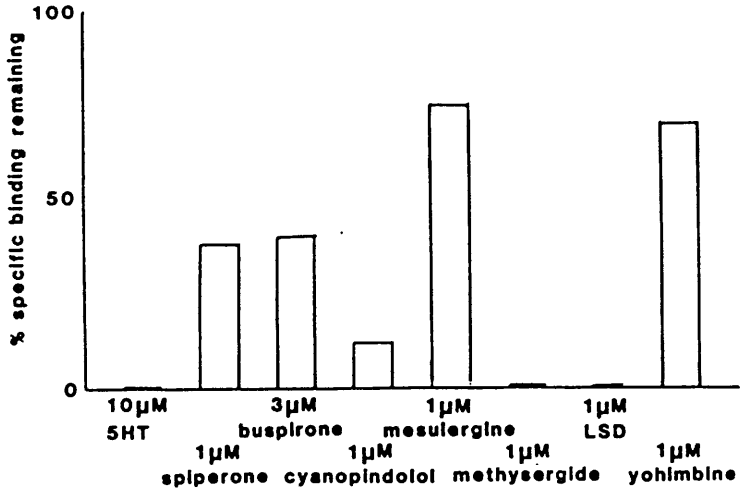


Fig. 3.11 Remaining specifc [^{3}H]-5HT binding to rat cerebral membranes in the presence of:- 1. 10μm 5HT = 0% used to determine nsb in [^{3}H]-5HT binding assay; 2. 1μm spiperone = 40% used to define specific binding to 5HT$_{1A}$ binding sites; 3. 3μm buspirone = 40% also used to define specific 5HT$_{1A}$ binding; 4. 1μm cyanopindolol = 10% blocks 5HT$_{1A}$ and 5HT$_{1B}$ sites; 5. 1μm mesulergine = 80% blocks 5HT$_{1C}$ binding sites; 6. 1μm methysergide = 0% blocks all specific [^{3}H]-5HT binding; 7. 1μm LSD = 0% blocks all specific [^{3}H]-5HT binding; 8. 1μm yohimbine = 75% equal to its IC$_{50}$ concentration at 5HT$_{1A}$ binding sites.

EFFECT OF GTP ON BUSPIRONE INHIBITION OF [^{3}H]-8-OH-DPAT BINDING TO RAT CEREBROCORTICAL MEMBRANES

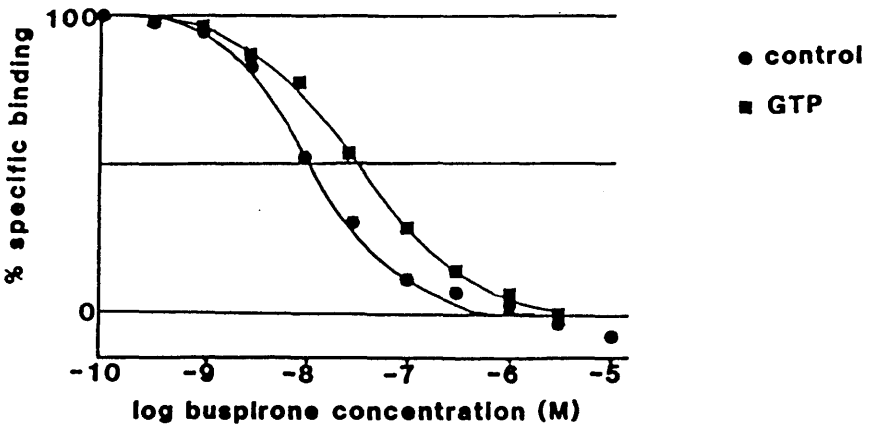


Fig. 3.12 Membranes were incubated with [^{3}H]-8-OH-DPAT (1nM) and buspirone ($1x10^{-10}$ -$1x10^{-5}$), at 37^{o}C for 10min in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4), in the absence or presence of 1mM GTP. Non-specific binding was determined in the presence of 10μm 5HT.

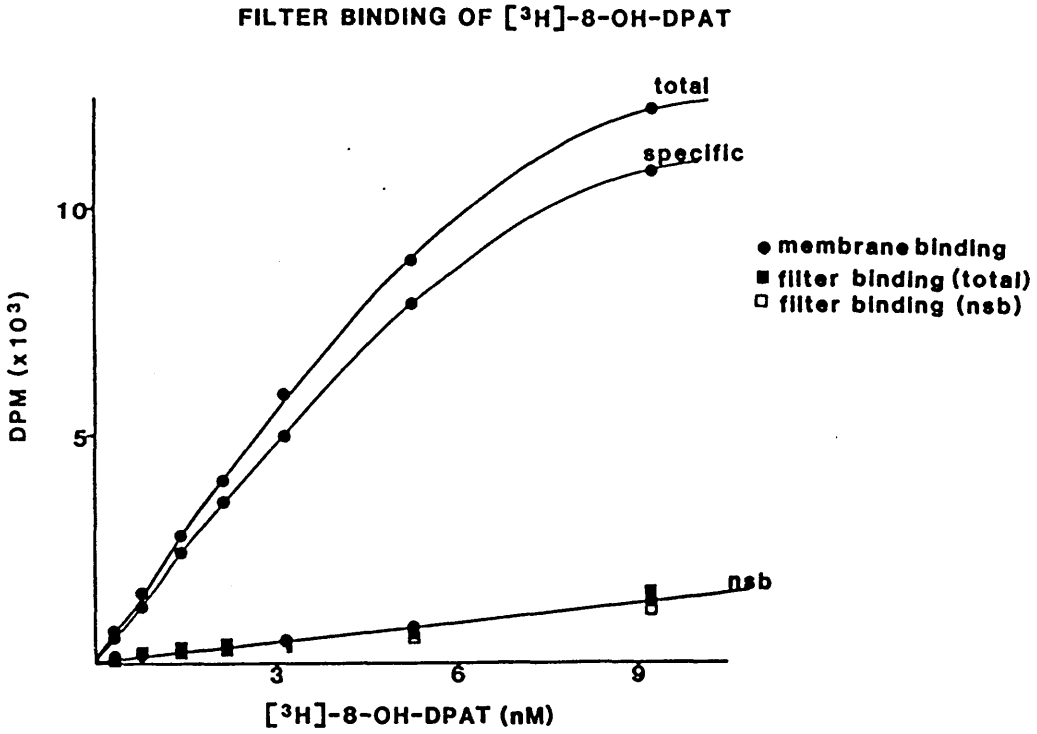


Fig. 3.13 Relationship between total, nsb and specific binding and ligand concentration. [^{3}H]-8-OH-DPAT concentrations of 0.05-9nM were incubated with control or inactivated rat hippocampal membranes at 37^{o}C for 10min in Tris HCl buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm 5HT.

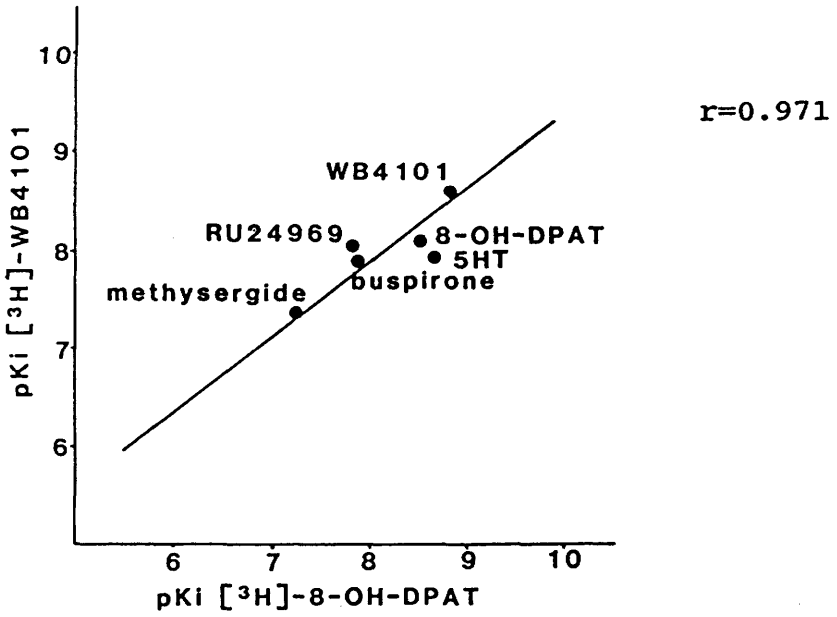


Fig. 3.14 Correlation between binding affinities of compounds for [^{3}H]-8-OH-DPAT and [^{3}H]-WB4101 binding to rat cerebrocortical membranes.

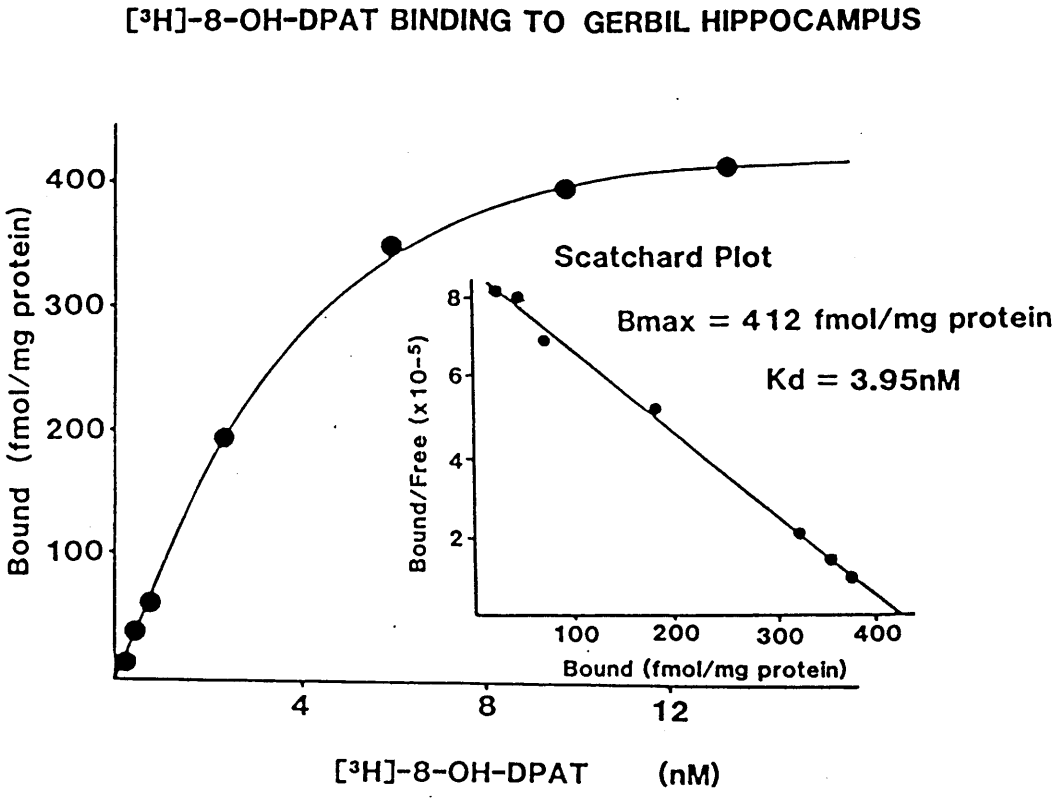


Fig 3.15 Specific binding of [^{3}H]-8-OH-DPAT to gerbil hippocampus Specific binding is defined as that binding displaced by 10μm 5HT, and the data describes a single experiment performed in duplicate.

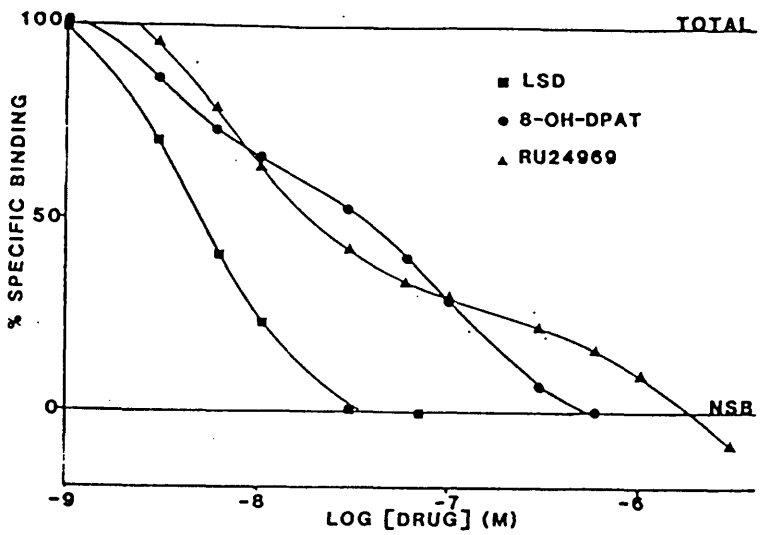


Fig. 3.16 Inhibition studies of [^{3}H]-5HT to gerbil cerebrocortical membranes were conducted over a concentration range of $1x10^{-9}$ -$3x10^{-6}$ M 8-OH-DPAT, RU24969 and d-LSD. Incubations were performed for 10min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH7.4). Non-specific binding was determined in the presence of 10μm 5HT. The data represents a single experiment performed in duplicate.

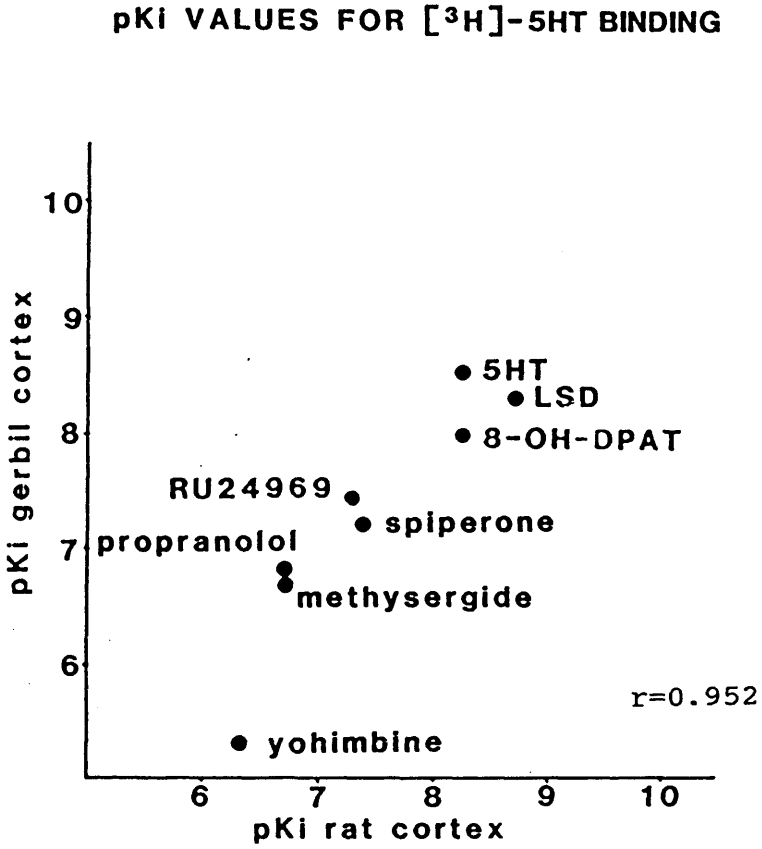


Fig. 3.17 Correlation between binding affinities of compounds for [^{3}H]-5HT binding to $5HT_{1A}$ sites in rat and gerbil cortical membranes.

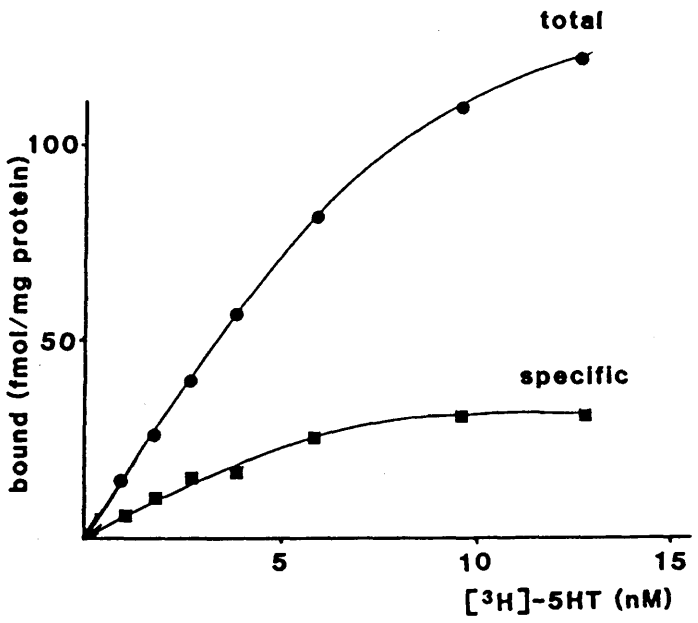


Fig. 3.18 Specific binding of [^{3}H]-5HT to rat lung membranes. Specific binding is defined as that binding displaced by 10μm 5HT. The data describes a single experiment performed in duplicate.

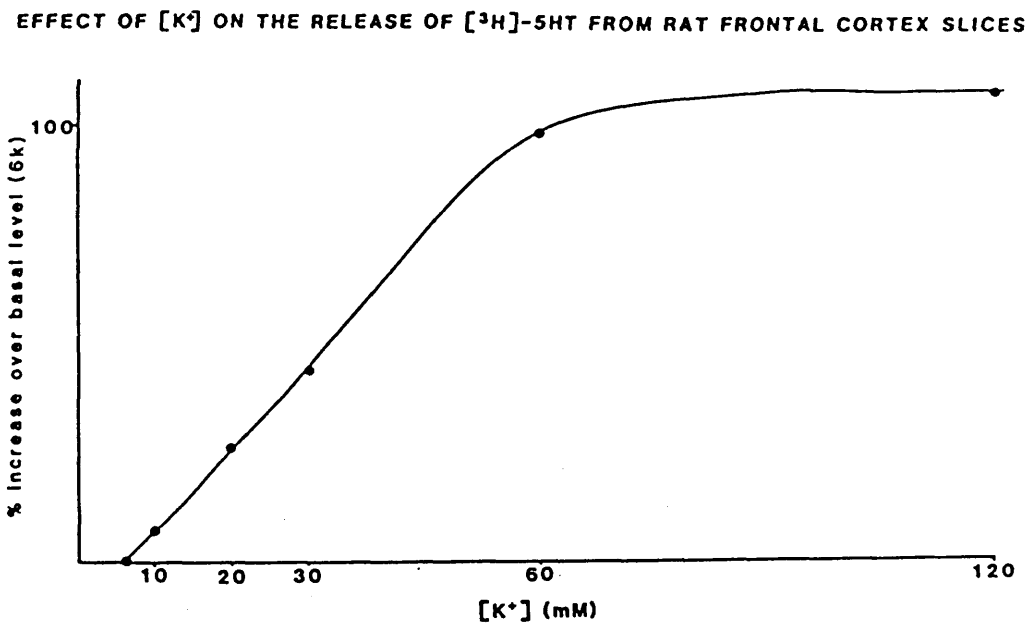


Fig. 3.19 Effect of $[K^+]$ on the % release of $[^3H]$-5HT from rat frontal cortex slices over a 5min time period.

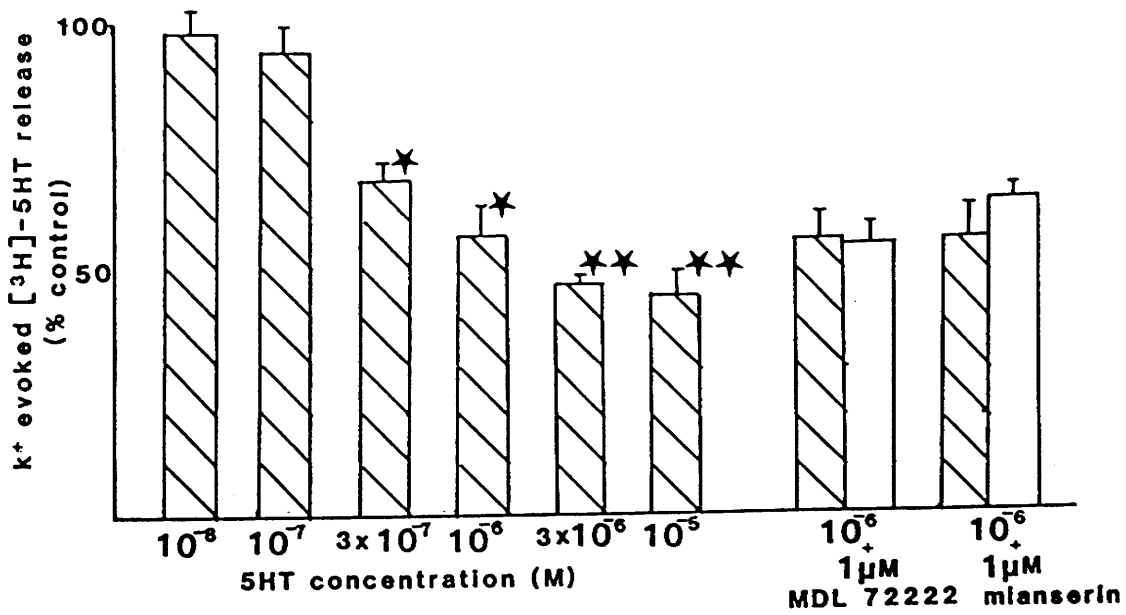


Fig. 3.20 Effect of 5HT, MDL 72222 and mianserin on K^+-evoked release of [^{3}H]-5HT from frontal cortex slices. Data is the mean ± s.e.m. of 3 determinations. Statistical significance with respect to control was assessed using a paired Student's t test. *$p<0.01$, **$p<0.001$.

Table 3.1 Effect of GTP on affinity values for [^{3}H]-5HT binding to rat cerebrocortical membranes

	IC_{50} (nM)	
	control	1mM GTP
5HT	2.5 ± 0.5	5.1 ± 0.9
5CT	0.5 ± 0.09	1.3 ± 0.1
8-OH-DPAT	8 ± 0.8	12 ± 1.2
methysergide	120 ± 5.6	135 ± 12
spiperone	220 ± 23	280 ± 20

Membranes were incubated with [^{3}H]-5HT (2nM) and a range of concentrations of the competing ligand in Tris buffer (50mM Tris HCl containing 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 10min at 37^{o}C, in the presence or absence of 1mM GTP. Nsb was defined using 10μm 5HT. Data represents the mean ± s.e.m. of 2-5 experiments performed in duplicate.

Table 3.2 The effect of incubation medium on the specific binding of [^{3}H]-5HT

Additions	Specific binding of [^{3}H]-5HT (%control)
Control (Ascorbate + $CaCl_2$)	100
Ascorbate	65 ± 5
EDTA	95 ± 4
Ascorbate + EDTA	91 ± 4
$CaCl_2$	83 ± 3
Ascorbate + EDTA + $CaCl_2$	92 ± 5

Membranes (500μg protein) were incubated with [^{3}H]-5HT (2nM) in Tris buffer (50mM Tris HCl; pH 7.4) containing ascorbate (5.7mM), EDTA (0.5mM) and $CaCl_2$ (4mM) where indicated, for 10min at 37^{o}C. Nsb was defined using 10μm 5HT.

Table 3.3 Effect of equilibrium temperature on [^{3}H]-5HT binding to rat cerebral cortex

Incubation Temperature	Kd (nM)	Bmax (fmol/mg protein)
22°C	4.65 ± 0.8	286 ± 15
37°C	4.80 ± 0.4	290 ± 6

Membranes were incubated to equilibrium (10min at 37°C; 30min at 22°C) with [^{3}H]-5HT (0.2-12nM) in a tris assay buffer (50mM Tris HCL, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4). Data represents the mean ± s.e.m. of three experiments performed in duplicate.

Table 3.4 Affinity values determined for the inhibition of [^{3}H]-5HT binding in the rat striatal membranes

	IC_{50} (nM)	
	[^{3}H]-5HT	[^{3}H]-5HT±1μm spiperone
	($5HT_{1A}$)	($5HT_{1B}$)
5HT antagonists		
ketanserin	900 ± 50	>10,000
methysergide	125 ± 20	350 ± 45
cyproheptadine	1,000	1,000
mianserin	1,995	990
D-LSD	4 ± 0.5	6 ± 0.8
Dopamine antagonists		
spiperone	80 ± 20	18,000
α-Adrenoceptor antagonists		
prazosin	>10,000	>10,000
phentolamine	2,000	>10,000
yohimbine	1,250	>10,000
imiloxan	>10,000	>10,000
β-Adrenoceptor antagonists		
propranolol	360 ± 20	160 ± 25
pindolol	66 ± 10	30 ± 8
Agonists		
5HT	6 ± 2	20 ± 3
dopamine	>10,000	>10,000
noradrenaline	>10,000	>10,000
histamine	>10,000	>10,000

Membranes were incubated with [^{3}H]-5HT (2nM) in the absence, $5HT_{1A}$ binding, or presence, $5HT_{1B}$ binding, of 1μm spiperone, in a Tris buffer system (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 10min at 37°C. Specific binding was determined by parallel incubations in the presence of 1μm spiperone for $5HT_{1A}$ binding or 10μm 5HT for $5HT_{1B}$ binding. Data represents the mean ± s.e.m. of 2-5 experiments performed in duplicate.

Table 3.5 [^{3}H]-5HT binding to rat cerebrocortical membranes

	Affinity (nM)		
compound	Site 1	Site 2	Site 3
5HT	6.3 ± 0.6 (100%)		
5CT	0.5 ± 0.3 (43%)	10.6 ± 5 (39%)	40000 (18%)
8-OH-DPAT	8.0 ± 1 (42%)	>10000 (58%)	
RU24969	42 ± 10 (33%)	1.5 ± 0.5 (37%)	500 (30%)
Spiperone	220 ± 15 (40%)	>10000 (60%)	

Membranes were incubated with [^{3}H]-5HT (2nM) and a range of concentrations of the competing ligand (12-22) in Tris buffer (50mM Tris HCl containing 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 10 min at 37^{o}C. Specific binding was determined from parallel incubations with 10μm 5HT. Data was anaylsed using computer-assisted curve fitting procedures, a two or three site fit was only accepted if statistically significant. Results represents the mean ± s.e.m. of 3-5 experiments performed in duplicate.

Table 3.6 Affinity values of imipramine, panuramine, desipramine and zimeldine for $5HT_{1A}$, $5HT_{1B}$ binding sites and [^{3}H]-5HT uptake sites

compound	Affinity value (pKi) $5HT_{1A}$	$5HT_{1B}$	[^{3}H]-5HT*
imipramine	4.5 ± 0.1	<5	7.55 ± 0.06
panuramine	4.2 ± 0.2	<5	7.41 ± 0.04
desipramine	<4	<5	6.84 ± 0.03
zimeldine	<4	<5	6.28 ± 0.03

Membranes were incubated with [^{3}H]-5HT (2nM) in the absence, $5HT_{1A}$ binding, or presence, $5HT_{1B}$ binding, of 1μm spiperone, in a Tris buffer system (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 10min at 37^{o}C. Specific binding was determined from parallel incubations in the presence of 1μm spiperone for $5HT_{1A}$ binding or 10μm 5HT for $5HT_{1B}$ binding. Data represents 2 experiments performed in duplicate.

* Inhibitory affinity constants for [^{3}H]-5HT uptake sites in the guinea-pig corpus striatum. Values quoted from Kilpartick, Goodwin and Brown, (1986).

Table 3.7 A comparison of the $5HT_{1A}$ binding sites labelled by WB4101 in the rat cortex and rat hippocampus

	pKi	
compound	rat cortex	rat hippocampus
5HT	7.98 ± 0.1	8.75 ± 0.2*
8-OH-DPAT	8.06 ± 0.15	8.50 ± 0.25
Quipazine	6.12 ± 0.5	5.80 ± 0.3
TFMPP	6.83 ± 0.3	
RU24969	8.01 ± 0.2	7.95 ± 0.2
Buspirone	7.92 ± 0.1	8.21 ± 0.3
WB4101	8.60 ± 0.2	8.90 ± 0.4
Methysergide	7.33 ± 0.2	7.45 ± 0.2
Ketanserin	<5	
Yohimbine	6.10 ± 0.1	6.90 ± 0.1**
Rauwolscine	6.77 ± 0.2	7.35 ± 0.2
WY26703	6.6 ± 0.3	6.95 ± 0.1
Idazoxan	5.9 ± 0.2	5.7 ± 0.1

Membranes were incubated with [^{3}H]-WB4101 (2nM) for 40min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4 at 37^{o}C). Nsb was defined using 10μm 5HT. [^{3}H]-WB4101 assays were all performed in the presence of 30nM prazosin to mask α_1-adrenoceptors. The concentration of drug to inhibit specific binding by 50% was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. The Hill slopes for all compounds tested were not significantly different from unity. Values shown are the mean Ki ±s.e.m. of at least three experiments performed in duplicate.

*P<0.02, **P<0.01 relative to rat cortex

Table 3.8 pKi values for $5HT_{1A}$ binding site in the rat hippocampus labelled by [^{3}H]-8-OH-DPAT

Ligand	pKi
5HT ligands	
5HT	8.65
8-OH-DPAT	8.52
Quipazine	5.47
RU24969	8.01
Buspirone	8.10
WB4101	8.75
Methysergide	7.15
Ketanserin	5.38
Mesulergine	<5
α-adrenoceptor antagonists	
Yohimbine	6.77
Rauwolscine	7.27
WY26703	6.88
Idazoxan	5.60

Membranes were incubated with [^{3}H]-8-OH-DPAT (1nM) for 10min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4 at 37^{o}C). Nsb was defined using 10μm 5HT. The concentration of drug to inhibit specific binding by 50% was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. The Hill slopes for all compounds tested were not significantly different from unity. Values shown are the mean Ki (s.e.m fall within 5%) of at least three experiments performed in duplicate.

Table 3.9 Affinity values for $5HT_{1B}$ binding sites labelled by [^{125}I]-CYP in the rat striatum

compound	pKi
5HT	7.9±0.15
5CT	8.2±0.2
RU24969	8.5±0.2
8-OH-DPAT	<5
Mesulergine	<5
Propranolol	7.1±0.15
Pindolol	6.9±0.1

Membranes were incubated with [^{125}I]-CYP (150pM) for 90min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 250ul Tris assay buffer. Nsb was defined using 10μm 5HT. The concentration of drug to inhibit specific binding by 50% was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. The Hill slopes for all compounds tested were not significantly different from unity. Values shown are the mean Ki ±s.e.m. of at least three experiments performed in duplicate.

Table 3.10 A comparison of the binding of [^{3}H]-8-OH-DPAT and [^{3}H]-WB4101 to rat cerebral cortex

compound	pKi [^{3}H]-8-OH-DPAT	pKi [^{3}H]-WB4101
5HT	8.52 ± 0.1	7.98 ± 0.1
8-OH-DPAT	8.50 ± 0.2	8.06 ± 0.15
Buspirone	7.85 ± 0.15	7.92 ± 0.10
WB4101	8.80 ± 0.3	8.60 ± 0.2
Methysergide	7.20 ± 0.35	7.33 ± 0.2
RU24969	7.80 ± 0.2	8.01 ± 0.2
Ketanserin	<5	<5
Mesulergine	5.60 ± 0.3	-
Quipazine	-	6.12 ± 0.5
TFMPP	-	6.83 ± 0.3

Assays were performed as described in methods and in the legends to tables 3.7 and 3.8. A diagramatic representation of the correlation between the binding affinities of the above compounds for [^{3}H]-WB4101 and [^{3}H]-8-OH-DPAT binding sites in the rat cerebral cortex can be seen in fig. 3.16.

Table 3.11 Saturation data for 5HT binding sites in different brain areas in the rat

	F. Cortex		Striatum		Hippocampus	
	Bmax	Kd	Bmax	Kd	Bmax	Kd
LIGAND	*	**				
$5HT_{1A}$						
5HT	135±8	4.8±1	158±10	4.5±1.5	-	-
WB4101	121±20	1.9±0.6	-	-	420±30	1.25±0.3
8-OH-DPAT	102±10	1.3±0.8	-	-	543±18	1.10±0.7
$5HT_{1B}$						
5HT	140±8	4.7±0.9	250±8	4.6±2.0	-	-
CYP	-	-	190±12	0.1±0.05	-	-

* Bmax fmol/mg protein

** Kd nM

Saturation binding assays were performed using membranes for rat cortex, striatum or hippocampus, incubated to equilibrium with a variety of radioligands; [^{3}H]-5HT, [^{3}H]-WB4101, [^{3}H]-8-OH-DPAT or [^{125}I]-CYP. Assays were performed as described in methods section 2.1.3. Values shown were obtained using the non-linear itterative curve fitting programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.m. of 3-5 experiments performed in duplicate.

Table 3.12 Comparison of the affinities of several drugs for $5HT_{1A}$, $5HT_{1B}$ and $5HT_{1C}$ binding sites in rat cerebral cortex

Compound	Affinity (nM) $5HT_{1A}$	$5HT_{1B}$	$5HT_{1C}$
5HT	8.25	7.71	
5,6 diOHT	6.22	6.22	
5CT	9.30	7.97	5.40
8-OH-DPAT	8.09	<5	
RU24969	7.38	8.82	6.30
Buspirone	7.85	<5	
Ketanserin	5.65	5.65	
D-LSD	8.40	8.28	
Spiperone	7.10	<5	
Methysergide	6.70	-	
Mianserin	<5	6.05	
Pindolol	7.18	7.55	
Propanolol	6.70	6.95	
Phentolamine	5.30	5.40	
Yohimbine	6.30	<5	
Prazosin	<5	<5	

A profile of the binding characteristics of $5HT_1$ subtypes in the rat cerebral cortex.

Table 3.13 Saturation data for 5HT binding sites in different brain areas in the gerbil

	Cortex		Striatum		Hippocampus	
	Bmax *	Kd **	Bmax	Kd	Bmax	Kd
$5HT_{1A}$						
5HT	132±6	4.95±1.0	124±12	3.99±1.2	600±120	6.12±1.5
WB4101	-	-	-	-	347±43	1.43±0.2
8-OH-DPAT	-	-	-	-	458±16	4.49±0.9
$5HT_{1B}$						
5HT	145±10	5.30±0.8	267±12	4.50±0.9	140±12	4.75±0.65
CYP	-	-	308±20	0.77±0.2	-	-

* Bmax fmol/mg protein

** Kd nM

Membranes were incubated with [^{3}H]-5HT (0.2-12nM) in the absence, $5HT_{1A}$ binding, or presence, $5HT_{1B}$ binding, of 1μm spiperone, in a Tris buffer system (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 10min at 37^{o}C. Specific binding was determined by parallel incubations in the presence of 1μm spiperone for $5HT_{1A}$ binding or 10μm 5HT for $5HT_{1B}$ binding. Values shown were obtained using the non-linear itterative curve fitting programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.m. of 2-4 experiments performed in duplicate.

Table 3.14 Comparison of the affinities of several drugs for $5HT_{1A}$, $5HT_{1B}$ and $5HT_2$ binding sites in gerbil frontal cortex

Compound	Affinity (nM) $5HT_{1A}$	$5HT_{1B}$
5HT	8.50	7.85
5CT	9.45	8.45
8-OH-DPAT	7.95	<5
RU24969	7.31	8.74
Buspirone	7.62	<5
Ketanserin	5.50	5.73
D-LSD	8.60	8.55
Spiperone	7.30	<5.00
Cyproheptadine	6.20	-
Methysergide	6.95	-
Mianserin	5.80	5.65
Propanolol	6.40	6.95
Phentolamine	<5	-
Yohimbine	5.30	-
Prazosin	<5	-

A profile of the binding characteristics of $5HT_1$ subtypes in the gerbil cerebral cortex.

Table 3.15 The effect of chronic dosing of desipramine on $5HT_{1A}$ binding sites in the rat hippocampus

Treatment	n	Kd nM	Bmax fmol/mg protein
Placebo	6	0.9 ± 0.05	533 ± 40
DMI (7.5mg/kg i.p. o.d. 14 days)	6	0.8 ± 0.07	350 ± 43*

*p<0.01

Animals were dosed with distilled water or DMI (7.5mg/kg i.p. o.d.) for 14 days. Hippocampal membranes (3/preparation) were incubated with [^{3}H]-8-OH-DPAT (0.2-12nM) for 10min at 37°C, in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5mM $MgSO_4$, 0.5mM EDTA; pH 7.4 at 37°C). Nsb was defined using 10μm 5HT. Values shown were obtained using the non-linear itterative curve fitting programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.m. of 6 experiments performed in duplicate.

3.4 Discussion

During the course of this thesis a vast amount of data has been published on $5HT_1$ receptor subtypes and their biological significance. A clearer, but as yet incomplete classification of $5HT_1$ receptors has emerged. This has occured through the increasing availability of potent and selective compounds for these sites. Binding studies with [^{3}H]-5HT indicate that there are at least four subtypes of "$5HT_1$-like" receptors each with a distinct pharmacology, which have been designated $5HT_{1A}$, $5HT_{1B}$ (Pedigo et al (1981), $5HT_{1C}$ (Pazos et al, 1984a), and $5HT_{1D}$ (Heuring & Peroutka, 1987). Functional correlates, implying biological significance, have now been established for each of these $5HT_1$ receptor subtypes. The experiments described in this chapter added to and have taken advantage of current knowledge and selective compounds, when available. The results were described in terms of the new binding classification wherever possible and this will be continued in the discussion.

The major finding of the present study is that three distinct $5HT_1$ subtypes can be identified in the rat cerebral cortex ($5HT_{1A}$, $5HT_{1B}$ and $5HT_{1C}$) whereas evidence of only two binding sites could be found in the rat striatum ($5HT_{1A}$ and $5HT_{1B}$). Rat hippocampus appears to predominantly contain $5HT_{1A}$ binding sites. In contrast no $5HT_{1C}$ binding sites were identified in the gerbil cerebral cortex, although $5HT_{1A}$ and $5HT_{1B}$ binding sites were found in all the regions examined in the gerbil, i.e. cortex, striatum and hippocampus. Studies on the regional distribution of $5HT_1$ binding sites in other species indicate that significant interspecies differences exist. The rat, guinea-pig and gerbil show large differences in the regional distribution of $5HT_1$ binding sites whereas in the mouse no significant difference is observed in the density of [^{3}H]-5HT binding in the hippocampal, cortex or striatal membranes (Hamon et al, 1984c). A quite different distribution is observed in the bovine brain where maximal

binding takes place in the substantia nigra and globus pallidum (Peroutka & Snyder, 1981); this has recently been described as a $5HT_{1D}$ binding site (Heuring and Peroutka, 1987). In the rat (Pazos et al, 1985) binding to the substantia nigra and globus pallidum is to a $5HT_{1B}$ binding site. In man the highest densities of binding sites are found in the frontal cortex, hippocampus, ($5HT_{1A}$, Hoyer et al, 1986) substantia nigra and pallidum (Pazos et al, 1987). The latter is most likely to be the $5HT_{1D}$ subtype (Heuring and Peroutka, 1987; Hoyer et al, 1987a). The $5HT_{1B}$ binding site is apparently absent from human, bovine and pig brains (Hoyer et al, 1985b, 1986). This is of interest since it would appear that CNS tissues lacking $5HT_{1B}$ sites may instead possess central $5HT_{1D}$ binding sites. A report by Middlemiss et al, (1986) on the effect of Alzheimer's disease on 5HT binding sites suggested that there are $5HT_{1B}$ binding sites in the human brain. However, the $5HT_{1B}$ binding was defined using [^{3}H]-5HT in the presence of 1μM spiperone and therefore could be labelling potentially any of the $5HT_{1}$ binding sites with the exception of the $5HT_{1A}$ binding site. Furthermore only a limited pharmacological profile was given: the $5HT_{1A}$, $5HT_{2}$ and $5HT_{3}$ ligands tested were inactive although 8-OH-DPAT showed a log order higher affinity than would be predicted for its inhibition of $5HT_{1B}$ binding sites.

The binding of [^{3}H]-5HT to rat cerebral cortex membranes was complex. Analysis of radioligand binding data generally assumes a reversible bimolecular reaction occuring between the ligand and receptor and that this interaction obeys the law of mass action. One radioligand and one site interaction should produce linear Scatchard plots and competition curves for competitive antagonists, with Hill coefficients equal to unity. However, more complex results are often found. On the assumption that no experimental artefacts contribute to the complex binding curves, then alternative explanations of the results must be found. The ability of guanine nucleotides and divalent ions to modulate ^{3}H-agonist binding may lead to complex drug competition curves. Alternatively non-sigmoidal curves of

^{3}H-ligands may indicate that multiple and distinct binding sites are being labelled. Both of these models for interpreting the results will be discussed.

The addition of 1mM GTP to the assay shifted the binding of [^{3}H]-5HT (to membranes of rat cerebral cortex) to the right thus decreasing the affinity of the ligand as reported by Sills et al, (1984). This suggests that, like the α_2-adrenoceptor and the β-adrenoceptor, the agonist binds to a high affinity state of the $5HT_{1A}$ receptor which is induced by GTP, through a guanine nucleotide binding protein. In the present studies multiple affinity states could not be determined in saturation studies up to 25nM [^{3}H]-5HT, although slight curvilinearity of the Scatchard plots was noted. A relatively small shift in the affinity (1-3 fold) was observed in this study, which was considerably less than the 10-100 fold shifts reported for α_2-adrenoceptor agonists (U'Prichard, 1981). Therefore the resolution at present may not be sufficient to distinguish two affinity states from saturation data. The addition of 1mM GTP did not alter the Bmax of $5HT_{1A}$ binding. This contrasts with the GTP-induced significant increases in α_2-adrenoceptor density reported by (U'Prichard, 1981). Thus it would appear that the $5HT_1$ receptor unlike the α_2-adrenoceptor which exists predominantly in a low affinity state, must already exist to a greater extent in a high affinity state. All competing drugs tested in the presence of GTP showed a decrease in affinity but interestingly still showed Hill slopes less than unity.

The effects of other modulators on the binding of [^{3}H]-5HT to rat cerebral cortex were also examined. [^{3}H]-5HT was found in these studies to be very unstable which suggested that an antioxidant is necessary. Andresen and Shih, (1986) showed that $\geq$1% oxidation of [^{3}H]-5HT can lead to increased specific binding which is not receptor-related but can be inhibited by the inclusion of ascorbate in the assay. Recently Hamblin et al, (1987) reported that the decomposition products of [^{3}H]-5HT appear to bind to two saturable sites on brain membranes which

mimic "specific" ligand-receptor binding.

Ascorbic acid is commonly used as an antioxidant agent for protecting biogenic amines. However, the inclusion of ascorbic acid in binding assays has been reported to be deleterious for several neurotransmitter binding sites, β-adrenoceptors (Heikkila, 1983), dopamine (Chan et al, 1982), opiate (Leslie et al, 1980) and 5HT (Weiner et al, 1982). When included in the assay ascorbate was found to cause a significant decrease in specific binding. A reduction in both the affinity and number of [^{3}H]-5HT binding sites was shown by Muakkassah-Kelly et al, (1982), which has been attributed to the ability of ascorbate to enhance iron-dependent lipid peroxidase activity in membrane preparations. It is proposed that disturbances in the lipid matrix environment of the receptor, as a consequence of the enhanced peroxidase activity, leads to the observed loss of binding capacity and a shift in the operating and cryptic forms of the receptor available to the radioligand. The effect of ascorbic acid is complex, displaying a bell-shaped concentration effect curve: lipid peroxidation accompanied by a marked decrease in [^{3}H]-5HT binding has been reported to occur at ascorbate concentrations as low as 0.025mM and reaching a maximum at 0.5mM. Increasing the ascorbate concentration above 0.5mM reverses the inhibition of [^{3}H]-5HT binding. However, above 6mM the binding decreases probably because of an auto-inhibitory effect of ascorbate on the peroxidising system (Muakkassah-Kelly et al, 1982).

Ascorbate (5.7mM) was found in these studies to decrease the amount of specific binding at 2nM [^{3}H]-5HT. The addition of $CaCl_2$ (4mM) was found to slightly inhibit the ascorbate-induced lipid peroxidation, which partly explains the effects of Ca^{2+} ions on the [^{3}H]-5HT binding, as did the lipid peroxidase inhibitor EDTA (0.5mM). The results of this study would argue that when ascorbate is used as antioxidant, the inclusion of $CaCl_2$ is necessary to maintain the specific binding of [^{3}H]-5HT. The antioxidant properties of EDTA were not investigated because this ligand chelates divalent ions which have been shown to be

beneficial to the binding of [^{3}H]-5HT.

In the present studies lipid peroxidase activity was not measured directly. In order to definitively determine if the observed effects of ascorbate were due to its ability to stimulate this enzyme, it would be necessary to measure peroxidase activity in the membrane preparations used and to examine the effects of ascorbate in the presence of specific inhibitors of lipid peroxidase (eg α-naphol or propyl gallate; Heikkila et al, 1982).

It should also be mentioned that ascorbate has also been reported to decrease ligand binding in several systems by a mechanism independent of lipid peroxidase (Heikkila et al, 1982).

In addition rat brain is a rich source of ascorbic acid. Thus the endogenous ascorbate may promote a degree of peroxidase-induced degradation of the [^{3}H]-5HT binding site or the anulus that surrounds the receptor altering the receptor integrity (eg lipid matrix) during membrane preparation.

Changing incubation temperature between 22^{o}C and 37^{o}C did not effect the specific binding of [^{3}H]-5HT to rat cerebral cortex membranes and no significant difference was found in the affinity or Bmax. This contrasts with the report of Hall et al, (1986) showing that in the rat striatum and rat hippocampus a significant increase in affinity for [^{3}H]-5HT was found at 23^{o}C compared with 37^{o}C with no change in Bmax. Therefore the differences found in the Kd values for [^{3}H]-5HT in different brain regions could partly be a result of the differential effects of temperature. All experiments in the present study were carried out at 37^{o}C.

Specific [^{3}H]-5HT binding to rat cortex showed the same pH dependency as has been reported for the rat striatum and hippocampus (Hall et al, 1986), i.e. an increase from pH 6.5-7.8, and then a decline at a pH greater than 8.0.

In would appear that multiple affinity states cannot explain the complex competition curves seen for [^{3}H]-5HT binding. Therefore the exsistence of subtypes of 5HT$_1$

binding sites was investigated. [^{3}H]-5HT was shown to label three subpopulations of $5HT_1$ binding sites in the rat cortex as shown by 5CT (present study), RU24969 (Peroutka, 1986) and (-)21-009 [(-)-4(3-ter-butyl-amino-2-hydroxypropoxy)indol-2-carbonic acid isopropylester) (Hoyer et al, 1985a & b). These results were determined using computer curve-fitting analysis which is limited in its ability to differentiate sites that have a less than 6 fold selectivity for a given compound (DeLean et al, 1982). In the present study affinity values determined from computer analysis of [^{3}H]-5HT binding were compared with values obtained in selective $5HT_{1A}$ and $5HT_{1B}$ binding assays. Compounds such as 5HT showed competitive inhibition of [^{3}H]-5HT binding but when tested in selective assays was found to have different affinities for the two sites. Interestingly compounds such as methysergide still showed biphasic displacement curves in the $5HT_{1B}$ assay, providing evidence for more than two binding sites. Important information can be gained from both computer analysis of competition curves and pharmacologically manipulated assays. However, a better pharmacological characterisation of the binding site usually awaits the development of more selective radioligands.

Spiperone and 8-OH-DPAT showed high affinity for one of the [^{3}H]-5HT binding sites, which was shown to be a homogeneous population by directly labelling with [^{3}H]-8-OH-DPAT (Gozlan et al, 1983) and [^{3}H]-WB4101 (Norman et al, 1985). [^{3}H]-5HT binding using 1μM spiperone to determine the nsb, correlated well with the binding of the new ligands. A series of selective ligands have been identified for this subpopulation of $5HT_{1A}$ binding sites including DP-5CT, buspirone and ipsapirone. All showed competitive inhibition of [^{3}H]-8-OH-DPAT binding which suggests that it binds to a homologous population of sites in the hippocampus.

Comparing the percentage inhibition of [^{3}H]-5HT binding is a complex way of determining the affinity of a compound at $5HT_{1A}$, $5HT_{1B}$ or $5HT_{1C}$ unless its affinity for one or more of these sites is already known. The $5HT_{1B}$ binding

site is difficult to study as assays have to be performed in the presence of a blocking agent; [^{3}H]-5HT in the presence of 1μM spiperone to block $5HT_{1A}$ binding sites or [^{125}I]-CYP in the presence of a β-blocker. The site has been defined as RU24969 > 5CT >>>>> 8-OH-DPAT as well as high affinity for the β-adrenoceptor antagonists cyanopindolol and (-)21-009. The $5HT_{1B}$ site identified in this study agrees with this classification. However, Peroutka, (1986) suggested that D-LSD has a relatively low affinity for the $5HT_{1B}$ site (170nM) compared with the $5HT_{1A}$ site (4nM) in the rat. The results of this study could not confirm the findings of Peroutka, (1986), D-LSD showed equal affinity for $5HT_{1B}$/$5HT_{1A}$ binding sites in the rat and gerbil.

$5HT_{1A}$ and $5HT_{1B}$ binding sites were characterised in the gerbil. Both subtypes showed a similar profile to that described for the rat, and a good correlation could be drawn between the binding to rat and gerbil cortex. So as in the mouse and rat a $5HT_{1B}$ binding site exists in the gerbil. However, no evidence of a $5HT_{1C}$ binding site was found in the gerbil cortex.

Preliminary investigations revealed a fourth 5HT binding site in bovine cortex, representing 30% of specific [^{3}H]-5HT binding, which was resistant to 100nM 8-OH-DPAT, and 100nM mesulergine, and therefore was unlikely to be $5HT_{1A}$ or $5HT_{1C}$. In addition the binding was not blocked by 100nM cyanopindolol, however, $5HT_{1B}$ binding sites are reported not to exsist in this tissue. The site showed moderate affinity for yohimbine (52nM). Heuring and Peroutka, (1987) have defined this site as a $5HT_{1D}$ binding site present in all regions of the bovine brain. It has also been identified in pig and human brain (Hoyer et al, 1987a), and suggested to be linked to adenylate cyclase in the calf substantia nigra (Hoyer and Schoeffter, 1988). In this study no evidence of this site could be found in the rat brain, although functional evidence of a $5HT_{1D}$-like receptor, based on its affinity of yohimbine, could be present in the rat fundus (Cohen and Fludzinski, 1987) and rat kidney (Charlton et al, 1986). Further characterisation

of this site must await the development of selective ligands.

Neither the $5HT_{1A}$ or $5HT_{1B}$ binding sites labelled by [^{3}H]-5HT showed any similarities with the 5HT uptake site. Indeed it is unlikely that [^{3}H]-5HT at a concentration of 2nM would label the uptake site as against [^{3}H]-paroxetine binding to 5HT uptake site on rat cerebrocortical membranes, 5HT has an affinity (Ki) value of 698nM (Habert et al, 1986). [^{3}H]-8-OH-DPAT has been shown to label the uptake site in rat striatal membranes. Although this is a low affinity site (Kd 34nM) compared with its affinity for the $5HT_{1A}$ receptor (Kd 1.1nM). Again 5HT has only moderate affinity for this site (IC_{50} 1530nM) (Schoemaker and Langer, 1986).

Preliminary investigations of the peripheral $5HT_1$ binding sites, showed that a high affinity, low capacity binding site exists in rat lung tissue. A full characterisation of the site was not carried out as only low percentage of specific binding was obtained with [^{3}H]-5HT, although 5HT was shown to inhibit the binding in a concentration-dependent manner (IC_{50} 22nM). 5HT is a potent bronchodilator in man, and has been shown to be inactivated during circulation through the pulmonary vascular bed by an active transport process. Autoradiographic localisation has confirmed that pulmonary endothelial cells are the site of 5HT uptake. Therefore presumably a 5HT uptake site should be present on the lung endothelial cells. However, it is unlikely that the uptake site was labelled in this study as imipramine and DMI were inactive. 5CT did show moderate inhibition (IC_{50} 200nM) and therefore it would interesting to repeat the study with the more selective radioligands with higher specific activities, to determine if this binding site represents a $5HT_1$ subtype. Das and Steinberg, (1985) identified two populations of [^{3}H]-5HT binding sites in a purified lung mitochondrial preparation and a high capacity site in lung microsomal membranes; this finding suggests that in addition to the lung serving as a metabolic site for 5HT it

may also have a physiological role. In order to confirm this, delineation of 5HT receptor types and the identification of their location must occur.

In contrast to the limited data in the lung, a great deal is known about the interaction of 5HT on the myocardium. It has been suggested that 5HT acts on the cardiac tissue via a direct action on $5HT_1$ receptors, (Saxena et al, 1985), by an indirect action on $5HT_3$ receptors present on sympathetic ganglia (Fozard, 1984a) or by a direct action on β_1-adrenoceptors (Trendelenburg, 1960). The failure to detect specific [^{3}H]-5HT binding to guinea-pig or rabbit atria may be because either both tissues possess a very high affinity [^{3}H]-5HT binding site or the density of $5HT_1$ sites is so low that it is beyond the sensitivity of the binding assay.

Investigation in this laboratory have shown that behavioural tests may be the best functional correlate for central $5HT_{1A}$ receptors. In this study a significant decrease in $5HT_{1A}$ receptor number in the hippocampus following chronic antidepressant treatment may indicate a possible role for $5HT_{1A}$ receptors in the aetiology of depression, which should be further investigated.

A study of the 5HT autoreceptor was undertaken. Rat cerebrocortical slices were loaded with [^{3}H]-5HT. After washing, the slices released tritium at a low basal rate which was enhanced by elavated K^+ levels, and was markedly Ca^{2+} dependent. Reproducibilty was difficult to obtain, each concentration was incubated individually in an eppendorf test tube containing as far as possible the same amount of slices. It was felt that concentration response curves could have been better achieved in a perfusion system. Nevertheless 5HT and 5CT were shown to cause a concentration-dependent reduction in K^+-evoked release of [^{3}H]-5HT presumably by the interaction with a 5HT presynaptic autoreceptor. Mianserin and MDL72222 were inactive, which suggests the the response is not via a $5HT_2$, $5HT_{1C}$ or $5HT_3$ receptor. Engel et al, (1986) using a wide range of 5HT ligands, showed this response to be via a $5HT_{1B}$ receptor.

Chapter 4: STUDIES ON $5HT_2$ RECEPTORS

4. STUDIES ON $5HT_2$ RECEPTORS

4.1 Introduction

$5HT_2$ binding sites were first detected using the neuroleptic [^{3}H]-spiperone in rat frontal cortex (Leysen & Laduron, 1977). The sites were characterised by a high affinity for known 'classical' 5HT antagonists, including cyproheptadine, methysergide and LSD , (which are all high affinity but non-selective $5HT_2$ ligands) and low micromolar affinity for 5HT. However, [^{3}H]-spiperone has also been proposed as the ligand of choice for labelling dopamine (D_2) receptor binding sites (Leysen et al, 1977). Therefore, binding to $5HT_2$ sites had to be carried out in the presence of a dopamine antagonist. Other radioligands have been used, although all of them have the disadvantage of labelling with high affinity more than one type of binding site. [^{3}H]-LSD (Peroutka and Snyder, 1979; Burt et al, 1976) and [^{3}H]-metergoline (Hamon et al, 1981) were found to label $5HT_1$ and $5HT_2$ receptor binding sites and probably also dopamine binding sites. [^{3}H]-mianserin (Peroutka and Snyder, 1981) was shown to label $5HT_2$ and histamine$_1$ receptors. In contrast [^{3}H]-methiothepin (Nelson et al, 1979) showed predominantly nonspecific binding to the lipid component of membranes. The first relatively selective radioligand to be introduced was [^{3}H]-ketanserin (Leysen et al, 1982a), although this ligand has been reported to label α_1-adrenoceptors in human and pig brain membranes (Hoyer et al, 1987c). However, the introduction of [^{3}H]-ketanserin has led to numerous studies of $5HT_2$ binding sites. Correlates for $5HT_2$ binding sites have been shown in animal models from behavioural and pharmacological studies (Yap and Taylor, 1983; Lucki et al, 1984; Cohen et al, 1985).

This chapter principally describes the binding characteristics of [^{3}H]-ketanserin. In addition, earlier studies undertaken before the availability of

[^{3}H]-ketanserin will be discussed. A comparison of the binding characteristics of three radioligands, [^{3}H]-ketanserin, [^{3}H]-mianserin and [^{125}I]-LSD, was made in rat frontal cortex. Limited regional distribution studies of $5HT_2$ binding sites in four regions of the rat and gerbil brain; frontal cortex, striatum, hippocampus and cerebellum were also undertaken.

Peripheral $5HT_2$ binding sites were also studied and where possible compared with functional preparations in the same tissue. A pharmacological profile of $5HT_2$ sites on human platelets was determined using [^{3}H]-LSD and compared with a functional platelet aggregation test. Binding sites in the rat lung membranes was also examined using [^{3}H]-ketanserin. [^{125}I]-LSD was used to study $5HT_2$ sites on vascular smooth muscle, namely human umbilical artery (HUA) and pig coronary artery (PCA). The PCA was also examined in an 'in vitro' organ bath preparation in which 5HT mediated contraction of vascular smooth muscle via $5HT_2$ receptors.

During the course of this study an interesting Syntex compound emerged with high $5HT_2$ affinity but showing low Hill slopes in competition assays, so a more detailed investigation of binding of this, compound X, was undertaken.

The figures and tables are included at the end of the results section.

4.2 Results

4.2.1a [^{3}H]-Ketanserin binding studies

Initial results indicated that [^{3}H]-ketanserin bound to a single population of saturable binding sites on rat frontal cortex membranes with nanomolar affinity. Preliminary kinetic experiments indicated a relatively slow rate of dissociation of [^{3}H]-ketanserin from washed cerebral membranes suggesting the suitability of using vacuum filtration procedures for separating bound from free ligand. In addition filter binding of [^{3}H]-ketanserin represented less than 1% of the total binding through the saturation range of 0.05-5nM. Specific binding of [^{3}H]-ketanserin was not significantly different when filtration was performed using 2 or 3 x 10ml washes with buffer at room temperature. The most favourable conditions employed 2 x 10ml washes. Washing with ice cold buffer slightly increased the amount of recoverable specific binding. In all experiments the washing buffer used was the same as the assay buffer. The methods for applying vacuum filtration were performed as described in results section 3.2.1b.

In later studies separation of bound from free was achieved using the Brandel cell harvester. 2 x 10s washes (flow rate 1.5L/min) were found to be optimal.

4.2.1b Tissue linearity

Specific binding of [^{3}H]-ketanserin (0.5nM) was linear when the assay protein concentration was increased from 50-1250μg and in all studies a protein concentration well within this range was used.

4.2.1c Kinetic experiments

Association

[^{3}H]-Ketanserin (1nM) bound rapidly to rat frontal cortex membranes (300μg) at 37^{o}C reaching half maximal binding in 2min (fig. 4.1) and remained constant for a period >20min. All subsequent experiments were performed using a 15min incubation period.

Dissociation

The binding was completely reversible. Addition of excess methysergide (2μM) resulted in complete dissociation of the bound [^{3}H]-ketanserin within 5min (fig. 4.1). The dissociation was temperature dependent, occurring more rapidly at higher temperatures which probably explains why slightly more specific binding was recovered with the use of ice-cold washing buffer.

4.2.1d Determination of non-specific binding

After investigation of a number of different compounds, methysergide was selected to define non-specific binding. Ketanserin itself displaced [^{3}H]-ketanserin binding from $1x10^{-11}$ $-3x10^{-6}$ M, resulting in approximately 85% specific binding (fig. 4.2). At concentrations in excess of 0.1μM, it was also found to displace the binding to extensively boiled tissue preparations, which probably represented a 'ketanserin binding site' i.e. displaceable non-specific binding. Therefore an underestimation of the true affinity values would result from using ketanserin to define nsb. No displacement from boiled membranes was obtained with methysergide at concentration up to 10^{-4} M. Methysergide concentration curves reached a plateau at 0.3μM and remained constant up to 100μM. A concentration of 2μM methysergide was selected to define nsb in all subsequent experiments. Specific binding at 0.5nM [^{3}H]-ketanserin represented approximately 75%. 5HT (see fig. 4.4) showed similar percentage of specific binding to methysergide. However, a concentration of 300μM would be required to define nsb.

4.2.1e Saturation experiments

[^{3}H]-Ketanserin was found to bind with high affinity (0.42±0.05nM) to a single class of saturable binding sites (Bmax 240±25fmol/mg protein) on rat frontal cortex membranes when assayed at 37^{o}C using a concentration range of 0.05-5nM (fig. 4.3). When the concentration range of [^{3}H]-ketanserin was extented to 20nM a more complex binding isotherm was produced, which could be resolved into two or more sites. This could indicate that above 5nM [^{3}H]-ketanserin labels a significant percentage of other

site(s) in the frontal cortex. Ketanserin was shown to have high affinity for H_1 receptors, (pKi 8.0) (Leysen et al, 1981) α_1-adrenoceptor receptors (pKi 7.7) (section 6.2.2) and $5HT_{1C}$ binding sites (pKi 7.2) (section 3.2.3a). Therefore all subsequent experiments were carried out at concentration $\leq$ 5nM.

4.2.1f Competition curves

The site was characterised by a high nanomolar affinity for 'classical' 5HT antagonists belonging to different chemical classes. 5HT itself shows micromolar binding affinity but was found to bind at least 100 fold more potently to this site than did other neurotransmitters tested. A summary of the affinity values obtained for a wide range of compounds is shown in table 4.1.

4.2.2 Modifications to the basic technique

4.2.2a Effect of guanine nucleotides

Agonist inhibition curves performed in the presence of 1mM GTP showed a significant decrease in affinity. The inhibition curve for 5HT in the absence (IC_{50} 260nM) and presence (IC_{50} 2490nM) of GTP is shown in fig. 4.4. Bufotenine (IC_{50} 690nM) also showed a significant decrease in affinity in the presence of GTP (IC_{50} 1790nM) with a slight increase in the Hill slope (table 4.2). No significant difference was seen in the IC_{50} values determined for the antagonists ketanserin, spiperone, and cyproheptadine in the absence or presence of GTP.

4.2.2b Effect of ions

As shown in table 4.3 the binding of [^{3}H]-ketanserin to rat frontal cortex membranes was influenced by additions to the Tris assay buffer. All [^{3}H]-ketanserin assays in this study were performed in a Tris HCl buffer (pH 7.4 at 37^{o}C) containing 5.7mM ascorbate, 4mM $CaCl_2$ and 10μM pargyline (control). In the presence of only ascorbate a significant decrease in the affinity was found. A decrease in the Bmax was also seen, although this did not reach significance. A similar decrease in the binding was found when assays were performed in a physiological salt buffer (NaCl 118mM, KCl 4.7mM, $MgSO_4$ 1.2mM, KH_2PO_4 1.2mM, $NaHCO_3$ 25mM, $CaCl_2$ 1.3mM).

4.2.2c Equilibrium temperature

[^{3}H]-ketanserin binding to rat frontal cortex membranes reached equilibrium after 15min at 37^{o}C in a final volume of 1ml. Equilibrium was achieved even at the lowest ligand concentrations. At 22^{o}C equilibrium was reached within 25min with no significant difference in the Kd or Bmax (table 4.4). For consistency 37^{o}C was chosen for all subsequent experiments.

4.2.2d Effect of pH

Specific [^{3}H]-ketanserin binding to rat frontal cortical membranes is not significantly different over the pH range of 7.0-8.0 (fig. 4.5). A pH of 7.4 was used in all subsequent studies. Similarly no significant change was seen in affinity values for cold ketanserin over the pH range 7.2-7.8; pKi values determined were:- 8.72±0.07 at pH 7.2, 8.72±0.06 at pH 7.4, 8.75±0.06 at pH 7.6 and 8.74±0.07 at pH 7.8. The Hill slopes were determined and found not to be significantly different from unity.

4.2.3 Specific 5HT$_2$ binding assays

The initial studies of 5HT$_2$ binding sites were made using [^{3}H]-ketanserin binding to rat frontal cortex, as described above. However, a number of other ligands were used to characterise central 5HT$_2$ binding sites.

4.2.3a [^{3}H]-mianserin binding to rat frontal cortex

[^{3}H]-Mianserin saturation curves were best fitted to a two site model; a high affinity site (4nM) and a lower affinity 5HT$_2$ site (11nM). The high affinity site was shown to be a H$_1$ binding site by Peroutka and Snyder, (1981). Therefore all [^{3}H]-mianserin assays were carried out in the presence of a histamine blocker, 0.3μM triprolidine. Under these conditions [^{3}H]-mianserin was found to label a saturable population of high affinity binding sites in the rat frontal cortex (Kd 9.2±0.9nM, Bmax 160±25fmol/mg protein) (see table 4.6). Only 45% specific binding was available at the Kd concentration. The 5HT$_2$ binding site is the 'lower' affinity site for [^{3}H]-mianserin, which has only a low percentage of specific binding and labels fewer binding sites than [^{3}H]-ketanserin.

4.2.3b [^{125}I]-LSD binding to rat frontal cortex

[^{125}I]-LSD was found to label a single population of high affinity sites (Kd 0.7±0.1nM, Bmax 237±30fmol/mg protein), i.e. a similar number of binding site to those labelled by [^{3}H]-ketanserin (see table 4.6).

4.2.4 A comparison of $5HT_2$ radioligands

A comparison of the binding of [^{3}H]-ketanserin, [^{3}H]-mianserin and [^{125}I]-LSD to rat frontal cortex was carried out. The affinity values for a wide range of compounds for the site labelled by these different ligands can be seen in table 4.5. A good correlation was apparent between [^{3}H]-ketanserin and [^{3}H]-mianserin ($r=0.828$; $p<0.05$) and between [^{3}H]-ketanserin and [^{125}I]-LSD ($r=0.877$; $p<0.05$). This significant correlation suggests that the ligands label the same site namely the $5HT_2$ binding site.

4.2.5 A comparison of $5HT_2$ binding in different rat brain regions

Saturation experiments were carried out to assess [^{3}H]-ketanserin's binding to different brain regions. Table 4.6 shows the Kd and Bmax values obtained in four regions of the rat brain; frontal cortex, striatum, hippocampus and cerebellum. The frontal cortex was rich in $5HT_2$ binding sites whereas only a low number of sites was found in the hippocampus and no binding was detected in the cerebellum. Competition curves for ketanserin and methysergide (see fig. 4.2) were performed in each tissue to assess the degree of displaceable non-specific binding; frontal cortex 10%, striatum 15%, hippocampus 5% and cerebellum 10%. Therefore the 'ketanserin binding site' was found to be present in all brain regions even in the cerebellum where significant specific $5HT_2$ binding could not be detected.

4.2.6 $5HT_2$ binding sites in gerbil brain

[^{3}H]-ketanserin saturation curves (0.05-5nM) to gerbil frontal cortex membranes (300μg membrane protein) were performed in a Tris HCl buffer system (50mM Tris HCl; pH 7.4 containing 5.7mM ascorbate, 4mM $CaCl_2$ and 10μm pargyline). The binding was saturable, reversible and of high affinity. Iterative non-linear analysis of the binding isotherms demonstrated a single class of high affinity sites with a Kd of 0.48±0.03nM and a Bmax of 206±20fmol/mg protein (n=5). A representative experiment is shown in fig. 4.6. Scatchard transformations of the specific binding isotherm revealed linear plots, which suggests that in the gerbil, as in the rat, [^{3}H]-ketanserin binds to a single population of high affinity sites over this concentration range.

[^{3}H]-ketanserin (1nM) bound rapidly to gerbil frontal cortex membranes (300μg) at 37^{o}C reaching full association within 3min and maintained this level for >30min. The binding was found to be completely reversible: when 2μM methysergide was added the bound [^{3}H]-ketanserin was completely dissociated within 6min. All conditions determined for [^{3}H]-ketanserin binding to rat frontal cortex were applied in the study of $5HT_2$ binding sites on gerbil cerebral membranes.

The displacement of [^{3}H]-ketanserin by various 5HT antagonists and agonists is shown in table 4.7. The antagonists ketanserin, spiperone, cyproheptadine, methysergide, mianserin and ritanserin were potent inhibitors of [^{3}H]-ketanserin binding with Ki values in the nanomolar range. The $5HT_{1A}$ agonist, 8-OH-DPAT and the $5HT_{1A}$ and $5HT_{1B}$ agonists 5CT and RU24969, as well as 5HT itself, showed micromolar affinity for [^{3}H]-ketanserin binding. The results of such displacement studies provide evidence that [^{3}H]-ketanserin is binding to a $5HT_2$ site in the frontal cortex of the gerbil. [^{3}H]-ketanserin has been shown to bind to an α_1-adrenoceptor binding site. However, in this study the α_1-adrenoceptor antagonist prazosin showed only low affinity (pKi 5.3). A good correlation can be made between the affinity of the above compounds for the $5HT_2$

site on gerbil frontal cortex and the values obtained for rat frontal cortex (fig. 4.7). Interestingly 5HT showed a 5 fold lower affinity for the $5HT_2$ binding site on gerbil frontal cortex compared with rat frontal cortex.

In the gerbil, as in the rat, regional differences in the density of $5HT_2$ binding sites were found. The highest density found in the frontal cortex > striatum >> hippocampus but no significant specific binding in the cerebellum (table 4.8). The Kd value obtained for the hippocampus was similar to that determined for the frontal cortex. However, a slight but significant decrease in affinity was found in the striatum ($p<0.05$).

4.2.7 $5HT_2$ binding sites in peripheral tissues

4.2.7a Platelets

Preliminary investigations of peripheral $5HT_2$ binding sites in this study were undertaken using [^{3}H]-LSD binding to human platelet membranes. [^{3}H]-LSD was found to bind to a saturable population of high affinity sites (Kd 0.83±0.07; Bmax 81±8fmol/mg protein).

The $5HT_2$ antagonists, ketanserin, methysergide and cyproheptadine were all found to be potent inhibitors of [^{3}H]-LSD binding to human platelet membranes (table 4.9), with Hill slopes not significantly different from unity. Similar binding affinities were found for compounds inhibiting the binding of [^{3}H]-LSD to human platelet membranes and [^{3}H]-ketanserin binding to rat frontal cortex (table 4.9). Yohimbine showed only low affinity for this binding site (10 fold less than it's affinity for $5HT_2$ binding sites on rat frontal cortex), 8-OH-DPAT and prazosin were inactive.

4.2.7b Pig coronary artery

The protein recovery in this membrane preparation was very low. The tissue was very fibrous, homogenisation was difficult and recovery of the membranes could only be made at a maximum centrifugation of 39000xg.

Initial studies with [^{3}H]-ketanserin binding to PCA were ineffective. Specific binding could be detected only above 10nM [^{3}H]-ketanserin (fig. 4.8) and represented ≤0.1% of

the free ligand concentration. High levels of nsb were found representing 90% of total binding at 10nM [^{3}H]-ketanserin. The binding could be inhibited only by high concentration of ketanserin (>10^{-6}M) and therefore was probably to a displaceable non-specific site (ketanserin binding site) and not to a $5HT_2$ receptor binding site.

^{125}I-LSD, with a specific activity of >74TBq/mmol, was found to label with high affinity a $5HT_2$ binding site on PCA membranes (fig. 4.9). However, a large percentage nsb was present. Only a low number of sites was labelled, which may have been the result of a poor membrane preparation. Nevertheless, the binding was probably to a $5HT_2$ binding site, as it was inhibited by ketanserin (Ki 16nM) and methysergide (Ki 25nM).

4.2.7c Human umbilical artery

The binding to HUA proved more sucessful than to PCA for a number of reasons. The tissue was less fibrous, the arteries (6/ preparation) were finely chopped, ground using a glass pestal and mortar and then homogenised in ice cold 0.25M sucrose using a polytron PT10 homogeniser (5 x 10s bursts). After filtering through a single layer of cheesecloth the homogenate was spun at 15000xg for 10min at 4^{o}C. The supernatant was then respun at 60000xg for 30min at 4^{o}C. The resultant pellet was resuspended in assay buffer (50mM Tris HCl, pH 7.4 at 37^{o}C, containing 4mM $CaCl_2$, 5.7mM ascorbate and 10μm pargyline). This membrane preparation proved successful unlike those prepared using Tris HCl buffers or slow centrifugation speeds. The higher recovery of 5HT binding sites was sufficient for use in [^{125}I]-LSD binding assays. Therefore, a characterisation of the 5HT binding site(s) on HUA was undertaken.

5HT is a potent agonist in the HUA: at the physiological oxygen tension (pO_2=15mmHg) contraction is mediated via $5HT_2$ receptors. At higher pO_2 (120mmHg) contraction is via both "$5HT_2$-like" and $5HT_2$ receptors (MacLennan and McGrath, 1986). [^{125}I]-LSD was found to bind to a saturable population of high affinity sites (Kd 0.4nM; fig 4.10). This binding site was characteristic of the $5HT_2$ site, showing high affinity for the $5HT_2$ antagonists and low

affinity for the $5HT_{1A}$ ligand buspirone and the $5HT_{1B}/5HT_{1A}$ ligand pindolol (table 4.10, fig 4.11).

The affinity values obtained from binding studies for effective antagonists were in good agreement with the pA_2 values for functional antagonism of receptors that were previously defined as $5HT_2$ (Table 4.10). However, so far, no evidence was found in these binding studies for the existence of the "$5HT_1$-like" receptor which mediates contraction of the HUA under high pO_2 conditions (MacLennan and McGrath, 1986)

These investigations of HUA suggest that it is a useful tissue for the study of vascular $5HT_2$ receptors using ligand binding techniques.

4.2.7d [^{3}H]-ketanserin binding to rat lung membranes

No specific binding of [^{3}H]-ketanserin to rat lung membranes could be detected over the concentration range 0.05-5nM.

4.2.8 Complex interactions at the $5HT_2$ binding site

In competition studies to determine the affinity of compounds for the $5HT_2$ binding site labelled by [^{3}H]-ketanserin on rat frontal cortex, all the antagonists described above, e.g. methysergide, spiperone and ketanserin, showed monophasic inhibition curves with Hill slopes not significantly different from unity. However, during the course of the study a Syntex compound (compound X) was found to produce shallow competition curves (IC_{50} 28.1±6.2nM), with a low Hill slope, 0.67±0.05, n=6. Further investigation of this complex binding phenomena was undertaken. Several possible explanations were examined, including the influence of assay constituents and conditions, agonist activity of compound X or negative co-operativity. It is important to note that heterogeneous steady state binding phenomena, due to multiple, independent binding sites or to interconvertible affinity states, also resemble the situation of negative co-operativity.

Assay constituents and conditions

The competition curves for compound X were repeated in

different buffer systems containing no or a high (8mM) concentration of $CaCl_2$. A decrease in the specific binding was seen in buffer without Ca^{2+} ions (see table 4.3). However, no significant change was seen in the affinity or nH values for compound X in either buffer; no Ca^{2+} IC_{50} 29.1nM, nH 0.69 or high Ca^{2+} IC_{50} 27.6, nH 0.75.

Changes in pH over the range 7.2-7.8 had no effect on the affinity value for compound X, as previously reported for ketanserin, 4.2.2d.

Effect of GTP

Inclusion of GTP has been shown to decrease the affinity values for agonists in this assay (fig. 4.4, Table 4.2) but to have no effect on antagonist binding. The inclusion of 1mM GTP in the competition assays for compound X had no effect on the binding parameters determined, IC_{50} 28.6nM, nH 0.72. Therefore it is unlikely, although not impossible, that compound X is an agonist at $5HT_2$ sites. In addition, compound X was shown to have no agonist activity in the HUA (MacLennan, 1986).

Multiple binding sites

The competition curve for compound X could be statistically fitted to two sites; site 1 IC_{50} 5.2×10^{-9} M, 46% and site 2 IC_{50} 5.4×10^{-7} M, 54% (fig. 4.12). Significance of the two site fit was assessed by the error of the sum of the squares method (see methods). Earlier work showed that under these assay conditions 1nM [^{3}H]-ketanserin labels only $5HT_2$ sites and not α_1-adrenoceptor or histamine receptors, for which it has lower affinity (10nM). In any case compound X has been shown to have low affinity for α_1-adrenoceptor (pKi 5.9) and histamine receptors (pKb 5.56). It is possible that [^{3}H]-ketanserin labels an as yet uncharacterised site. However, a wide variety of compounds from very different classes of structure to ketanserin produce Hill coefficients close to unity (tables 4.1 & 4.5).

Saturation experiments

The type of inhibition [^{3}H]-ketanserin binding by compound X was further investigated. Specific [^{3}H]-ketanserin binding with increasing [^{3}H]-ketanserin

concentrations was assayed in the absence of drug or in the presence of of $1x10^{-8}$ M compound X. Experiments were performed under the usual incubation conditions. Results analysed in Scatchard plots are presented in fig. 4.13. Compound X caused a significant decrease in the number of binding sites:- control 240±25, $1x10^{-8}$ compound X 82±10 (n=4) ($p<0.001$), and an increase in the affinity:- control 0.42±0.05, $1x10^{-8}$ compound X 0.24±0.06 (n=4). Therefore the inhibition was non-competitive.

The results of these studies would suggest that compound X has a non-competitive interaction at the $5HT_2$ binding site on rat frontal cortex, labelled by [^{3}H]-ketanserin.

4.2.9 $5HT_2$ functional assays

4.2.9a Platelet aggregation

5HT activates platelets of various mammalian species. Human platelets respond to 5HT with a shape change and only weak reversible aggregation (Baumgartner and Born, 1968). However, 5HT amplifies the human platelet aggregation induced by other agonists, via; adenosine-5'-diphosphate (ADP), collagen, adrenaline and noradrenaline. De Clerck et al (1984a & b) showed that ketanserin causes potent and selective inhibition of 5HT-induced and 5HT-amplified human platelet aggregation, suggesting the presence of a functional $5HT_2$ receptor on the platelet. The aim of this part of the study was to show that inhibition of 5HT-induced platelet aggregation can be used as a simple functional test of $5HT_2$ antagonism.

$5HT_2$ antagonists inhibited in a concentration-dependent manner the 5HT amplification of platelet aggregation induced by collagen in human PRP. The α_1-adrenoceptor antagonist, prazosin, was inactive and the α_2-adrenoceptor antagonist, yohimbine, showed some activity at concentrations probably corresponding to its affinity for $5HT_2$ receptors (Ki 1,550nM; see table 4.9) (table 4.11 and fig. 4.14). There is a good correlation between the inhibition of [^{3}H]-LSD binding to human platelet membranes and inhibition of 5HT induced platelet

aggreggation which can be seen in fig. 4.15.

4.2.9b Pig Coronary Artery

Characterisation of the receptor which may mediate the response to 5HT in the isolated PCA was made using antagonists.

Cumulative additions of 5HT ($1x10^{-8}$ -$1x10^{-5}$ M) caused concentration-related contractions of the isolated PCA (see figs. 4.16 and 4.17). which was well maintained at each response. The pD_2 for 5HT was 6.8±0.1, n=8.

Following washout of the drug, there was a slow relaxation to baseline tension which was complete within 1h. The second concentration response curve (CRC) to 5HT was performed 2h after the first and was found to be not significantly different from the first. Successive CRC's to 5HT in the PCA could therefore be reproduced with minimal shift.

Methysergide

Methysergide was a potent antagonist of 5HT. CRC's to 5HT in the presence of methysergide ($1x10^{-7}$ -$1x10^{-5}$ M) were parallel (fig. 4.16). The results were plotted graphically in the form of a Schild plot for the estimation of pA_2 value, determined to be 8.4 (8.1-8.7). The slope was also assessed using linear regression and determined to be 0.95±0.05, which was not significantly different from unity.

Ketanserin

Ketanserin ($1x10^{-7}$ -$1x10^{-5}$ M) displaced the 5HT CRC in a parallel manner at all concentrations (fig. 4.17). The Schild plot had a slope of 0.98±0.05 and the estimated pA_2 for ketanserin against 5HT was 8.9 (8.7-9.1).

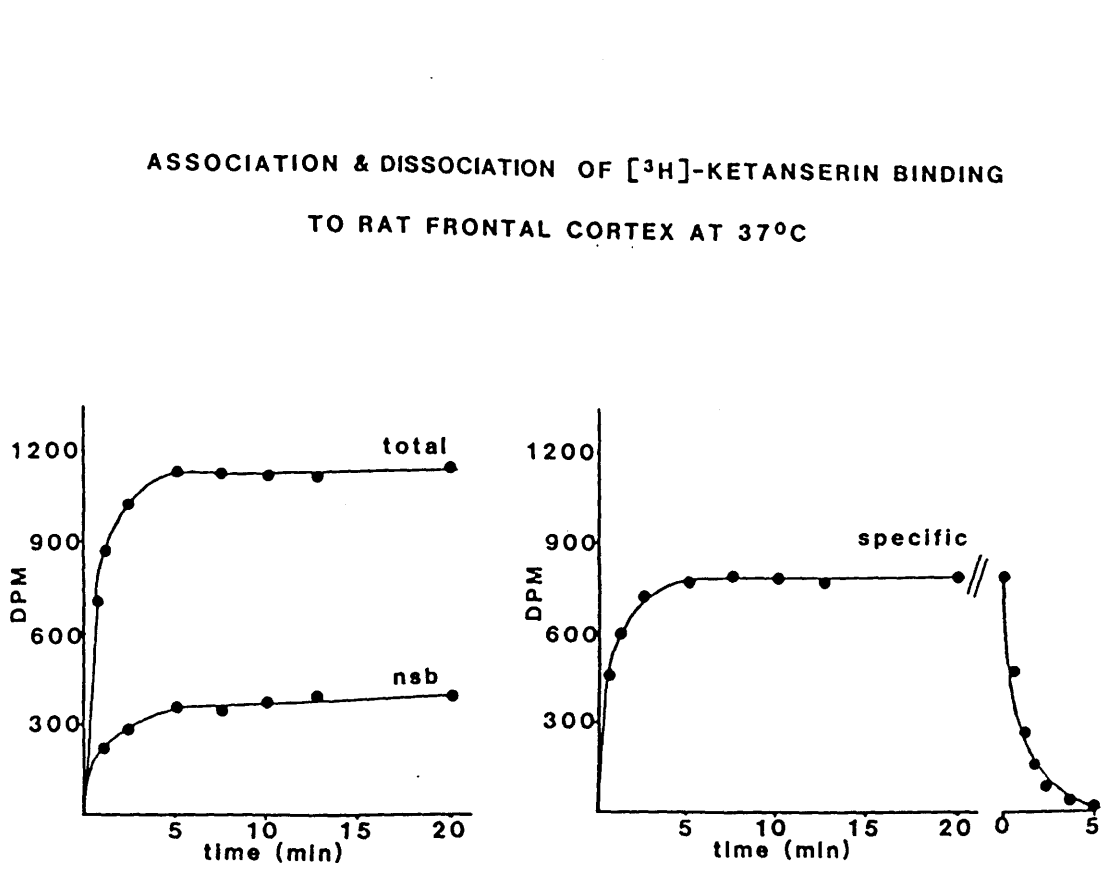


Fig. 4.1 Association and dissociation of [^{3}H]-ketanserin binding.

Association; membranes were incubated with [^{3}H]-ketanserin (1nM) at 37^{o}C for varying time intervals, before separating bound from free ligand as described in methods.

Dissociation; membranes were incubated with [^{3}H]-ketanserin (1nM) at 37^{o}C to equilibrium before dissociation was initiated by adding 2μM methysergide. Aliquots were then removed at varying time intervals and the bound radioactivity separated from free.

Specific binding was determined by subtracting nsb from total.

METHYSERGIDE & KETANSERIN INHIBITION OF [^{3}H]-KETANSERIN BINDING

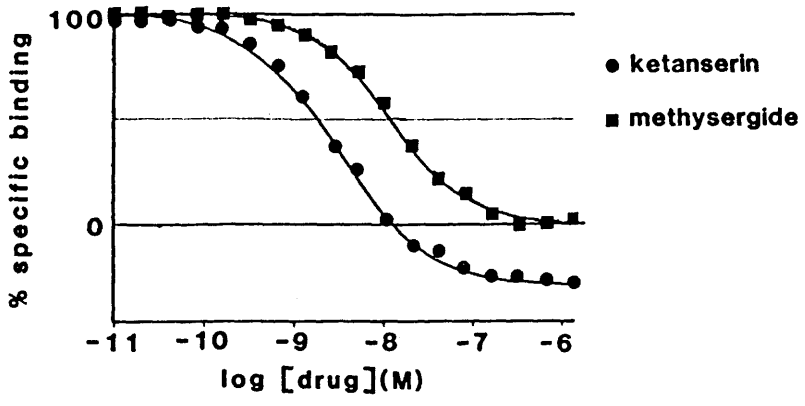


Fig. 4.2 Inhibition studies of [^{3}H]-ketanserin (1nM) to rat frontal cortical membranes were conducted over a concentration range of $1x10^{-11}$ -$1.31x10^{-6}$ M methysergide or ketanserin. Incubations were performed for 15min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μM pargyline; pH 7.4). The data describes a single experiment for each competitor performed in duplicate.

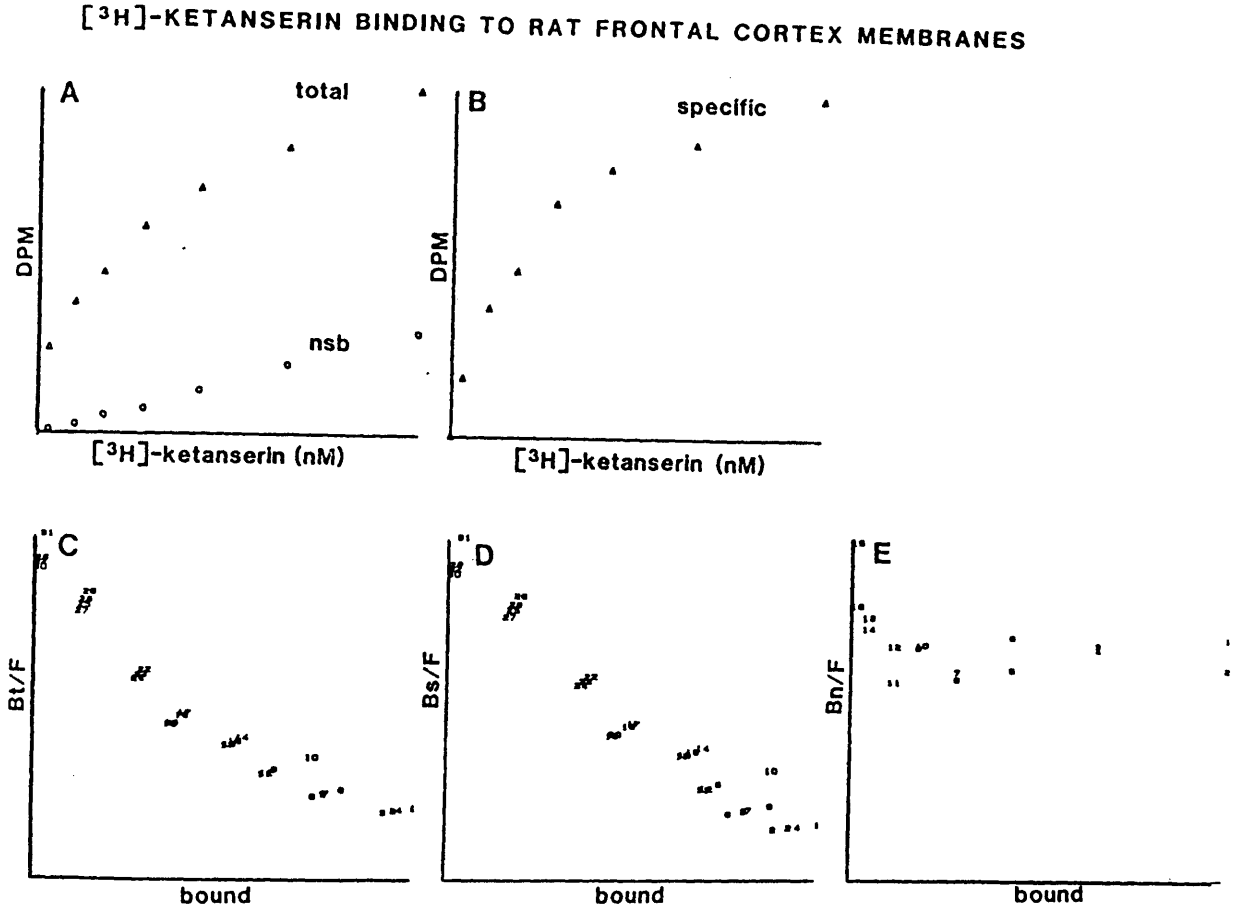


Fig. 4.3 Saturation binding of [^{3}H]-ketanserin to rat frontal cortical membranes. Specific binding is defined as that binding displaced by 2μM methysergide. The data describes a single experiment. A and B show total, nsb and specific binding as a function of the free ligand concentration. C,D and E show the Scatchard tranformations of the total, specific and nsb, respectively. Graphs like this were produced for all saturation assays performed in this study, and allowed the identification of rogue points before analysing the data via 'Ligand'.

EFFECT OF GTP ON 5HT INHIBITION OF [^{3}H]-KETANSERIN BINDING

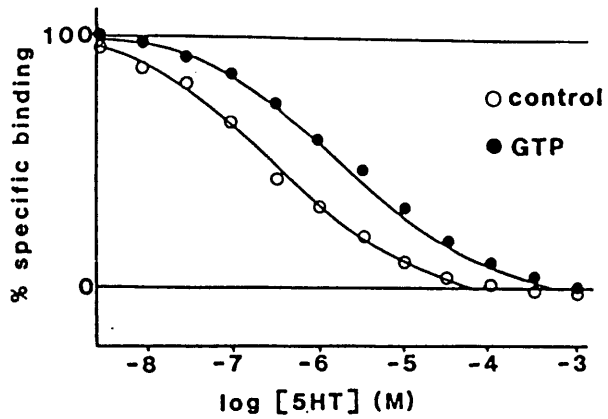


Fig. 4.4 Membranes were incubated with [^{3}H]-ketanserin (1nM) and 5HT (3×10^{-9} -1×10^{-3}), at 37^{o}C for 15min in a final volume of 1ml Tris assay buffer (50mM Tris HCl, 4mM $CaCl_2$, 5.7mM ascorbate, 10μM pargyline; pH 7.4), in the absence or presence of 1mM GTP. Non-specific binding was determined in the presence of 2μM methysergide.

EFFECT OF INCUBATION pH ON SPECIFIC BINDING OF [^{3}H]-KETANSERIN TO RAT FRONTAL CORTEX

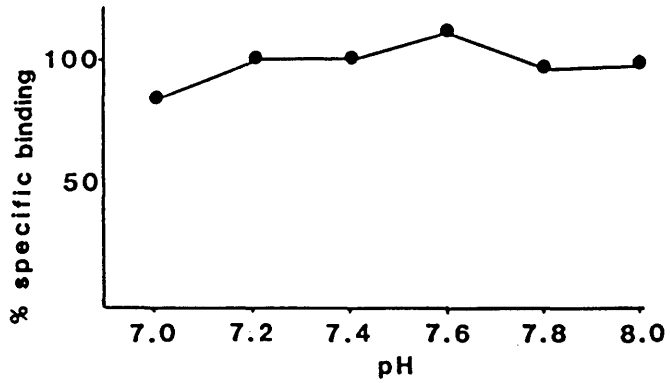


Fig. 4.5 Membranes were incubated with [^{3}H]-ketanserin (1nM) for 15min at 37^{o}C in 50mM Tris HCl assay buffer, pH 7.0-8.0. The specific binding is expressed as a percentage of that determined at pH 7.4. Data represents the mean of two experiments performed in duplicate.

[³H]–KETANSERIN BINDING TO GERBIL FRONTAL CORTEX

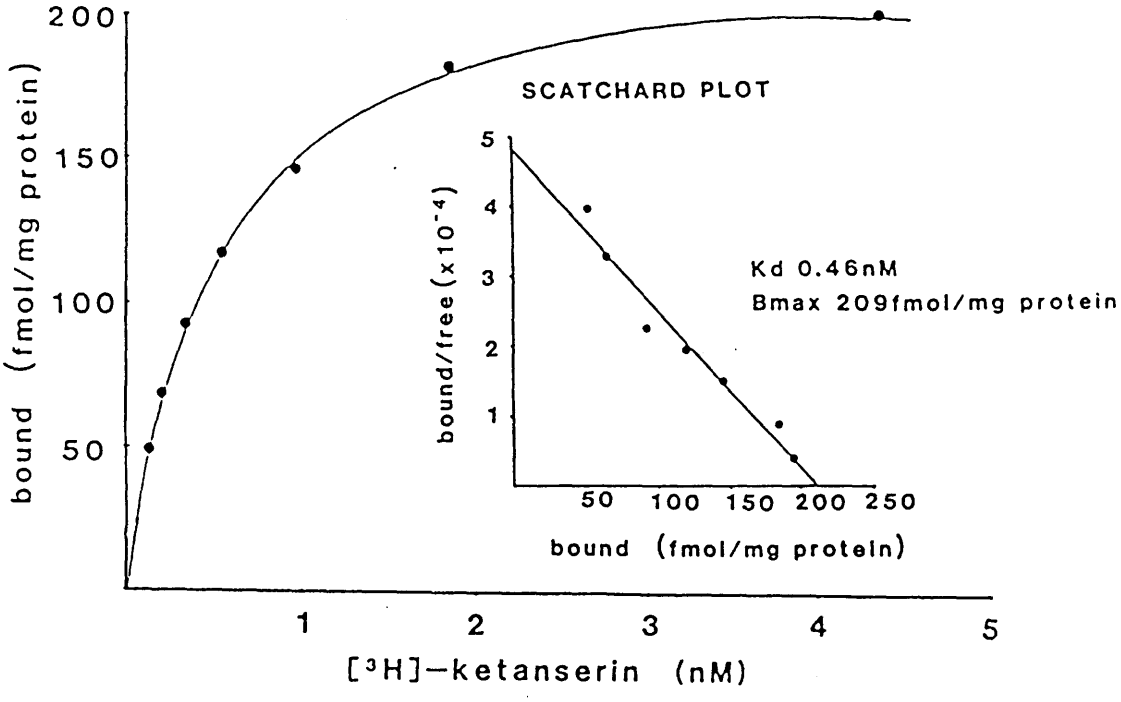


Fig. 4.6 Specific binding of [^{3}H]-ketanserin to gerbil frontal cortex. Specific binding is defined as that binding displaced by 2μm methysergide, and the data describes a single experiment performed in duplicate.

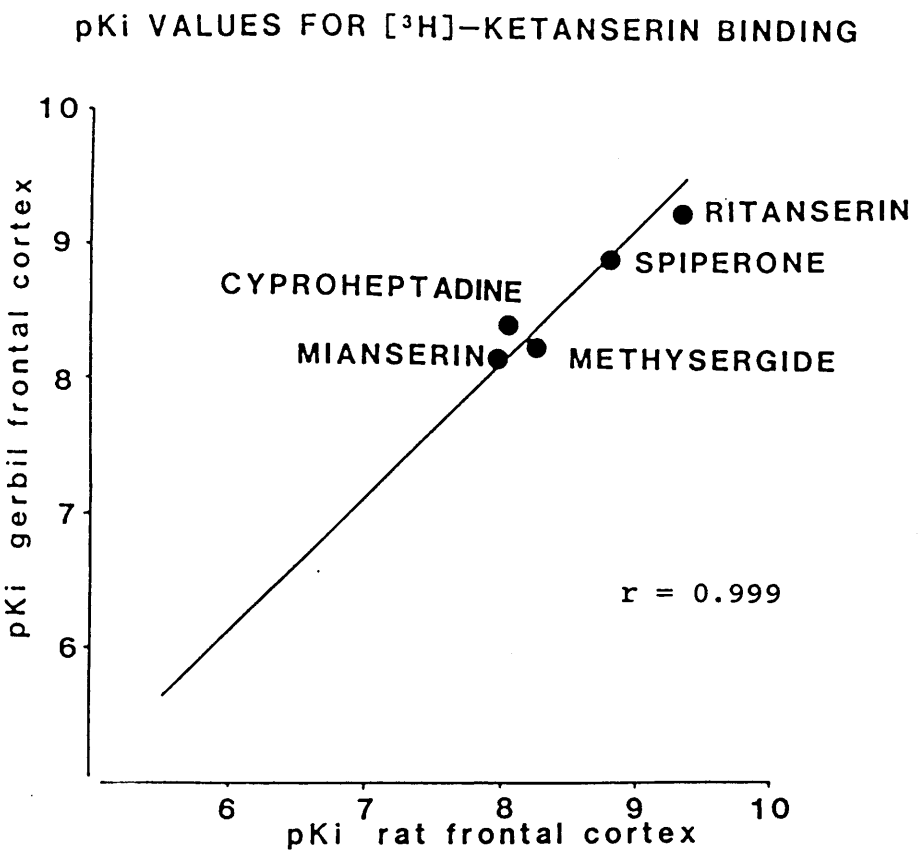


Fig. 4.7 Correlation between binding affinities of compounds for [^{3}H]-ketanserin binding to rat and gerbil frontal cortical membranes.

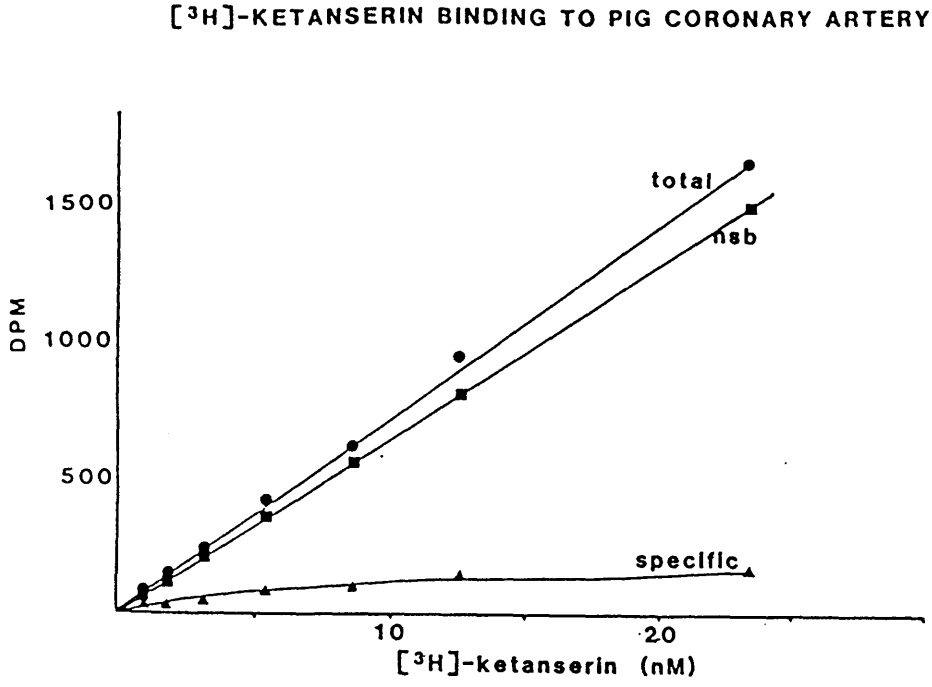


Fig. 4.8 Relationship between total, nsb and specific binding and ligand concentration. [^{3}H]-ketanserin concentrations of 0.05-25nM were incubated with pig coronary artery membranes at 37^{o}C for 15min in Tris HCl buffer (50mM Tris HCl, 4mM $CaCl_2$, 5.7mM ascorbate, 10μm pargyline; pH 7.4). Non-specific binding was determined in the presence of 2μm methysergide.

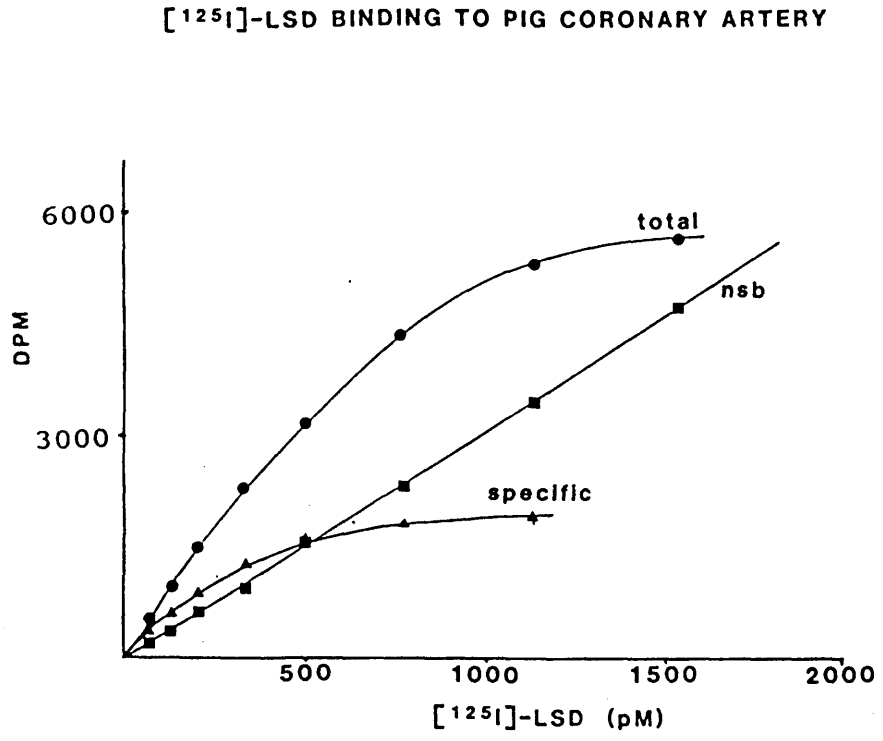


Fig. 4.9 Relationship between total, nsb and specific binding and ligand concentration. [^{125}I]-LSD concentrations of 0.01-4nM were incubated with pig coronary artery membranes at 37^{o}C for 60min in Tris HCl buffer (50mM Tris HCl, 4mM $CaCl_2$, 5.7mM ascorbate, 10μm pargyline; pH 7.4). Non-specific binding was determined in the presence of 2μm methysergide.

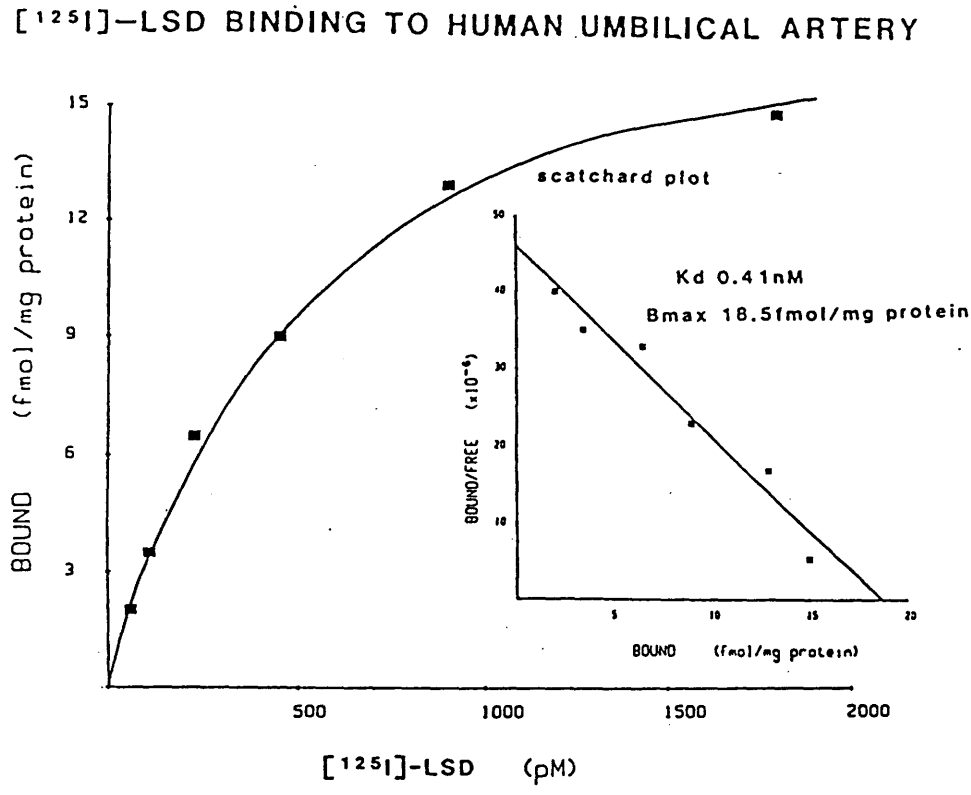


Fig. 4.10 Specific binding of [^{125}I]-LSD to human umbilical artery membranes. Membranes were incubated with [^{125}I]-LSD (0.01-4nM) at 37°C for 60min in Tris HCl buffer (50mM Tris HCl, 4mM $CaCl_2$, 5.7mM ascorbate, 10μm pargyline; pH 7.4). Non-specific binding was determined in the presence of 2μm methysergide. The data describes a single experiment performed in duplicate.

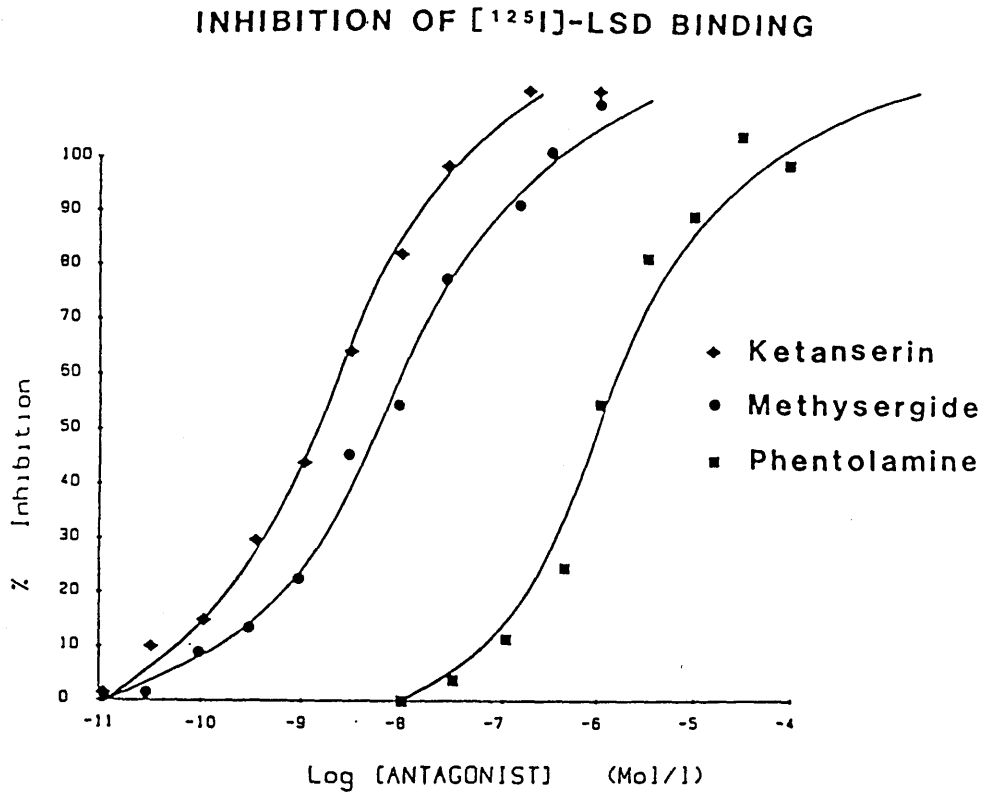


Fig. 4.11 Inhibition studies of [^{125}I]-LSD binding to HUA membranes were conducted over a concentration range of $1x10^{-11}$ -$1x10^{-4}$ M ketanserin, methysergide and phentolamine. Incubations were performed for 15min at 37 ^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline, pH 7.4). Non-specific binding was determined in the presence of 2μm methysergide. The data describes a single experiment for each competitor performed in duplicate.

COMPOUND X INHIBITION OF [^{3}H]-KETANSERIN BINDING

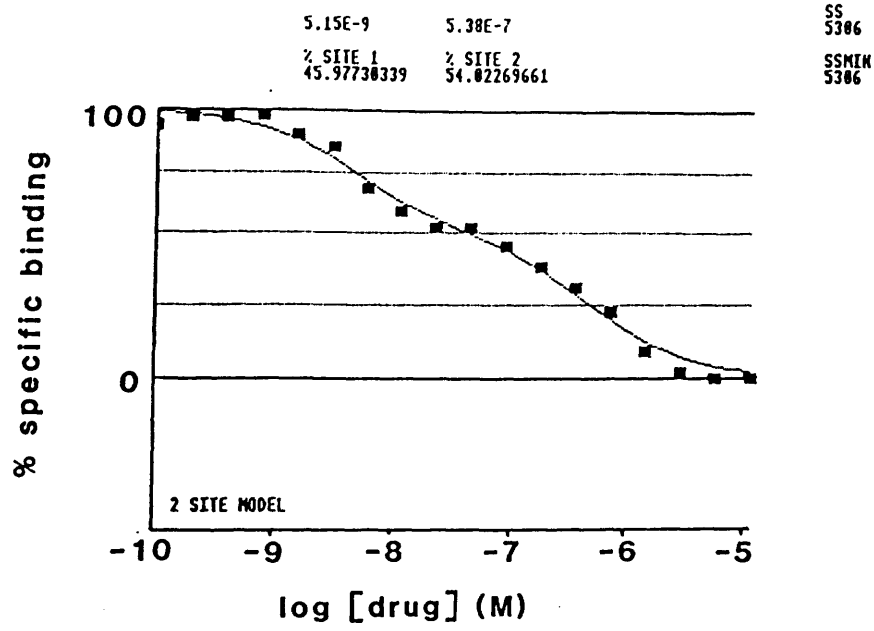


Fig. 4.12 Inhibition studies of [^{3}H]-ketanserin to rat frontal cortical membranes were conducted over a concentration range of $1x10^{-10}$ -$1x10^{-5}$ M compound X. Incubations were performed for 15min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4). Non-specific binding was determined in the presence of 2μm methysergide. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.001$.

Scatchard plots of specific [^{3}H]-ketanserin binding to rat cerebrocortical membranes in the presence or absence of compound X

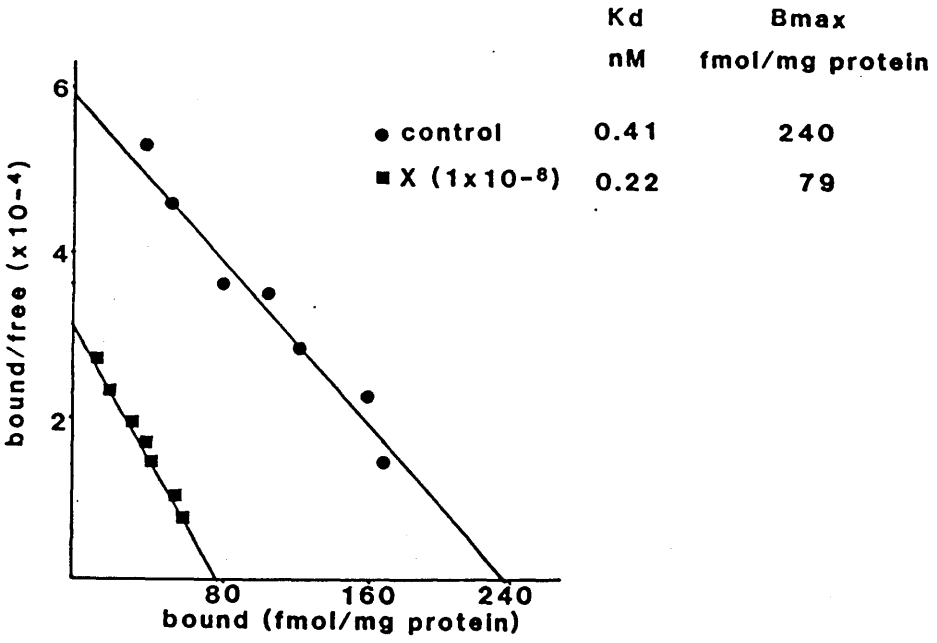


Fig. 4.13 Scatchard plots of specific [^{3}H]-ketanserin binding to rat frontal cortical membranes in the absence and presence of compound X. Incubations were performed for 15min at 37^{o}C in a Tris HCl buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4). Non-specific binding was determined in the presence of 2μm methysergide. The data represents a single experiment.

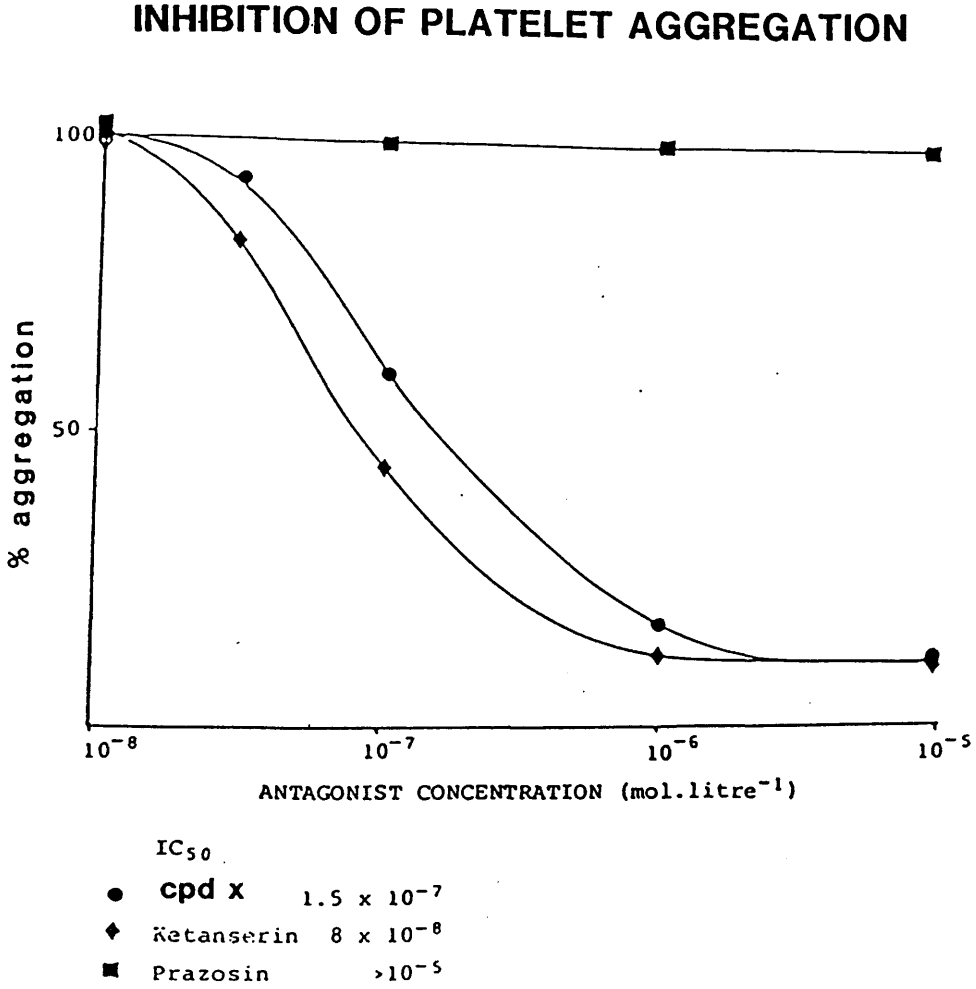


Fig. 4.14 Platelet aggregation was carried out in response to 5HT (10μm) and collagen (160nM) in the absence or presence of increasing concentrations of compound X, ketanserin or prazosin.

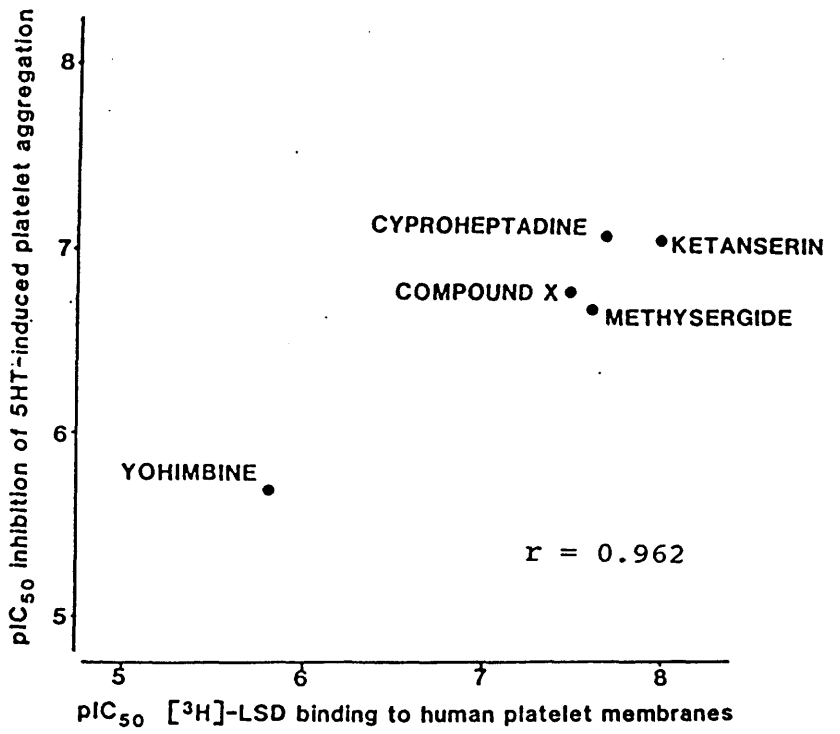


Fig. 4.15 Correlation of affinity of compounds for [^{3}H]-LSD binding to human platelet membranes and inhibition of 5HT-induced platelet aggregation.

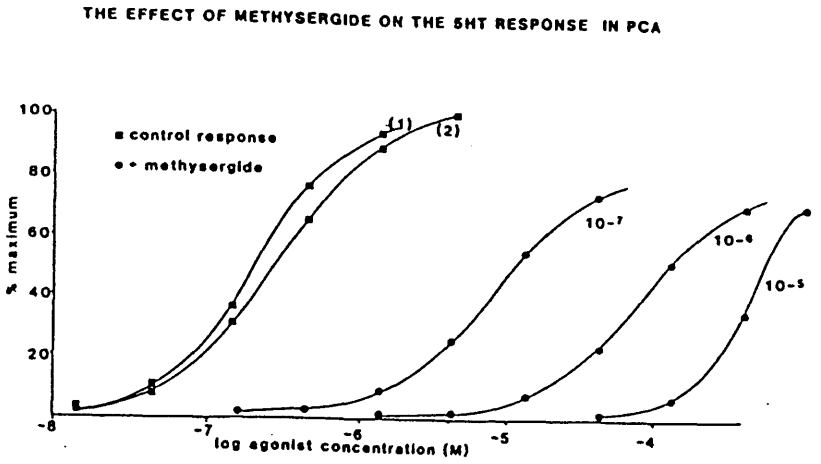


Fig. 4.16 Log concenration-response curves (CRC's) to 5HT in the presence of methysergide in longitudinal strips of pig coronary artery (n=4). CRC's to 5HT were constructed twice in each of four preparations from the same artery. Response (ordinate) was calculated as a % of the maximal response to 5HT of the first curve. In three of the four preparations the second CRC was repeated in the presence of one concentration of methysergide. In the fourth (control) preparation the first and second CRC's were constructed without antagonist in order to assess the change in sensitivity to 5HT with time. Vertical bars are the mean + s.e.m.

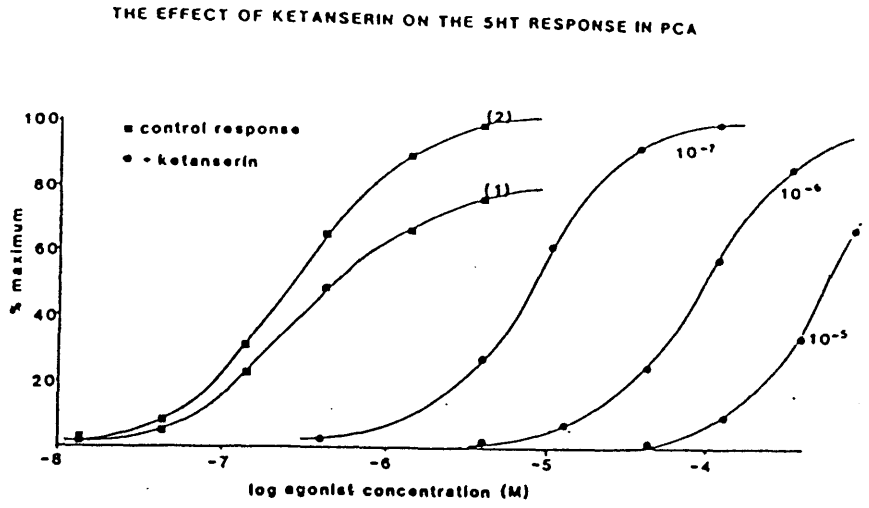


Fig. 4.17 Log concenration-response curves (CRC's) to 5HT in the presence of ketanserin in longitudinal strips of pig coronary artery (n=4). CRC's to 5HT were constructed twice in each of four preparations from the same artery. Response (ordinate) was calculated as a % of the maximal response to 5HT of the first curve. In three of the four preparations the second CRC was repeated in the presence of one concentration of ketanserin. In the fourth (control) preparation the first and second CRC's were constructed without antagonist in order to assess the change in sensitivity to 5HT with time.

Table 4.1. The affinity of selective antagonists and agonists for the $5HT_2$ binding site

	pKi (nM)
ketanserin	8.72 ± 0.06
methysergide	8.00 ± 0.15
cyproheptadine	8.20 ± 0.10
mianserin	7.95 ± 0.08
imiloxan	<5
yohimbine	6.7 ± 0.15
prazosin	<5
domperidone	<5
propranolol	<5
triprolidine	<5
5HT	6.32 ± 0.11
noradrenaline	<4
dopamine	<4
isoprenaline	<4

Membranes were incubated with [^{3}H]-ketanserin (0.5nM) for 15min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4 at 37^{o}C). Nsb was defined using 2μm methysergide. The concentration of drug to inhibit specific binding by 50% was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. The Hill slopes for all compounds tested were not significantly different from unity. Values shown are the mean Ki of at least three experiments performed in duplicate.

Table 4.2 Effect of GTP on affinity values for [^{3}H]-ketanserin binding to rat frontal cortical membranes

	IC_{50} (nM) control	nH	1mM GTP	nH
5HT	260 ± 35	0.85±0.05	2490 ± 70*	0.88±0.06
bufotenine	690 ± 20	0.75±0.03	1790 ± 85*	0.92±0.08
ketanserin	2 ± 0.08	0.90±0.03	1.8 ± 0.1	0.92±0.04
spiperone	1.6 ± 0.05	0.95±0.04	1.6 ± 0.09	0.94±0.05
cyproheptadine	6 ± 0.15	0.99±0.04	6 ± 0.15	1.02±0.03
methysergide	10 ± 0.2	0.93±0.03	13 ± 0.2	0.96±0.04

Membranes were incubated with [^{3}H]-ketanserin (0.5nM) for 15min at 37°C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4 at 37°C) in the presence or absence of 1mM GTP. Specific binding was determined by parallel incubations in the presence of 2μm methysergide. Data represents the mean ± s.e.m. of 2-5 experiments performed in duplicate.

*P<0.001 relative to control

Table 4.3 Effect of ascorbate and buffer ions on [^{3}H]-ketanserin binding to rat frontal cortical membranes

	Kd nM	Bmax fmol/mg protein
Control	0.42±0.05	240±25
5.7mM ascorbate	0.68±0.08*	173±20
physiological	0.86±0.07**	183±30

Membranes were incubated to equilibrium with [^{3}H]-ketanserin (0.5nM) in a 50mM Tris HCl buffer (pH 7.4 at 37^{o}C) containing 5.7mM ascorbate, 4mM $CaCl_2$ and 10μM pargyline (control), or containing only 5.7mM ascorbate or a physiological buffer as described in section 4.2.2b. Data represents the mean ± s.e.m. of three experiments performed in duplicate.

Statistical significance, relative to control.

*p<0.05; **p<0.01.

Table 4.4 Effect of equilibrium temperature on [^{3}H]-ketanserin binding to rat frontal cortical membranes

Incubation Temperature	Kd (nM)	Bmax (fmol/mg protein)
22^{o}C	0.48±0.08	222±18
37^{o}C	0.42±0.05	240±25

Membranes were incubated to equilibrium (15min at 37^{o}C; 25min at 22^{o}C) with [^{3}H]-ketanserin (0.05-5nM) in a Tris assay buffer (50mM Tris HCL, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4). Data represents the mean ± s.e.m. of three experiments performed in duplicate. No significant difference was found in any group.

Table 4.5 Characterisation of the $5HT_2$ binding site in the rat frontal cortical membranes

	Ki (nM)		
	LSD	ketanserin	mianserin
5HT antagonists			
ketanserin	1.9	2	2.5
methysergide	7	10	12
cyproheptadine	4	6	3
mianserin	15	11	5
Dopamine antagonist			
spiperone	1.7	1.6	1.4
α-Adrenoceptor antagonists			
prazosin	-	1,259	-
phentolamine	-	1,000	-
yohimbine	-	200	-
imiloxan	-	>10,000	-
β-Adrenoceptor antagonist			
propranolol	-	8,000	-
Agonists			
5HT	694	501	562
dopamine	>10,000	>10,000	>10,000
noradrenaline	>10,000	>10,000	>10,000
histamine	>10,000	>10,000	>10,000

Membranes were incubated with [^{3}H]-ketanserin (0.5nM) for 15min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4 at 37^{o}C). Nsb was defined using 2μm methysergide. The concentration of drug to inhibit specific binding by 50% was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. The Hill slopes for all compounds tested were not significantly different from unity. Values shown are the mean Ki (s.e.m. fall within 5%) of at least three experiments performed in duplicate.

Table 4.6 Regional distribution of $5HT_2$ binding sites in the rat brain

	Radioligand	Bmax (fmol/mg protein)	Kd (nM)
Frontal cortex	ketanserin	240±25	0.42±0.05
	mianserin	160±25	9.2 ±0.9
	LSD	237±30	0.7 ±0.1
Striatum	ketanserin	175±15	0.61±0.7
Hippocampus	ketanserin	86±7	0.52±0.8
Cerebellum	ketanserin	ND	ND

ND No detectable specific binding

Saturation assays were performed as described in methods section 2.1.3, using rat membranes; frontal cortex, striatum, hippocampus or cerebellum, a variety of radioligands; [^{3}H]-ketanserin, [^{3}H]-mianserin or [^{125}H]-LSD. Values shown were obtained using the non-linear itterative curve fitting programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.m. of 3-5 experiments performed in duplicate.

Table 4.7 Affinity values for the $5HT_2$ binding site on gerbil frontal cortical membranes

	pKi (nM)
ketanserin	8.65 ± 0.09
spiperone	8.90 ± 0.05
ritanserin	9.20 ± 0.08
methysergide	8.25 ± 0.09
cyproheptadine	8.35 ± 0.05
mianserin	8.20 ± 0.04
5HT	5.62 ± 0.04
8-OH-DPAT	<5
RU24969	5.98 ± 0.05
5CT	5.70 ± 0.05
Prazosin	5.30 ± 0.05

Membranes were incubated with [^{3}H]-ketanserin (0.5nM) for 15min at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4 at 37^{o}C). Nsb was defined using 2μm methysergide. The concentration of drug to inhibit specific binding by 50% was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. The Hill slopes for all compounds tested were not significantly different from unity. Values shown are the mean Ki ± s.e.m. of at least three experiments performed in duplicate.

Table 4.8 Regional distribution of $5HT_2$ binding sites in the gerbil brain

	Bmax (fmol/mg protein)	Kd (nM)
Frontal cortex	206 ± 25	0.48 ± 0.03
Striatum	190 ± 10	1.90 ± 0.6*
Hippocampus	50 ± 14	0.76 ± 1.8
Cerebellum	ND	ND

ND No detectable specific binding

Membranes were incubated with [^{3}H]-ketanserin (0.05-5nM) in a Tris buffer system (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 15min at 37^{o}C. Specific binding was determined by parallel incubations in the presence of 2μm methysergide. Values shown were obtained using the non-linear itterative curve fitting programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.m. of 2-5 experiments performed in duplicate.

Statistical significance, relative to frontal cortex.

*$p<0.05$.

Table 4.9 Affinity values for $5HT_2$ binding sites on human platelet membranes

	Ki (nM)	nH
Ketanserin	10	0.88
cyproheptadine	20	0.90
methysergide	25	0.97
5HT	205	0.80
8-OH-DPAT	>10,000	-
yohimbine	1,550	0.79
prazosin	>10,000	-

Membranes were incubated with [^{3}H]-LSD (0.5nM) for 4h at 37^{o}C, in the presence or absence of 12 concentrations of the competing drug in a final volume of 2ml Tris assay buffer (50mM Tris HCl, 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4 at 37^{o}C). Nsb was defined using 2μm methysergide. The concentration of drug to inhibit specific binding by 50% was obtained graphically and corrected for free ligand concentration. Values shown are the mean Ki (s.e.m fall within 5%) of at least three experiments performed in duplicate.

Table 4.10 Inhibition of [^{125}I]-LSD binding to human umbilical artery membranes

Antagonist	pKi (nM)	n	*pA_2	n
Ketanserin	8.88 ± 0.06	4	8.92 (8.70-9.14)	6
Methysergide	8.35 ± 0.10	3	8.52 (8.32-8.72)	5
Phentolamine	6.25 ± 0.05	4	6.37 (5.88-6.86)	6
Buspirone	6.05 ± 0.11	3	<7	5
Pindolol	<5	3	<5	5

values are the mean (95% confidence limits) or mean ± s.e.mean

*pA_2 values determined by S MacLennon and taken from Brown, MacLennon and McGrath (1987).

Membranes were incubated with [^{125}I]-LSD (0.2nM) and a range of concentrations of the competing ligand in Tris buffer (50mM Tris HCl containing 5.7mM ascorbate, 4mM $CaCl_2$, 10μm pargyline; pH 7.4) for 60min at 37^{o}C in a final volume of 250μl. Nsb was defined using 2μm methysergide.

Table 4.11 Inhibition of 5HT-induced platelet aggregation

	IC_{50} (M)
ketanserin	8.0×10^{-8}
compound X	1.5×10^{-7}
methysergide	2.0×10^{-7}
cyproheptadine	7.5×10^{-8}
yohimbine	2.0×10^{-6}
prazosin	$>10^{-5}$

Platelet aggregation was carried out in response to 5HT (10μm) and collagen (160nM) in the absence or presence of increasing concentrations of the competing ligands.

4.4 Discussion

The studies performed in this section were designed to characterise the binding of [^{3}H]-ketanserin to rat and gerbil brain membranes and to examine the influence of experimental conditions. Investigations of peripheral [^{3}H]-ketanserin binding were also undertaken and the results compared with functional studies of the $5HT_2$ receptors.

The results obtained confirmed that [^{3}H]-ketanserin is an effective radioligand for the study of $5HT_2$ receptor binding sites, showing both high affinity for the site and only a low percentage of non-specific binding (approximately 25% around the Kd of the ligand). The slow dissociation rate of the ligand-receptor complex means that bound radioactivity can be separated from free by vacuum filtration.

The kinetics of [^{3}H]-ketanserin binding were consistent with it binding to a single population of sites up to 5 nM. However, above this concentration a second component to the binding was identified in both total and specific binding isotherms. This second component also showed relatively high affinity and low capacity. It was assumed to be binding to a second neurotransmitter site as ketanserin has been shown to have nanomolar affinity for α_1-adrenoceptors and histamine receptors (Leysen and Laduron, 1977). Therefore studies of $5HT_2$ receptor binding sites were performed at concentrations less than 5nM. [^{3}H]-Ketanserin has been shown to label α_1-adrenoceptors in human and pig brain membranes (Hoyer et al, 1987c). This probably explains the reports in earlier studies that some antagonists show biphasic inhibition curves for [^{3}H]-ketanserin binding in the pig cortex (Hoyer et al, 1985b).

Difficulty was encountered in finding a drug to define nsb. Ketanserin itself was found to displace nsb, probably due to its high lipophilic nature. The structurally unrelated antagonist methysergide (2×10^{-6} M) was chosen.

Several compounds including 5HT, showed a plateau at the same level of specific binding as methysergide which suggests that the specific binding is accurately defined and that interaction with other receptor sites or with chemical recognition sites of the ketanserin structure 'ketanserin binding site' is not involved. Compounds of the same chemical structure to the radioligand have previously been shown to displace nsb (see Burt, 1985). [^{3}H]-ketanserin has been shown to label displaceable non-serotonergic sites in striatal membranes and on platelets (Leysen 1985), which were found to be selectively inhibited by tetrabenazine and proposed to be involved in the release of mainly oxidized metabolites of catecholamines and 5HT (Leysen et al, 1987).

There were considerable differences in the binding of [^{3}H]-ketanserin under various conditions. When included in the assay to prevent oxidation of indolamine ligands, ascorbate caused a reduction in both the affinity and number (although not significant in this study) of $5HT_2$ binding sites. This has previously been shown by Muakkassah-Kelly et al, (1982) for the binding of [^{3}H]-spiperone and Leysen et al, (1982a) for the binding of [^{3}H]-ketanserin. The reduction in binding in the presence of ascorbate could be cause by lipid peroxidation induced by ascorbate (Muakkassah-Kelly et al, 1982). This phenomena was described at some length in the discussion to chapter 3 and therefore will not be repeated here.

In a physiological buffer a significant decrease in affinity and a decrease in Bmax, although not significant was found for the binding of [^{3}H]-ketanserin. This was suggested by Leysen et al, (1982a) to be due to an increase in the dissociation rate of the [^{3}H]-ketanserin receptor complex in physiological compared with Tris HCl buffers. Monovalent ions have been shown to have very little effect on the specific binding of [^{3}H]-ketanserin. Sodium which is usually the most potent monovalent ion in shifting agonist curves (Limbird, 1981) has little effect on agonist inhibition of [^{3}H]-ketanserin binding. However, Battaglia et al, (1983) showed that Li^+ was 10 fold more

potent in reducing the apparent affinity of 5HT for [^{3}H]-ketanserin.

GTP was found to cause a rightward shift in agonist concentration response curves, similar to those reported for receptors coupled to adenylate cyclase. However, Conn and Sanders-Bush (1985, 1986) showed a good correlation between the $5HT_2$ binding affinity in the rat frontal cortex and the inhibition of PI turnover. Therefore, as suggested by Rodbell (1980), it would appear that not only sites linked to adenylate cyclase are influenced by GTP. The involvement of a guanine nucleotide binding protein in the coupling of the $5HT_2$ binding site to its effector systems has been proposed (Berridge and Irvine, 1984).

The site labelled by [^{3}H]-ketanserin in the gerbil frontal cortex would appear to be $5HT_2$ specific as adjudged by the moderate affinity of 5HT and high affinity of $5HT_2$ antagonists but low affinity of NA, dopamine, isoprenaline and histamine and of prazosin, domperidone, propranolol and triprolidine. Although only a limited study of the distribution of $5HT_2$ binding sites in the gerbil brain was undertaken. The highest density of binding sites was seen in the frontal cortex as in the rat (this study, Leysen and Laduron, 1977) and guinea-pig (Leysen et al, 1982b). In human brain a high density of $5HT_2$ binding sites is found in layers 111 and V of several cortical areas including the frontal cortex (Pazos et al, 1987). No significant binding was seen in the cerebellum. This is similar to that observed in the rat but in contrast with that reported for the guinea-pig (Leysen et al, 1982b). The distribution of $5HT_2$ binding sites markedly differs from the distribution of $5HT_1$ binding sites in the gerbil, see section 3.2.6.

A high affinity, saturable [^{3}H]-LSD binding site was identified on human platelet membranes, with drug affinities comparable to the $5HT_2$ binding site in rat frontal cortex membranes. The characterisation of this site was similar to the site described by McBride et al (1983) for the binding of [^{3}H]-spiperone to intact human platelets

and by Geaney et al, (1984) for the binding of [^{3}H]-LSD. [^{3}H]-5HT also labels a 5HT site on human platelets with relatively high affinity (20nM) and high capacity (800fmol/mg protein) which has been shown to be the 5HT uptake site (Pletscher and Affolter, 1983). The affinity values of a number of antagonists, for the human platelet $5HT_2$ binding site were found to correlate with their ablility to inhibit 5HT-induced platelet aggregation. Therefore this could provide a useful functional test for $5HT_2$ antagonists. This information would be especially useful as changes in platelet aggregation are implicated in the aetiology of many vascular disorders.

A high affinity, saturable [^{125}I]-LSD binding site was identified for the first time on human umbilical artery membranes. Binding was displaced potently by $5HT_2$ antagonists with affinities comparable to the rat brain and not by $5HT_1$ ligands. The failure to identify $5HT_2$ binding sites on PCA using [^{3}H]-ketanserin was probably due to the low specific activity of the radioligand. Studies of [^{125}I]-LSD to PCA showed only limited success because of the difficulties encountered in the membrane preparation, the tissue was very fibrous and at the time of these studies only a relatively low spin speed was available. However, these problems were overcome in the HUA studies and the binding methods used here may prove useful for the further characterisation of human vascular $5HT_2$ receptors. They also show a useful integration of binding and functional techniques.

Compound X, using in vitro binding assays, was found to bind with high affinity to $5HT_2$ receptor sites; however the competition curves showed low Hill slopes. A shallow Hill slope can indicate negative co-operativity. The binding of compound X lessens the apparent affinity that the rest of the binding sites display for the competing drug; i.e. there is an allosteric modulation on the binding of the compound. This observation prompted a more thorough investigation of the type of inhibition caused by compound

X on [^{3}H]-ketanserin binding to $5HT_2$ sites on rat frontal cortex.

Compound X was shown to have no intrinsic agonist activity on 'in vitro' smooth muscle preparations. Displacement of [^{3}H]-ketanserin binding to rat frontal cortex membranes by compound X was unaffected by 1mM GTP, unlike 5HT and bufotenine, therefore it is unlikely to have agonist activity.

Interestingly, the experiments indicated that compound X was non-competitive at $5HT_2$ binding sites in the rat frontal cortex. Frenkel and Kaumann, (1985) suggested a model for allosterically controlled $5HT_2$ receptors. According to the model 5HT and ketanserin compete for the $5HT_2$ receptor in the active R state whereas methysergide and ritanserin (Frenkel and Kaumann 1987) favour isomerization of the receptor towards the less active R' state. Ketanserin competes not only with 5HT for the $5HT_2$ receptor in the R state but also competes with methysergide for the allosteric site thus causing R' - R. When the $5HT_2$ receptor is in the R' state, 5HT at high micromolar concentrations can evoke merely a residual low-sensitivity component of contraction.

Therefore, functional experiments suggest that ketanserin binds with high affinity to both the $5HT_2$ receptor and an allosteric site, whereas methysergide and ritanserin bind apparently only to the allosteric site. This would imply that [^{3}H]-ketanserin should label both sites and that ritanserin and methysergide should bind only to the allosteric site. Ritanserin and methysergide therefore should only partially remove ketanserin from specific sites. However, this does not hold true for the binding of [^{3}H]-ketanserin to rat frontal cortex. Methysergide appears to be a competitive antagonist which displaces the same percentage of specific binding as 5HT. The extra binding displaced by cold ketanserin has been attributed to a 'ketanserin binding site' i.e. displaceble nsb.

Following preincubation with 1nM ritanserin (Leysen et al, 1985) or 10nM compound X [^{3}H]-ketanserin binding is reduced. Conceivably these compounds, by allosterically

favouring the R' state of the $5HT_2$ receptor prevents the access of [^{3}H]-ketanserin to both the allosteric site (by competition) and to the R state of the $5HT_2$ receptor (by allosterism). However, the non-competitive inhibition by ritanserin has been ascribed to the slow dissociation of the compound from $5HT_2$ sites in the rat frontal cortex (Leysen et al, 1985). This cannot explain the apparent non-competitive inhibition by compound X, as it rapidly dissociates from $5HT_2$ binding sites, (MacLennan, 1986).

A further complication is that compound X appears to be competitive in PCA and HUA preparations (MacLennan, 1986). It would be useful to examine the binding kinetics of these compounds in tissues where they have been shown to be non-competitive in functional studies, in order to further examine the proposed 'states' of the $5HT_2$ receptor. Compound X may well prove a useful tool in these studies.

Chapter 5: THE EFFECT OF CEREBRAL ISCHAEMIA ON 5HT NEUROPHARMACOLOGY IN THE MONGOLIAN GERBIL

5. THE EFFECT OF CEREBRAL ISCHAEMIA ON 5HT NEUROPHARMACOLOGY IN THE MONGOLIAN GERBIL

5.1 Introduction

A marked reduction in the supply of oxygen or glucose to the brain leads to a cascade of events that proceed from neuronal dysfunction to cell death depending on the severity and duration of the insult. Although cerebral ischaemia is generally related to disturbances within the circulation, its impact on the tissue is determined by neurochemical events at the cellular and subcellular level. The absence of all blood supply results in an immediate reduction in high energy phosphates and glucose (Goldberg et al, 1966) with a reciprocal increase in lactate (Rehncrona et al, 1981) and free fatty acids (Bazan, 1970). If a critical period of ischaemia is reached then uncontrolled entry of calcium occurs leading to cell death (Hass, 1983).

In addition to changes in cellular metabolism, cerebral ischaemia alters the level of the monoamines, noradrenaline (NA) dopamine (DA) and 5HT; neurotransmitter release could be associated with calcium entry. Cerebral infarction in the Mongolian gerbil, Meriones unguiculatus, reduces the level of DA in the neostriatum, hippocampus and hypothalamus (Zervas et al, 1974) and corpus striatum (Alps et al, 1984), and 5HT in the cerebral cortex (Welch et al, 1977; Harrison et al, 1979) and striatum (Alps et al, 1985). Low levels of 5HT have also been reported in human cerebral infarcts at autopsy (Jellinger et al, 1978). The reduction in DA and 5HT has been attributed to their release from neurons situated in the penumbra of the ischaemic insult (Lavyne et al, 1975a; Welch et al, 1972). The increased level of extraneuronal 5HT has been suggested as a contributing factor in the pathogenesis of progressive cerebral ischaemia (Welch et al, 1972; 1973), possibly leading to secondary deleterious effects on cerebral blood flow and metabolism (Wurtman and Zervas, 1974).

In order to investigate further the effects of cerebral ischaemia on the 5HT system, studies were carried out in the gerbil, using a number of models of ischaemia. Although it is known that certain types of neurons are selectively vulnerable to ischaemia, such as the hippocampus pyramidal cells in the CA1 subfield (Kirino, 1982),. very few studies have focused on the alterations in the receptor populations following ischaemia. This section describes the effect that ischaemia has on 5HT binding sites in the gerbil, namely $5HT_2$ sites in the frontal cortex, $5HT_{1B}$ sites in the striatum and $5HT_{1A}$ sites in the hippocampus. The latter population are particularly interesting as they are located on the pyramidal cells (Pazos and Palacios, 1985). The effect of cerebral ischaemia on the level of 5HT in various regions of the brain was also examined to determine, if possible, its relation to the alterations in the receptor populations. Finally, preliminary studies were undertaken to examine the functional relevance of the changes observed.

The figures and tables are included at the end of the results section.

5.2 RESULTS

5.2.1 Identification of stroke-prone animals

Initial studies into the effects of cerebral ischaemia on 5HT binding sites were undertaken using the 3h unilateral non-recovery model of ischaemia described in section 2.3.1a. One disadvantage to the model is the low yield of ischaemic animals. The Mongolian gerbil was introduced as a stroke model because of its incomplete circulus arteriosus; unilateral carotid ligation produces ischaemia in 30-40% of the gerbils. The failure to induce cerebral ischaemia in all animals made it necessary to select out the stroke-prone from stroke-resistant animals. Selective breeding in some laboratories has increased the yield of stroke-prone animals. Selection is most commonly achieved using an assessment of clinical signs, circling behaviour, extreme rotation of hind limb, eyes fixed open, ptosis, paucity of movements and piloerection-tremor. However, this is only possible in recovery models of ischaemia and therefore could not be used in these studies. A method for rapidly assessing the anatomical defects in the circulus arteriosus was devised for determining stroke-prone animals.

5.2.1a Anatomical determination of stroke-prone animals

A schematic representation of the circulus arteriosus in the Mongolian gerbil is shown in fig. 5.1.

The observation by Levine and Payan, (1966) that unilateral carotid ligation in the Mongolian gerbil caused a 20% mortality rate after 48h was suggested to be due to some inadequacy of the cerebral anastomotic circulation. Anatomical investigation by Levine and Sohn, (1969) established that in the gerbil, posterior communicating arteries between the carotid and vertebro-basilar circulatory systems were often absent. This finding was confirmed by Kahn, (1972) and was extended by Harrison et al, (1973) who also reported a variable anatomy of the anterior cerebral arteries.

GERBIL CEREBRAL VASCULATURE

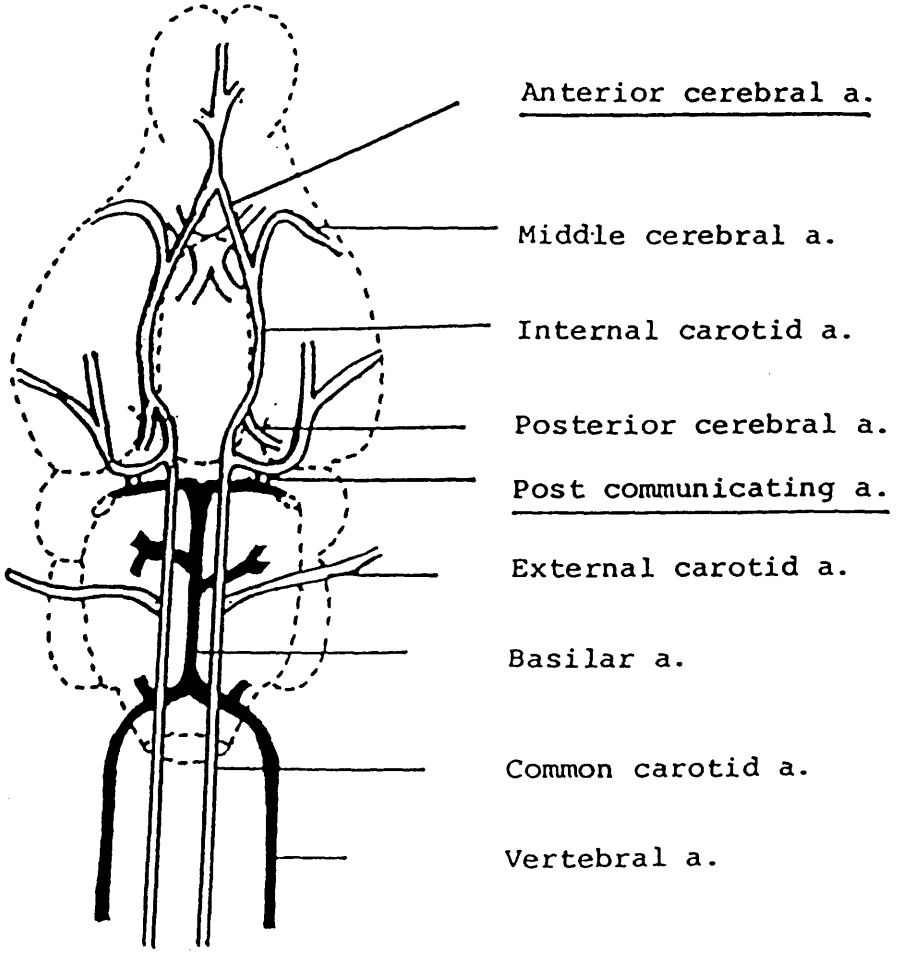


Fig. 5.1 Diagramatic representation of the gerbil cerebral vasculature, showing the arteries making up the circulus arteriosus. a=artery.

Initial results in this study indicated that determination of whether an animal succumbed to unilateral carotid ligation was independent of the lack of anastomosis between the carotid and vertebro-basilar circulation as the majority of the animals failed to show anastomosis. The significant feature of the blood supply was the degree of anastomosis between the anterior cerebral arteries. Thus, the anterior neurovasculature could be used to predict the outcome of the surgical procedure.

On the basis of the anterior vasculature arrangement a method for identifying stroke-prone and stroke-resistant animals was established. From a detailed microscopic examination of the anterior cerebral arteries of 500 animals, the following classification was devised:-

Group 1 - No anterior cerebral anastomosis. The two anterior cerebral arteries continued as separate vessels to a point where they could no longer be traced beyond the olfactory bulbs.
Aproximately 5-10% of animals

Group 2 - Small, probably insignificant, branch between the two anterior cerebral arteries which again remain separate. This connecting branch is distal to the optic chiasma.
Aproximately 25-30% of animals

In groups 1 and 2 the critical point for effective anastomotic supply to the opposite anterior and middle cerebral arteries has been passed. Together they form the stroke-prone group of animals.

Group 3 - The anterior cerebral arteries fuse to form a single vessel close to the optic chiasma. The blood supply is greatest from the unligated side.

Group 4 - The anterior cerebral arteries fuse close to the optic chiasma and form many collaterals. The blood supply is even from both sides. Often a communicating artery was found between the anterior and posterior circulation.

Groups 3 and 4 make up the stroke-resistant group of gerbils which is >60% of the total number of animals. A diagrammatical representation of the four groups is shown in fig 5.2.

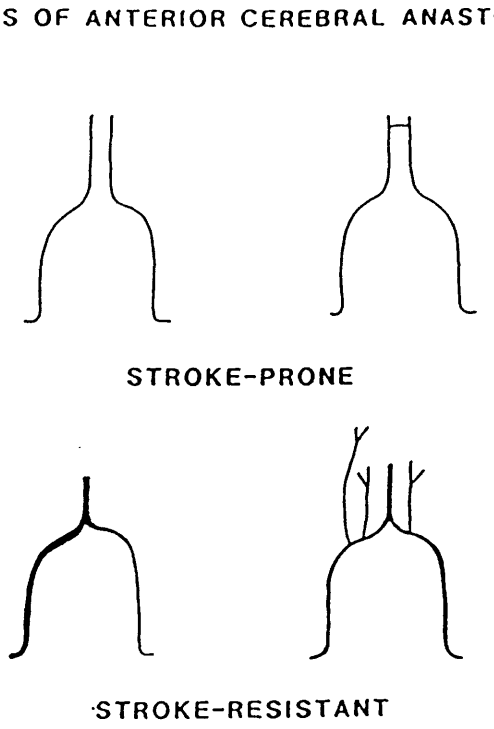
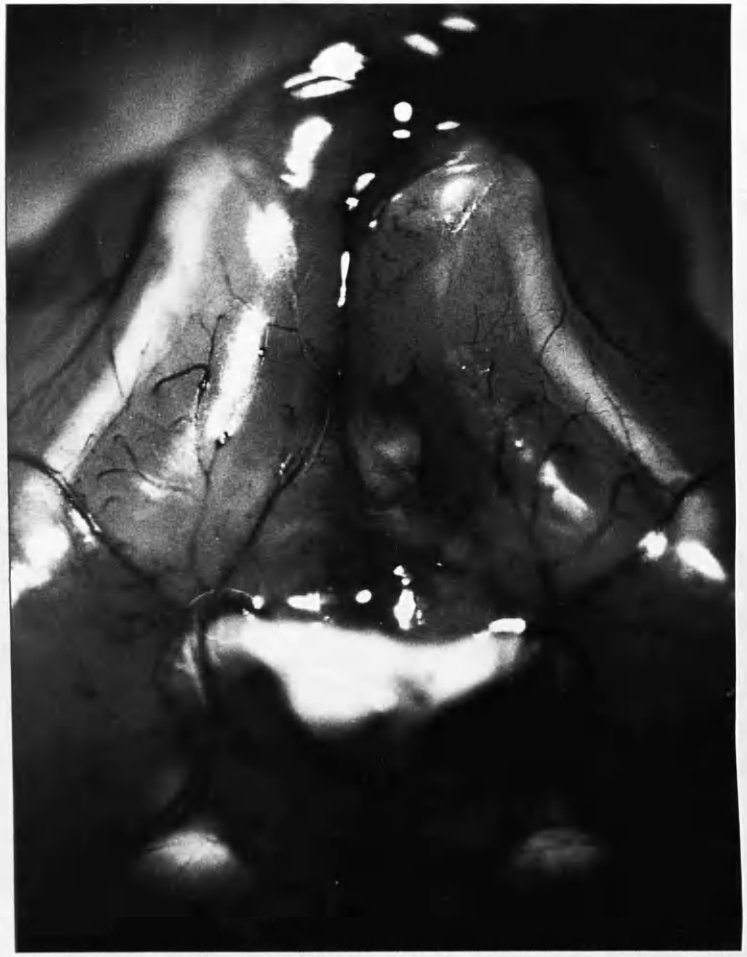
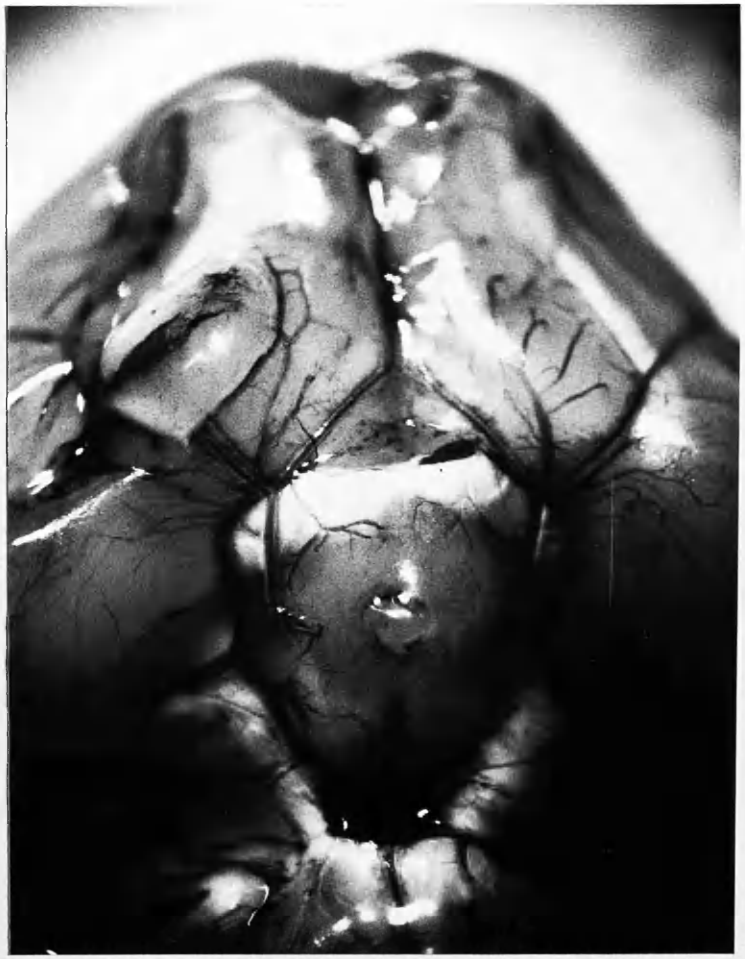


Fig. 5.2 Diagramatic representation of the possible types of anastomoses between the anterior cerebral arteries in stroke-prone and stroke-resistant gerbils.

The anterior cerebral vasculature of stroke-prone and stroke-resistant animals, stained with black ink, can be seen in photographs 5.1 and 5.2.



Photograph 5.1 Anterior neurovasculature of a stroke-prone gerbil.



Photograph 5.2 Anterior neurovasculature of a stroke-resistant gerbil.

5.2.1b EEG determination of stroke-prone animals

A second method of determining stroke-prone animals was investigated, namely examination of the electroencephalogram (EEG) of the animal. Animals were anaesthetised with halothane. No change in the magnitude of the signal was apparent before or after surgery. Following unilateral occlusion of the right common carotid artery the EEG became isoelectric. On removal of the occlusion normal EEG activity returned indicating the reversible nature of the effect. Animals showing ipsilateral or bilateral isoelectric EEGs within 10s of unilateral occlusion of the right common carotid artery were designated stroke-prone (fig 5.3). Anatomical investigation of the cerebral vasculature of these animals indicated an incomplete circulus arteriosus and poor collateral circulation. This suggested that a correlation existed between neurovascular anatomical defficiency and the occurence of an isoelectric EEG. However, only a subpopulation of animals described as stroke-prone in anatomical studies developed isoelectric EEGs. This subpopulation (8%) was shown to correspond to the group 1 stroke-prone animals showing severe vascular insufficiency. Bilateral EEG flattening in many of these animals emphasised that the poor blood flow was unable to support any electrical activity. Stroke-resistant animals were not susceptible to unilateral occlusion and showed no EEG flattening (fig. 5.3).

The use of EEG flattening to determine stroke-prone animals was rapid and less invasive than the anatomical examination of the circulus arteriosus. However, this method indentified only a subpopulation of the previously designated stroke-prone animals. Interestingly all animals under pentobarbital anaesthesia showed some degree of EEG flattening. However, pentobarbital was used in unilateral ligation studies because gaseous anaesthesia was impractical for large numbers of animals.

In the following studies stroke-prone animals were identified for an anatomical assessment of the circulus arteriosus.

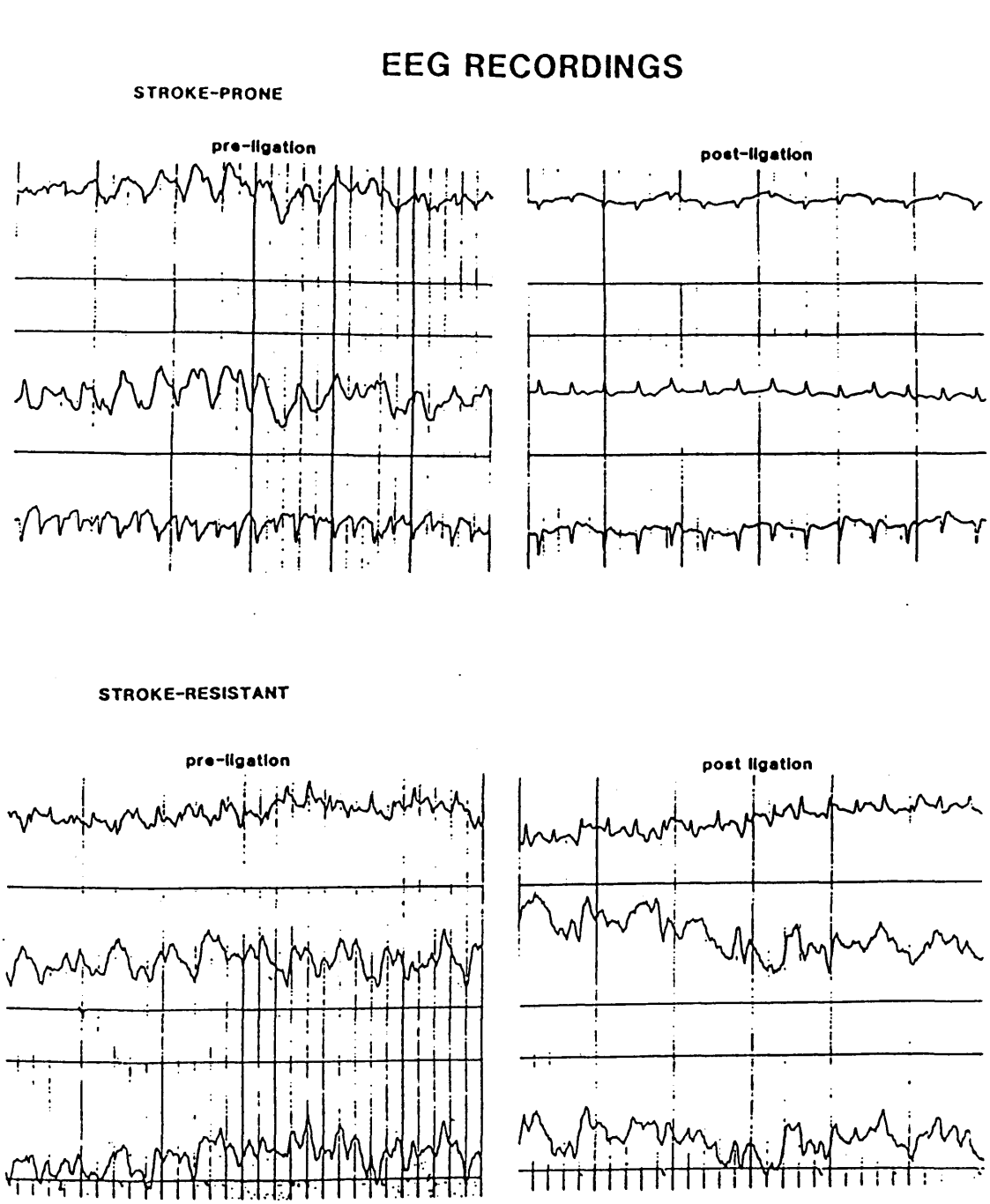


Fig. 5.3 EEG recordings from a stroke-prone and stroke-resistant animal pre and post-ligation. The electrodes were postioned subcutaneously in the left cortex, right cortex and hindbrain areas, roughly equivalent to EEG positions 13,14 & 20 in the human. Recordings were made from left hemisphere to hindbrain, right hemisphere to hindbrain and left to right hemisphere. A flattening of the EEG recording can be seen post-ligation in the stroke-prone gerbil.

5.2.2 Effect of cerebral ischaemia on 5HT levels

5.2.2a Determination of 5HT levels in the left and right corpus striatum following 3h unilateral ligation

Examination of stroke-prone animals, Groups 1 and 2, showed that in sham-operated animals there was no difference between the 5HT levels in the left and right striatum; whereas in ligated animals a fall in 5HT was observed. This reduction in tissue 5HT was only statistically significant in the occluded hemisphere (table 5.1).

In animals classified as stroke-resistant, Groups 3 and 4, ligation of one common carotid artery did not result in a statistically significant decrease in the level of 5HT in either the left or right hemisphere of the corpus striatum, (table 5.1).

Table 5.2 shows the percentage change in corpus striatal 5HT in the occluded relative to the non-occluded hemisphere for both experimental groups. The data for each individual animal is represented in fig 5.4.

5.2.2b Determination of 5HT levels in the left and right corpus striatum following 30 and 60min unilateral ligation

The fall in 5HT was dependent upon the duration of the ischaemic insult, a decrease in the 5HT level in the ligated hemisphere was seen at 30min and 60min (fig 5.5), however, it was not significant until 3h.

5.2.2c Determination of 5HT and 5HIAA in the frontal cortex following 3h unilateral ligation

In stroke-prone animals a significant decrease in the level of 5HT was observed in the ischaemic hemisphere of the frontal cortex (table 5.3). The alteration in the level of 5HT as a result of global ischaemia is therefore not restricted to the corpus striatum. A corresponding increase, although not significant, in the level of 5HIAA was found in the ischaemic hemisphere.

5.2.2d Determination of 5HT and 5HIAA levels in the frontal cortex following 5min bilateral ischaemia

A significant decrease in 5HT levels was seen in the frontal cortex following 5min bilateral ischaemia (table

5.4). A slight although not significant decrease in 5-hydroxyindole acetic acid (5HIAA) was also found.

An example of the chromatograph (fig 5.6) shows the separation of 5HT and internal standard produced under conditions used in this study.

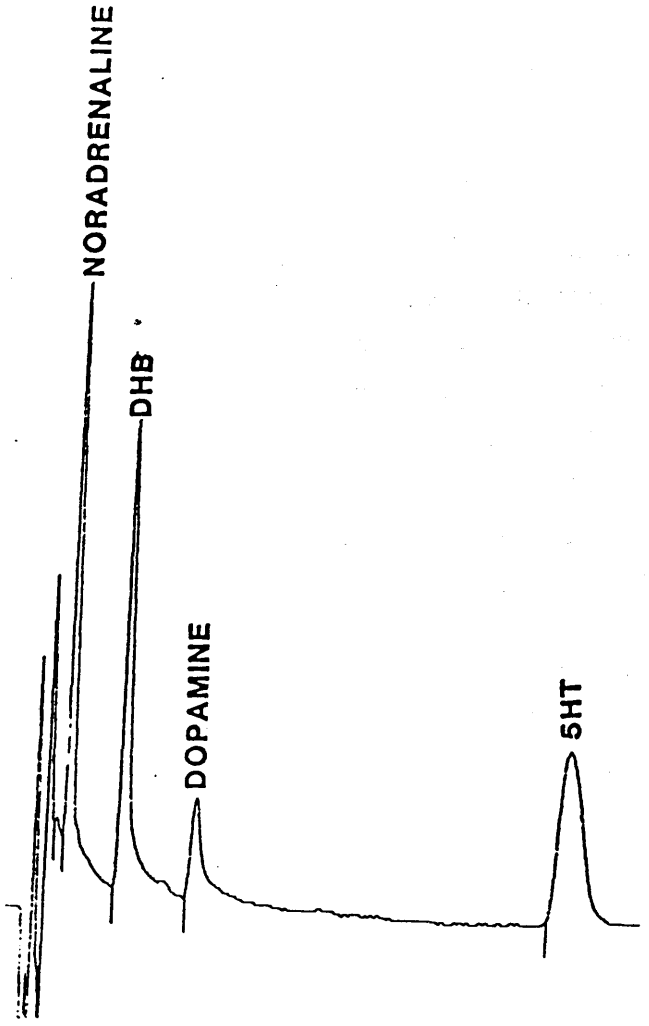


Fig. 5.6 A standard chromatogram for NA (2ng/100μl), DA (2ng/100μl), 5HT (2ng/100μl) and the internal standard DHB (1ng/100μl). Analysis by HPLC was performed as described in methods.

Thus global unilateral ischaemia (up to 3h) and brief bilateral ischaemia have pronounced effects upon the level of 5HT in at least two areas of the gerbil brain (frontal cortex and corpus striatum).

5.2.3 Effect of cerebral ischaemia on 5HT binding sites

5.2.3a Effect of ischaemia on $5HT_2$ binding site parameters

The binding of [^{3}H]-ketanserin to membranes of gerbil frontal cortex was saturable, reversible and of high affinity. Iterative non-linear analysis of the binding isotherms demonstrated a single class of high affinity sites with a Kd of 0.48±0.03nM and a Bmax of 206±20 fmol/mg protein (see section 4.2.6).

The effect of ischaemia on [^{3}H]-ketanserin binding in the non-recovery model is shown in table 5.5. Ligation of the right common carotid artery for 3h without reperfusion caused significant reduction in [^{3}H]-ketanserin binding to the right hemisphere of the frontal cortex. The binding of [^{3}H]-ketanserin to the left hemisphere was unaffected by unilateral ligation. Iterative non-linear analysis of the binding isotherms indicated that the change in [^{3}H]-ketanserin binding to the right hemisphere was due to a decrease in the density of binding sites and not a change in affinity. Also see fig 5.7.

[^{3}H]-Ketanserin binding was also affected in the less severe model of cerebral ischaemia. Analysis of the saturation isotherms for [^{3}H]-ketanserin binding to frontal cortical membranes prepared from animals 3 days after a 5min period of cerebral ischaemia revealed a significant decrease in the total number of [^{3}H]-ketanserin binding sites without an apparent change in affinity (table 5.6).

[^{3}H]-ketanserin binding to ischaemic frontal cortex membranes showed a similar pharmacological profile to non-ischaemic tissue. In competition studies affinity values were determined for ketanserin (pKi 8.6), methysergide (pKi 7.9) and 5HT (pKi 5.4), which are similar to the affinities determined for the binding of these compounds to non-ischaemic (control) gerbil cortex (see table 4.7).

5.2.3b The effect of different methods of inducing death on the $5HT_2$ binding site parameters after 3h

The 3h unilateral ligation of the right common carotid artery is a very severe model of cerebral ischaemia. Therefore, in order to provide additional evidence that cerebral ischaemia does selectively alter the binding parameters of the $5HT_2$ binding site, a comparative study was carried out to assess 3h hemispheric ischaemia and 3h postmortem changes.

In this study all animals were anaesthetised with 6mg pentobarbital, and then killed by decapitation, air embolism or cervical dislocation. The brains were left 'in situ' for 3h and the animals maintained at 37°C, for direct comparison with the 3h ischaemia model. Membranes were then prepared and assays performed as described in section 2.1.1.

The binding of [^{3}H]-ketanserin to membranes prepared 3h after death was found to be highly variable (table 5.7). Death by cervical dislocation resulted in animals showing either 'normal' binding characteristics, ischaemia-like binding characteristics (i.e. decrease in Bmax without a change in Kd) or minimal specific binding. Death by decapitation resulted in ischaemia-like binding characteristics or in most animals no detectable specific binding. Death induced by air embolism again resulted in the loss of specific binding in most of the animals tested. It is possible that cervical dislocation does not immediately stop all blood flow to the brain, unlike the other two methods. However, no dose-dependent inhibition of [^{3}H]-ketanserin binding by methysergide could be measured in membranes from 3h postmortem tissue. Therefore any binding detected in saturation studies is probably nsb. The results of this study suggest that $5HT_2$ binding sites in the gerbil frontal cortex are destroyed 3h postmortem and are selectivly vulnerable to 3h hemispheric ischaemia.

5.2.3c Effect of ischaemia on $5HT_{1A}$ binding site parameters

Preliminary experiments carried out using [^{3}H]-5HT (table 5.8), showed an increase in the number of $5HT_1$ binding sites. The increase was not seen immediately following 10min bilateral ligation but was apparent after 2h reperfusion ($p<0.02$). A significant decrease in affinity was also found ($p<0.05$).

The binding site labelled by [^{3}H]-8-OH-DPAT in the gerbil hippocampal membranes is characteristic of the $5HT_{1A}$ binding site, having high affinity for 5HT, 8-OH-DPAT, buspirone and WB4101 and low affinity for the $5HT_2$ antagonist ketanserin or the selective $5HT_{1C}/5HT_2$ compound mesulergine (see section 3.2.6).

Iterative non-linear analysis of the saturation isotherms indicated that transient ischaemia followed by 72h recovery resulted in a significant increase in the density of $5HT_{1A}$ binding sites in the hippocampus (table 5.9). This increase in $5HT_{1A}$ binding sites was not accompanied by a change in the affinity of [^{3}H]-8-OH-DPAT for the binding site.

Similar results were obtained using the $5HT_{1A}$ ligand [^{3}H]-WB4101. An increase was found in the number of $5HT_{1A}$ binding sites in the hippocampus without an apparent change in affinity (table 5.10).

5.2.3d $5HT_{1B}$ binding site

In contrast to the ischaemia-induced increase in $5HT_{1A}$ binding sites in the hippocampus and decrease in $5HT_2$ binding sites in the frontal cortex, 3h unilateral ischaemia had no significant effect upon $5HT_{1B}$ binding sites labelled by [^{125}I]-CYP in the corpus striatum, (table 5.11). A slight but not significant increase in affinity was noted.

5.2.4 Functional correlates for changes in 5HT binding

5.2.4a The effect of the 5min bilateral ligation followed by 72h recovery on the number of quipazine-induced head shakes

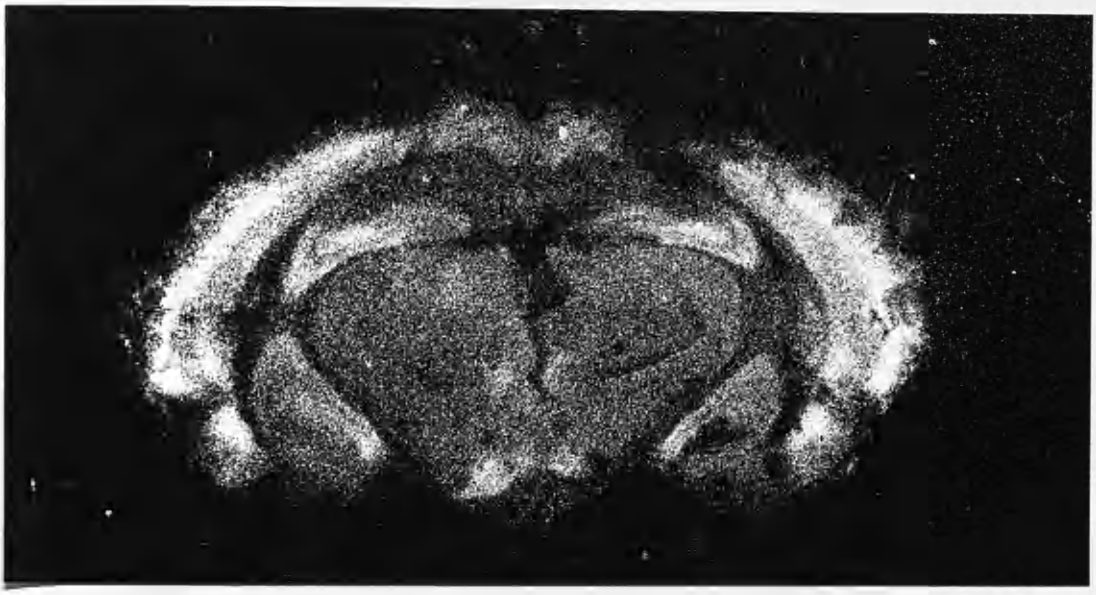
Agonist interactions at $5HT_2$ binding sites induce a head twitch behaviour in mice and rats which can be inhibited by $5HT_2$ antagonists (Ortmann et al, 1982; Green et al, 1983; Green and Heal, 1985). In these studies the $5HT_2$ agonist quipazine was also shown to induce head shakes in the gerbil.

The number of quipazine-induced head shakes seen pre- and post-ischaemia is shown in table 5.12. No significant change in the number of head shakes in the sham-operated or ligated groups of animals was seen 24 or 72h following ischaemia. This could be as a result of the low number of head shakes recorded for the gerbil, 13±3/group of three animals /15min, or the degree of interanimal variability. Simiarly no significant change was seen in the locomotor activity of the sham-operated or ligated animals following ischaemia (table 5.13).

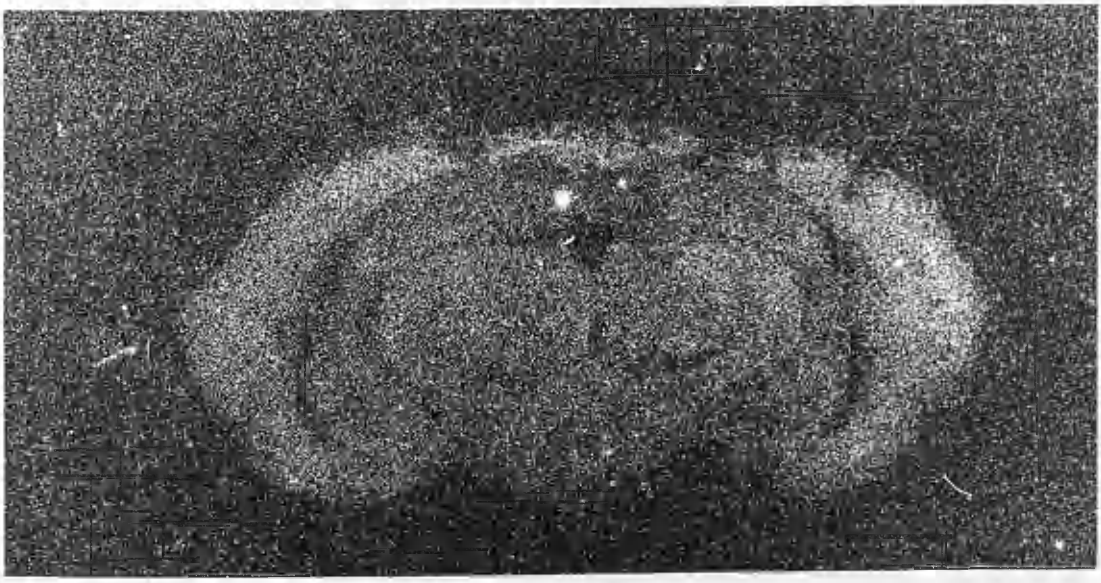
5.2.3b Autoradiographic location of 5HT binding sites in the gerbil

The localisation of $5HT_2$ binding sites labelled by [^{125}I]-LSD in coronal sections through the frontal cortex area of the gerbil brain can be seen in photograph 5.3. The binding was assessed to be specific for the $5HT_2$ binding site as in it could be displaced by 2μM methysergide leaving only non-specific binding (photograph 5.4). These results indicate a high density of $5HT_2$ binding sites in the frontal cortex of the gerbil brain as shown in the binding studies, section 4.2.6.

Unfortunately no method of quantifying the density of binding was available, therefore it was difficult in these studies to assess the effect of ischaemia. However, this could be a very useful technique for future studies.



Photograph 5.3 Autoradiograph of total [^{3}H]-ketanserin binding to a section through the gerbil frontal cortex.



Photograph 5.4 Autoradiograph of nsb [^{3}H]-ketanserin binding to a section through the gerbil frontal cortex.

5.2.3c Behavioural observations

Ipsilateral rotational behaviour was observed in some of the recovered animals undergoing unilateral ligation, these animals were later assessed as stroke-prone by anatomical investigation of the circulus arteriosus. However, this rotational behaviour is unlikely to be associated with the release of 5HT. In the rat, rotational behaviour induced by 5HT agonists has been shown to act via $5HT_1$ receptors, probably located in the substantia nigra, an area not affected in this model, and the rotation is contralateral to the lesion (Blackburn et al, 1984). The release of large amounts of DA has been suggested to be the cause of ipsilateral rotational behaviour in stroked gerbils (Lavyne et al, 1975b), a phenomenon which in rats is also related to asymmetric DA release (Anden et al, 1966).

A few animals recovering from bilateral ligation showed flat body posture. However, other 5HT behavioural signs described for the rat (Tricklebank, 1984a;b) such as head weaves, front paw treading and head shakes were not observed.

The effect on Corpus Striatal 5HT levels of unilateral ligation of the common carotid artery

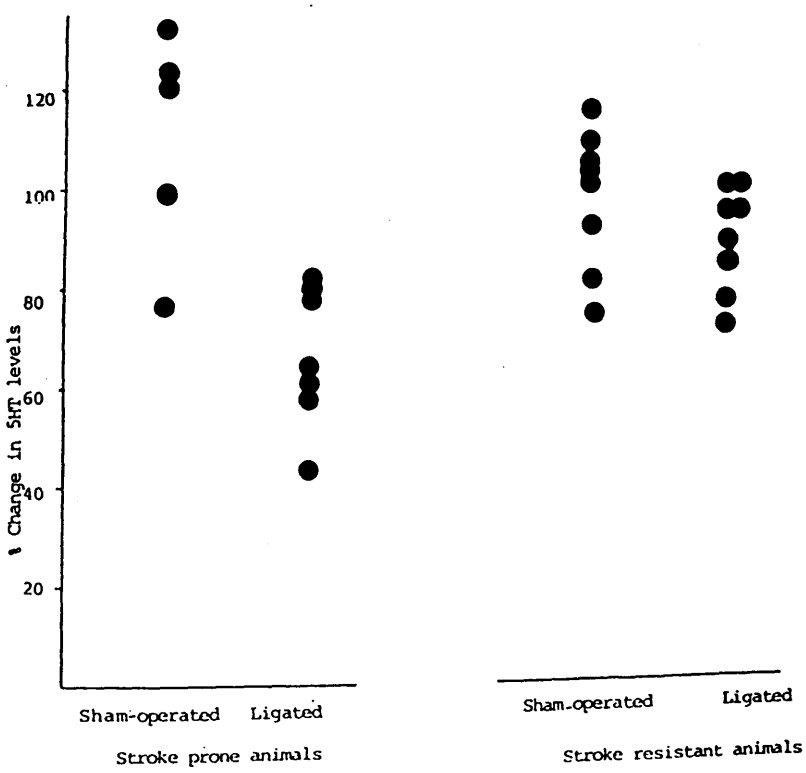


Fig. 5.4 Diagramatic representation of the results presented in table 5.2, showing the individual data points. The results are expressed as the % change in 5HT levels in the ligated relative to the unligated hemisphere.

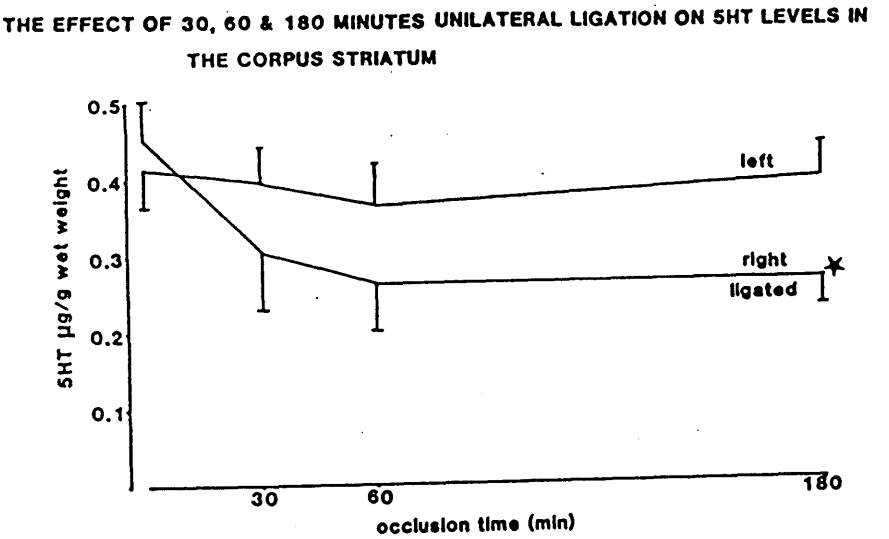
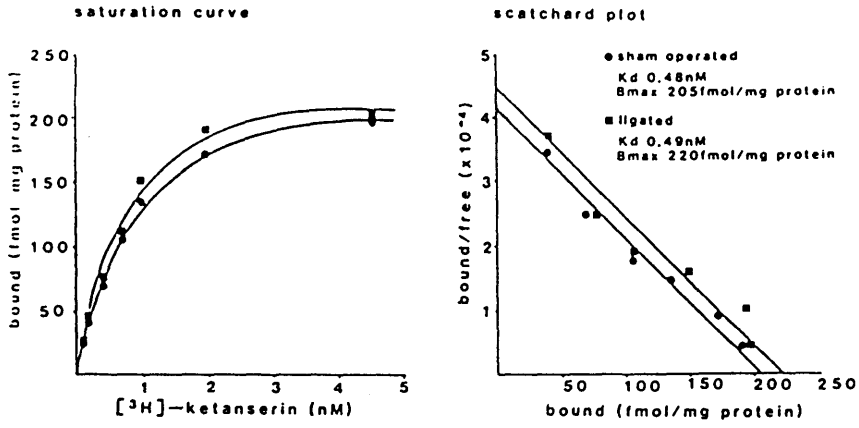


Fig. 5.5 Ischaemia was induced by unilateral ligation of the right common carotid artery for periods of 30-180min. A statistically significant decrease in the level of 5HT was seen in the right ligated hemisphere after 3h, *$p<0.05$.

[^{3}H]-KETANSERIN BINDING TO GERBIL LEFT FRONTAL CORTEX



[^{3}H]—KETANSERIN BINDING TO GERBIL RIGHT FRONTAL CORTEX

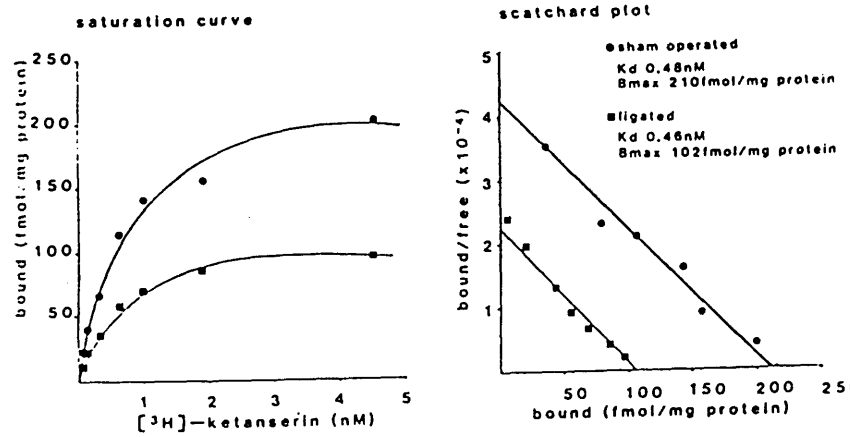


Fig. 5.7 Saturation experiments were carried out on the left or right frontal cortical membranes (2 cortex/membrane preparation) from ischaemic and non-ischaemic brains. Unilateral ischaemia was induced by a 3h ligation of the right common carotid artery. The data describes a single experiment in each group.

Table 5.1 The effect of unilateral ligation on 5HT levels in the corpus striatum

A. Stroke-prone gerbils

experimental group	n	5HT (μg/g wet weight) left hemishere	right hemisphere (ligated)
Sham-operated	5	0.414 ± 0.049	0.464 ± 0.053
Ligated	7	0.392 ± 0.040	0.261 ± 0.043 *@

5HT was measured by HPLC as described under methods. Unilateral ischaemia was induced by a 3h ligation of the right common carotid artery.

Statistical significance

* p<0.05 relative to left hemisphere

@ p<0.02 relative to sham-operated right hemisphere

B. Stroke-resistant gerbils

experimental group	n	5HT (μg/g wet weight) left hemishere	right hemisphere (ligated)
Sham-operated	8	0.435 ± 0.025	0.422 ± 0.036
Ligated	8	0.392 ± 0.045	0.347 ± 0.072

5HT was measured by HPLC as described under methods. Unilateral ischaemia was induced by a 3h ligation of the right common carotid artery.

No significance difference between any group

Table 5.2 Percentage change in 5HT levels in the corpus striatum

	right (ligated) hemisphere	
experimental group	Stroke-resistant animals	Stroke-prone animals
Sham-operated	97.0 ± 5.1 (8)	112.2 ± 10.8 (5)
Ligated	88.4 ± 3.8 (8)	66.6 ± 5.8 (7) *@

Statistical significance

* $p<0.01$ relative to stroke-prone sham-operated

@ $p<0.01$ relative to stroke-resistant ligated

number of animals/group shown in brackets

Percentage change in 5HT levels in the right (ligated) hemisphere, following 3h unilateral ischaemia. Data are derived from the absolute values shown in table 5.1.

Table 5.3 The effect of unilateral ligation on 5HT levels in the frontal cortex

	(μg/g wet weight)	
	left hemishere	right hemisphere (ligated)
5HT	0.89 ± 0.05	0.38 ± 0.05*
5HIAA	5.63 ± 0.07	6.19 ± 0.05

5HT and 5HIAA were measured by HPLC as described under methods. Unilateral ischaemia was induced by a 3h ligation of the right common carotid artery. Each value represents the mean ± s.e.m. of twelve seperate determinations. Significant difference from the non-ischaemic hemisphere
*p<0.001.

Table 5.4 The effect of bilateral ligation on 5HT levels in the frontal cortex

	(μg/g wet weight)	
	Sham-operated	Ligated
5HT	0.93 ± 0.22	0.40 ± 0.11*
5HIAA	6.78 ± 0.52	6.33 ± 0.27

5HT and 5HIAA were measured by HPLC as described under methods. Ischaemia was induced by a 5min ligation of the left and right common carotid arteries. Each value represents the mean±s.e.m. of 16 separate determinations. Significant difference from the non-ischaemic sham-operated animals.
*p<0.05.

Table 5.5 Effect of unilateral cerebral ischaemia on 5HT$_2$ binding sites in gerbil frontal cortex labelled by [^{3}H]-ketanserin

Left hemisphere		Right hemisphere	
Kd nM	Bmax fmol/mg protein	Kd nM	Bmax fmol/mg protein
NON-ISCHAEMIC CORTEX			
0.48 ± 0.03	203 ± 10.4	0.47 ± 0.03	209.4 ± 18.9
ISCHAEMIC CORTEX			
0.51 ± 0.03	218.4 ± 7.0	0.45 ± 0.02	95.5 ± 10.0*

Saturation experiments were carried out on the left and right frontal cortical membranes (2 cortex/membrane preparation) from ischaemic and non-ischaemic brains. Unilateral ischaemia was induced by a 3h ligation of the right common carotid artery. Each value represents the mean ± s.e.m. of five seperate determinations. Significant difference from the non-ischaemic sham-operated animals *$p<0.001$.

Table 5.6 The effect of bilateral cerebral ischaemia on $5HT_2$ binding sites in gerbil frontal cortex labelled with [^{3}H]-ketanserin

	Kd nM	Bmax fmol/mg protein
NON-ISCHAEMIC	0.42 ± 0.06	192.9 ± 9.8
ISCHAEMIC	0.51 ± 0.06	136.6 ± 18.9*

Saturation experiments were carried out on frontal cortex membranes prepared from ischaemic and non-ischaemic brains. Ischaemia was induced by a 5min ligation of the left and right common carotid arteries followed by 72h recovery. Each value represents the mean±s.e.m. of 7 separate determinations. Significant difference from the non-ischaemic sham-operated animals *$p<0.01$.

Table 5.7 3h ischaemic or 3h postmortem changes in $5HT_2$ binding sites on gerbil frontal cortex

	Kd nM	Bmax fmol/mg protein
Control	0.47 ± 0.03	209.4 ± 18.9
Control hemisphere* (3h hemisheric ischaemia)	0.51 ± 0.03	218.4 ± 7.0
ligated hemisphere (3h hemispheric ischaemia)	0.45 ± 0.02	95.5 ± 10.0
Decapitation (3h global ischaemia)	0.54	86.9
	no detectable specific binding	
	no detectable specific binding	
	no detectable specific binding	
3h death (air embolism)	0.28	98.4
	no detectable specific binding	
	no detectable specific binding	
	no detectable specific binding	
3h death (cervical dislocation)	0.23	70.2
	1.65	16.6
	0.45	222.0
	0.37	118.5

all experiments at 37°C

* left hemisphere

n=4, or individual results are shown.

[^{3}H]-Ketanserin binding parameters were determined in membranes from 3h ischaemic or 3h postmortem tissue as described in methods.

Table 5.8 The effect of cerebral ischaemia on 5HT$_{1A}$ binding sites in gerbil hippocampus labelled by [^{3}H]-5HT

	Kd nM	Bmax fmol/mg protein
non-ischaemic hippocampus	6.12 ± 1.5	600 ± 120
ischaemic hippocampus	5.18 ± 0.8	570 ± 80
ischaemia + 1h reperfusion	6.02 ± 1.0	650 ± 60
ischaemia + 2h reperfusion	11.80 ± 1.2*	1110 ± 50**

Saturation experiments were carried out on hippocampal membranes prepared from ischaemic and non-ischaemic brains. Ischaemia was induced by a 10min ligation of the left and right common carotid arteries. Each value represents the mean±s.e.m. of 3 separate determinations.

*P<0.05 relative to non-ischaemic hippocampus

**P<0.02 relative to non-ischaemic hippocampus

Table 5.9 The effect of cerebral ischaemia on $5HT_{1A}$ binding sites in gerbil hippocampus labelled by [^{3}H]-8-OH-DPAT.

	Kd nM	Bmax fmol/mg protein
non-ischaemic hippocampus	4.49 ± 0.89	458 ± 16
ischaemic hippocampus	4.49 ± 0.20	636 ± 27*

* $p<0.01$

Saturation experiments were carried out on hippocampal membranes prepared from ischaemic and non-ischaemic brains. Ischaemia was induced by a 5min ligation of the left and right common carotid arteries followed by 72h recovery. Each value represents the mean±s.e.m. of 4 separate determinations.

Table 5.10 The effect of cerebral ischaemia on $5HT_{1A}$ binding sites in gerbil hippocampus labelled by [^{3}H]-WB4101.

	Kd nM	Bmax fmol/mg protein
non-ischaemic hippocampus	1.43 ± 0.22	347 ± 43
ischaemic hippocampus	1.21 ± 0.07	538 ± 62*

* $p<0.05$

Saturation experiments were carried out on hippocampal membranes prepared from ischaemic and non-ischaemic brains. Ischaemia was induced by a 5min ligation of the left and right common carotid arteries followed by 72h recovery. Each value represents the mean±s.e.m. of 4 separate determinations.

Table 5.11 The effect of cerebral ischaemia on $5HT_{1B}$ binding sites in gerbil corpus striatum labelled by [^{125}I]-CYP

	Kd pM	Bmax fmol/mg protein
Sham-operated		
Stroke prone		
left	77 ± 21	310 ± 20
right	87 ± 20	314 ± 20
Stroke resistant		
left	69 ± 25	309 ± 12
right	65 ± 19	325 ± 10
Ligated		
Stroke prone		
left	37 ± 14	378 ± 21
right	35 ± 11	360 ± 15
Stroke resistant		
left	31 ± 11	350 ± 24
right	35 ± 15	338 ± 12

Saturation experiments were carried out on left and right frontal cortex membranes prepared from ischaemic and non-ischaemic brains. Ischaemia was induced by a 3h ligation of the right common carotid artery. Each value represents the mean±s.e.m. of 3 separate determinations.

Table 5.12 Headshakes pre- and post-ischaemia

HEADSHAKES

	Pre-ischaemia	1 day post-ischaemia	3 day post-ischaemia
Sham-operated			
group 1	8	10	14
group 3	14	5	4
group 5	16	7	5
ligated			
group 2	7	1	6
group 4	7	2	1
group 6	26	18	21

Ischaemia was induced by a 5min ligation of the left and right common carotid arteries followed by 72h recovery. Pre-ischaemia, 24 and 72h post-ischaemia the gerbils (3/group) were given quipazine (25mg/kg s.c.) and 30min later the number of headshakes determined over a 15min period.

Table 5.13 Locomotor activity pre- and post-ischaemia

	ACTIVITY		
	Pre-ischaemia	1 day post-ischaemia	3 day post-ischaemia
Sham-operated			
group 1	2849	2732	2876
group 3	3072	3046	3152
group 5	2830	2962	2881
ligated			
group 2	3013	3068	3026
group 4	3414	3304	3398
group 6	3497	3608	3820

Ischaemia was induced by a 5min ligation of the left and right common carotid arteries followed by 72h recovery. Pre-ischaemia, 24 and 72h post-ischaemia the gerbils (3/group) were given quipazine (25mg/kg s.c.) and 30min later their activity monitored over a 15min period.

5.3 Discussion

The use of the gerbil as an animal model of cerebral ischaemia is dependent on the identification of stroke-prone animals by anatomical investigation of the circulus arteriosus. Ligation of one common carotid artery produces cerebral ischaemia in 30-40% of animals (Kahn, 1972) whereas bilateral common carotid artery ligation produces ischaemia in 60% of animals (Levy and Brierley, 1974). In contrast ligation of both common carotids in the rat causes little morbidity and unilateral ligation is practically ineffective (Payan et al, 1965).

The absence of a significant posterior communicating artery has been suggested to account for the succeptibility of the gerbil (Levine and Sohn, 1969; Kahn, 1972). However, in this study the determination of whether the animals succumbed was apparently independent of the lack of anastomosis between the anterior and posterior circulation, as most of the animals failed to show any such anastomosis. This finding is in agreement with studies by Harrison et al, (1973). The significant feature of the blood supply appeared to be the degree of anastomosis between the anterior cerebral arteries. Microscopic examination of the anterior vasculature proved to be rapid, reliable and highly predictive.

EEG recordings can also be used to identify a subpopulation of stroke-prone animals equivalent to the Group 1 stroke-prone animals with severe vascular deficiencies. These animals showed an isoelectric EEG (often bilateral) following unilateral ligation. This assessment could only be used in halothane anaesthetised animals, pentobarbital was found to cause a degree of flattening in all animals. Barbiturate treated animals have been shown to lose corneal reflexes (Levy and Brierley 1979) and have been shown to be protected against stroke (see review by Smith, 1977). The incidence of stroke in pentobarbital anaesthetised gerbils following unilateral ligation has been reported to be less (Lightfoote et al, 1977) or the same (Harrison et al, 1973; and this study) as

using ketamine (Berry et al, 1975), ether (Levine and Payan, 1966) or halothane (Levy et al, 1975) anaesthesia.

Measurement of the common carotid artery pressure can also be useful, the lower threshold of the arterial blood pressure for the development of brain infarcts ranges from 22-25mm Hg (Laas, 1984). Recently occular fundus examination with direct ophthalmoscopy has been suggested as a suitable method to select stroke-prone gerbils (Delbarre et al, 1988).

The metabolism of 5HT appears particularly sensitive to the effects of even minimal ischaemia. The levels of 5HT were reduced in the ligated hemisphere, as early as 30min after unilateral ligation, which reached significance after 3h. The early reduction of 5HT may be a factor contributing to the development of seizure-like activity in Mongolian gerbils undergoing carotid artery ligation. Depletion or elevation of brain 5HT by pharmacological means respectively facilitates or decreases susceptibility to seizures in several animal models (Maynert et al, 1975). However, Welch et al, (1978) have suggested that DA and NA levels fall only in animals that develop seizures whereas 5HT falls in ischaemic tissue in the absence or presence of seizures. Further study is needed to establish if the change in 5HT associated with ischaemia-induced seizures is primary or secondary to the seizure activity itself. In should be added that pentobarbital has also been shown to significantly reduce seizure activity (Goldblatt et al, 1971).

Unilateral carotid artery occlusion resulted in a 44% decrease in the level of 5HT in the striatum and a 57% decrease in the level of 5HT in the frontal cortex after 3h. This is in good agreement with Welch et al, (1977) and Harrison et al, (1979); they reported decreases of 44% and 38% respectively. Both these studies were performed using cerebral cortex, where control levels of 5HT were reported to be between 0.35 and 0.45 μg/g wet weight, which is similar to the levels reported in this study, 0.46±0.05 μg/g wet weight in the corpus striatum and 0.89±0.06 μg/g

wet weight in the frontal cortex.

The exact cause of the reduction in 5HT is unknown. Neuronal release of monoamines early after the onset of ischaemia has also been reported in the baboon (Welch et al, 1972) and in patients suffering from stroke (Meyer et al, 1974). Release of 5HT could be attributed to membrane depolarization with ionic shifts and/or mitochondrial dysfunction (Lee and Yatsu, 1974). Other factors which would cause eventual intraneuronal monoamine depletion are failure of energy dependent synaptosomal reuptake (a 16% decrease in 5HT reuptake was found in this laboratory following 5min bilateral ligation) and synthesis impairment (Lavyne et al, 1975a). Inhibition of tryptophan hydroxylase activity prior to ischaemia, by PCPA treatment, results in an even greater depletion of 5HT than in untreated animals. The reduction in tissue 5HT is likely to be due in part to synthesis impairment (Welch et al, 1977).

The relationship between ischaemia-induced alteration in 5HT, changes in 5HT binding site characteristics or compounds affecting these parameters is likely to be complex. The experiments outlined in chapters 3 and 4 demonstrated the existence of 5HT binding sites in the brain of the Mongolian gerbil. The $5HT_2$ binding site in the frontal cortex and the $5HT_{1A}$ site in the hippocampus were characterised pre- and post-ischaemia. Ischaemia changed the Bmax but not the Kd of high affinity 5HT binding sites. However, the binding sites showed the same pharmacological profile as non-ischaemic (normal) membrane preparations, which suggests they maintain their specificity.

Cerebral ischaemia resulted in a significant decrease in the number of $5HT_2$ binding sites in the ipsilateral frontal cortex following 3h unilateral ligation with no apparent change in affinity. The precise mechanism is not known. It would appear not to depend on reperfusion, which is associated in other models with ischaemic changes, as the artery was occluded for the duration of the experiment. Long periods of reperfusion have been shown to increase

α_1-adrenoceptor density in the ischaemic cat myocardium (Corr et al, 1981).

The decrease in the density of $5HT_2$ binding sites may be as a consequence of a general disruption of neuronal membranes. Histological damage to the gerbil cortex has been shown to occur in severe models of ischaemia such as unilateral occlusion for periods in excess of 1h (Klatzo, 1975; Ito et al, 1975). The severity of this model was questioned. However, specific $5HT_2$ binding was lost in 3h postmortem tissue unlike 3h ischaemic tissue. There is a general increase in proteolysis in postmortem brains. Therefore it may be interesting to see what effect incubation with protease inhibitors e.g. PMSF, has on $5HT_2$ binding after ischaemia.

Ischaemia-induced changes in the number of $5HT_2$ binding sites could result from a sudden non-physiological release of 5HT which could, through excessive stimulation, alter the $5HT_2$ binding protein to such an extent that it cannot recover later when 5HT levels decrease; prehaps they are internalised and degraded. In vivo dialysis measurement of DA release has shown there is a sevenfold increase in DA release in the ischaemic hemisphere 15min after carotid ligation (Brannan et al, 1987). As yet similar studies to determine the extent of 5HT release have not been published. However, post-ischaemia changes in 5HT levels suggests that 5HT release could occur earlier and take the tissue level of 5HT longer to recover than DA.

Alternatively the changes observed could be specific and selective. Alterations in the 5HT system may be one of the factors exacerbating ischaemic damage.

To investigate further the possible selective effects of ischaemia on the $5HT_2$ sites a second ischaemic model was used, a 5min bilateral occlusion with 72h recovery. This eliminated some of the problems associated with the 3h unilateral model, i.e. it is less severe and relies less on a wasteful, subjective anatomical assessment for stroke-prone animals. In addition, this model allows a greater control over the anaesthesia used. However, even in this

model 40% of gerbils are resistant to bilateral ischaemia (Levy and Brierley, 1974). Therefore, flattening of the EEG recording was used to determine susceptibility to ischaemia.

A decrease in the density of $5HT_2$ sites in the gerbil frontal cortex with no apparent change in affinity was also found in this less severe model of ischaemia. Brief bilateral carotid artery occlusion does not produce the 'no reflow' phenomenon in the gerbil after removal of the clips (Levy et al, 1975) suggesting that a lack of blood flow to the cortex during the recovery phase of the bilateral model is unlikely to give rise to the reduction in $5HT_2$ binding site density. However reperfusion following the insult could expose the 'injured' cells to damaging oxy-radicals leading to increased lipid peroxidation and proteolysis etc. The ischaemic period is therefore very different from the the unilateral model. The long periods of reperfusion associated with bilateral ligation may cause physical disruption of the membrane. In this model histological changes have been shown to be restricted to the hippocampus (Suzuki et al, 1983; Kirino and Sano, 1984), and although spontaneous neuronal activity in the cortex and hippocampus ceases within 60sec of the ischaemic insult, it then returns to normal in the cortex (Suzuki et al, 1983). Therefore it is unlikely that the decrease in density of $5HT_2$ binding sites was due to a general disruption of membranes within the cortex but may represent an example of selective vulnerability of neurons containing the $5HT_2$ binding sites.

There is evidence to suggest that the 5HT system may be selectively vulnerable to cerebral ischaemia. Cyproheptadine and LSD, compounds with high affinity for $5HT_2$ binding sites, have been shown to exert a beneficial protective effect in a rabbit spinal cord ischaemia model which could be reversed by the 5HT agonist bufotenine (Zivin, 1985).

At present the functional significance of the decrease in $5HT_2$ binding sites in the gerbil cortex is unknown, It

has been suggested that alterations in the density of $5HT_2$ binding sites may play a role in the aetiology of depression (Blackshear and Saunders-Bush, 1982). A decrease in the density of $5HT_2$ binding sites has also been reported in the hippocampus of depressed suicide victims (Cheetham et al, 1986). In contrast Stanley and Mann, (1983) reproted increased $5HT_2$ binding in the frontal cortex of suicide victims. This may reflect heterogeneity in the nature of the psychiatric illness. Chronic administration of the $5HT_2$ antagonists ritanserin (Leysen et al, 1986) mianserin and ketanserin (Blackshear et al, 1983) and the $5HT_2$ antagonists/5HT reuptake blockers, zimelidine (Green, 1984) and amytriptyline (Peroutka and Snyder, 1980) apparently decrease the density of cortical $5HT_2$ binding sites. Agonist interactions at $5HT_2$ binding sites in mice induce a head twitch behaviour which can be inhibited by $5HT_2$ antagonists (Ortmann et al, 1982; Green et al, 1983). This behaviour has been used to investigate both acute and longer term effects of antidepressant drugs on 5HT function. Several antidepressant treatments have been shown to inhibit the head twitch response both acutely and chronically (Friedman et al, 1984; Goodwin et al, 1984).

This study showed that quipazine head shakes can be observed in the gerbil, which seem to resemble the rat rather than the mouse in frequency of responses. However, a significant correlation between the decrease in receptor binding sites and the decrease in head shakes could not be made.

As discussed above the 5min bilateral carotid artery occlusion reduces blood flow and suppresses EEG activity in the hippocampus of the gerbil (Suzuki et al, 1983); after 3 days severe destruction of neurons within the hippocampus is observed (Suzuki et al 1983; Kirino and Sano, 1984). Therefore further investigations into effect of ischaemia on 5HT binding sites were carried out in the gerbil hippocampus, an area shown in this study to be rich in $5HT_{1A}$ binding sites.

Although there was no change in the affinity of the

$5HT_{1A}$ binding sites for the ligand, the number of binding sites was increased following ischaemia. The finding that the number of sites increases in a brain area where death of selectively vulnerable cells occurs, suggests that the $5HT_{1A}$ sites are not located on vulnerable cells. However, it is well established that the CA1 pyramidal cells in the hippocampus are particularly vulnerable in this model of ischaemia (Kirino, 1982; Kirino and Sano, 1984) and it has been shown that the CA1 region is rich in $5HT_{1A}$ binding sites which are located on the pyramidal cells (Pazos and Palacios, 1985); Hall et al, 1985). These receptors mediate 5HT-induced hyperpolarisations and are directly coupled via a G protein to a K^+ channel (Andrade et al, 1986).

Cerebral ischaemia has been reported to alter adenosine receptors and GABA binding sites in a 15min bilateral model of ischaemia in the gerbil. Adenosine (A_1) receptors, also located on the pyramidal cells, are markedly reduced but not until 27 days post-ischaemia (Onodera and Kogure, 1985). $GABA_A$ binding, located on GABAergic interneurons in the CA1 region, showed a significant increase 3 days after ischaemia and decreased after 27 days (Onodera et al, 1987). It is possible that there are several different populations of $5HT_{1A}$ sites in the hippocampus and that the $5HT_{1A}$ sites influenced by ischaemia do not reside on the pyramidal cells. $5HT_{1A}$ sites have been suggested to be located both pre- and post-synaptically and have been linked to different second messenger systems in the hippocampus; adenylate cyclase (Shenker et al, 1985) and possibly PI metabolism (Janowsky et al, 1985).

Therefore the increase in the density of $5HT_{1A}$ binding sites is probably a compensatory change in response to the maturation of neuronal damage. In this respect destruction of the serotonergic raphe system with 5,7 dihydroxy-tryptamine increases the number of $5HT_{1A}$ sites in the hippocampus (Wang et al, 1985).

The affinity and number of sites on a membrane is variable according to the physiological state of the

tissue. The nature of a proteinaceous binding site is likely to be influenced by its microenvironment. The microviscosity of the membrane can influence the number of available and non-available (cryptic) sites to the ligand. For the 5HT system it has been calculated that 50-90% of the binding sites are not available to the agonist (Heron et al, 1980). Phospholipids, cholesterol and cytoskeletal components all contribute to the nature of the microenvironment which will be altered during ischaemia. The microviscosity of the membrane is controlled by the phospholipid composition and the composition of other membrane lipids. Early studies by Bazan, (1970) suggested that energy failure in the cell led to a sharp rise in free fatty acids which could be correlated with the duration and severity of ischaemia. If the liberated FFAs arise from membrane phospholipids, then it is probable that brain ischaemia results in marked changes in the viscosity of the cell membranes. Therefore it is possible that the change in number of 5HT binding sites could be a consequence of a change in membrane viscosity. The dependence of 5HT binding sites on phospholipid composition could be further studied by examining 5HT binding in membrane preparations where the phospholipid environment has been altered, e.g. treatment with phospholipase.

The changes in binding site density is unlikely to be due to endogenous 5HT in the membrane preparation. Analysis by HPLC of the membrane preparation failed to detect 5HT. However, endogenous inhibitors or activators for the 5HT binding site that are induced or removed during the ischaemic process could influence the number of available sites. The effect of divalent ions and other modulators on ischaemic membranes should be further studied: In the present studies Ca^{2+} or Mg^{2+} were always added back to the membrane preparation.

These findings encourage further study of the effects of ischaemia on all aspects of serotonergic function. It will be important to establish whether the changes in 5HT binding sites are related to functional changes which might

well be a contributing factor in the pathology of ischaemia. This could be achieved by further behavioural studies, preliminary results were presented here, or by biochemical assessment of functional response, i.e. inhibition of adenylate cyclase by $5HT_{1A}$ sites or hydrolysis of phosphatidylinositol by $5HT_2$ receptors.

Finally it will be important to show that these effects were not exclusive to the gerbil, and study the possible protective effects, if any, of selective 5HT compounds.

Chapter 6: 5HT/NA INTERACTIONS IN THE BRAIN AND ALPHA$_2$-ADRENOCEPTOR SUBTYPES

5HT/NA INTERACTIONS IN THE BRAIN AND α_2-ADRENOCEPTOR SUBTYPES

6.1 Introduction

The morphological organisation of monoamine systems in the brain and psychopharmacological studies have long suggested a functional linkage between noradrenergic and 5HT neuronal systems.

The 5HT neurons in the midbrain raphe nuclei receive one of the greatest noradrenergic innervations in the brain. This input has been demonstrated by histofluorescence (Fuxe, 1965) and by immunocytochemical (Grzanna and Molliver, 1980; Baraban and Aghajanian, 1981), biochemical (Levitt and Moore, 1979) and electrophysiological techniques (Baraban and Aghajanian, 1980). It has been suggested that the noradrenergic innervation plays an important role in maintaining the tonic firing activity and the transmitter release of 5HT neurons. Interruption of noradrenergic transmission by application of α-adrenoceptor antagonists suppresses 5HT nerve firing (Baraban and Aghajanian, 1980) and 5HT metabolism (Reinhard and Roth, 1982). Administration of the β-adrenergic agonists has been shown to increase 5HT synthesis and metabolism in several brain areas (Waldmeier, 1981) and to potentiate the behavioural syndrome elicited by 5-hydroxytryptophan (Ortmann et al, 1981).

Midbrain 5HT neurons have been reported to contain β-adrenoceptors coupled to adenylate cyclase (Dolphin et al, 1979) and cAMP-dependent protein kinase (Sawada et al, 1985) and it has been suggested that the activity of tryptophan hydroxylase is linked to a β-adrenoceptor related mechanism probably via an intracellular cAMP-dependent process. α-adrenoceptor agonists, such as the imidazoline clonidine, have no effect on tryptophan hydroxylase activity. However, antagonists of the yohimbine type reduce it to 78% (Sawada and Nagatsu, 1986).

Raiteri et al (1983) showed that in the rat cerebral

cortex, where from an anatomical point of view nothing is known about the NA/5HT interaction, two stereochemically different subtypes of α_2-adrenoceptors can be found which regulate NA and 5HT release and are located, respectively, on NA and 5HT presynaptic nerve terminals. Schlicker et al, (1987) suggested that the 5HT nerve terminals in the rat brain cortex are endowed with an adenylate cyclase, which is negatively coupled to the presynaptic α_2-adrenoceptors, but is not linked to the presynaptic 5HT autoreceptors.

Recent studies with antidepressants have provided new evidence for a 5HT/NA link in the brain, at the level of the β-adrenoceptor coupled adenylate cyclase system. Most antidepressant treatments, on repeated administration, decrease the number of β-adrenoceptors in the cortex, a change that requires an intact 5HT neuronal input, suggesting that 5HT neurons play a pivotal role in the change, and high-lighting the complex neurotransmitter interactions that are present (Manier et al, 1987). Later studies have shown NA to regulate the population of β-adrenoceptors in the agonist high affinity state and 5HT to regulate those in the low affinity conformation (Gillespie et al, 1988). The subtype of 5HT receptor involved in this response has so far not been identified.

A number of compounds show high affinity for both 5HT receptors and α-adrenoceptors. These include: LSD, mianserin, the yohimbine series and dibenzyline, which historically was the first compound used to define $5HT_2$ ('D') receptors in guinea-pig ileum (Gaddum and Picarelli, 1957), but has since been shown to be a very potent α-adrenoceptor antagonist (Drew, 1976). The specificity and selectivity of a wide range of structurally different compounds for both classes of receptors are examined in this chapter.

The hippocampus was shown to be rich in $5HT_{1A}$ binding sites (see chapter 3) but found in the present studies to have low numbers of α_2-adrenoceptor binding sites labelled by [^{3}H]-yohimbine. The ratio of $5HT_{1A}$:α_2-adrenoceptor binding sites was found to be 6:1. The hippocampus is an

important mood control center and maybe an important site of action of new $5HT_{1A}$ anxiolytic compounds such as buspirone and ipsapirone. These compounds have been shown to be effective anxiolytics in man (Goa and Ward, 1986). Interestingly they have a common active metabolite 1-(2-pyrimidinyl)-piperazine a compound with appreciable α_2-adrenoceptor affinity but devoid of $5HT_{1A}$ affinity. This study undertook to investigate if the α_2-adrenoceptor in the hippocampus is atypical or if some allosteric coupling exists between the α_2-adrenoceptor and $5HT_{1A}$ receptor binding sites in the hippocampus, such coupling might be important for the mechanism of action of these new anxiolytic compounds.

[^{3}H]-Yohimbine is routinely used in many laboratories to label α_2-adrenoceptors. However the binding data produced is often both diverse and complex. It has been suggested to be both monophasic (Cheung et al, 1982) and biphasic (Diop et al, 1983) in rat cortical membranes. Michel and Whiting, (1984) reported shallow antagonist displacements of [^{3}H]-yohimbine binding which indicates an even more complex binding isotherm. This study undertook a careful investigation of the binding characteristics of [^{3}H]-yohimbine in three tissues; rat cortex, rat hippocampus and human platelets. Displacement curves for a wide range of α and 5HT ligands were examined.

Some interesting differences were observed between the α_2-adrenoceptor populations in the three tissues. Therefore further studies were undertaken to study possible α_2-adrenoceptor subtypes. The α_2-adrenoceptor populations in rat cortex, rat hippocampus and human platelets were compared and contrasted using [^{3}H]-yohimbine and [^{3}H]-idazoxan radiolabels.

Brief history of α-adrenoceptor subtype binding assays

Early attempts to directly label α-adrenoceptors (May et al, 1967) were unsuccessful due mainly to the low affinity and specific activity of the radioligand employed and to the high levels of non-specific binding. The early use of [^{3}H]-catecholamines was also largely unsucessful (Lefkowitz

et al, 1973) as these studies failed to demonstrate the normal characteristics of ligand-receptor interactions.

The availability of high affinity ligands which could be labelled to high specific activity enabled direct labelling of α-adrenoceptors (Williams and Lefkowitz, 1976; Greenberg et al, 1976). Investigations in several laboratories led to the conclusion that α_1-adrenoceptors and α_2-adrenoceptor subtypes were present in the CNS (U'Prichard et al, 1979; U'Prichard and Snyder, 1979; Tanaka and Starke, 1980).

Following the widespread acceptance of α-adrenoceptor subtypes, a range of selective radioligands has been introduced (see review: Bylund and U'Prichard, 1983). [^{3}H]-prazosin (Greengrass and Bremner, 1979; Hornung et al, 1979) is regarded as the ligand of choice for the study of α_1-adrenoceptors. [^{3}H]-yohimbine (Diaguji et al, 1981), [^{3}H]-rauwolscine (Perry and U'Prichard, 1981) and [^{3}H]-RX 781094 ([^{3}H]-idazoxan) (Pimoule et al, 1983) have been used as antagonist radioligands for the study of α_2-adrenoceptors.

Evidence for further divisions of α_2-adrenoceptor subtypes

Bylund, (1981) provided a basis for further differentation of α_2-adrenoceptors by showing that the densities of [^{3}H]-yohimbine and [^{3}H]-clonidine binding sites varied considerably across several tissues and species. Since then a number of laboratories have demonstrated heterogeneity of α_2-adrenoceptors between species (Cheung et al, 1982; Feller and Bylund 1984; Neylon and Summers 1985; Kawahara and Bylund 1985; Dickinson et al, 1986). A classification of α_2-adrenoceptor binding site heterogeneity has been developed based on the differences in affinity and rank order of potency of a variety of compounds to inhibit ^{3}H-antagonist binding (Nahorski et al 1985, Bylund 1985) and ^{3}H-agonist binding (Randall et al 1983).

A number of clear differences between rodent and non-rodent α_2-isoreceptors have been demonstrated; the affinity of yohimbine and oxymetazoline is higher in

non-rodent species as compared with rodents whereas the α_1-adrenoceptor antagonists prazosin and indoramin show relatively high affinity for rodent α_2-adrenoceptors and low affinity for non-rodents. A study in kidney membranes from five different species reported marked differences not only between species but also in the rank order of potency of antagonists. The species potency series for yohimbine is man > rat > mouse > rabbit > dog and for idazoxan is rat > man > dog > rabbit > mouse (Neylon and Summers 1985), demonstrating that a rodent and non-rodent classification is clearly oversimplified. Other groups have also shown heterogeneity within the same species; rat (Bylund, 1985), human (Summers et al, 1983; Petrash and Bylund, 1986).

Further biochemical evidence that the rat brain contains two subtypes of α_2-adrenoceptor is based on the observation that (-)-mianserin acts as an antagonist at the α_2-adrenoceptor (heteroreceptor) which mediates 5HT release but is without effect on the α_2-adrenoceptor (autoreceptor) which mediates presynaptic regulation of NA release (Raiteri et al, 1983). This also suggests heterogeneity between α_2-adrenoceptors in different anatomical locations. One interesting development in support of this is the introduction of SK & F 104078 which is a compound claimed to be inactive at pre-junctional α_2-adrenoceptors but with approximately 100nM affinity for post-junctional α_2-adrenoceptors and platelet α_2-adrenoceptor binding sites (Ruffolo et al, 1987).

There is evidence to suggest that α_2-adrenoceptor -mediated responses are dependent on the physical environment. The α_2-adrenoceptor can exist in different conformational states depending on tissue or species, according to the presence of co-factors in the immediate vicinity of the receptor. However, the heterogeneities between human platelets and rat cerebral cortex (Karahara and Bylund 1985) and human and rabbit spleen (McKernan et al 1986) are maintained upon solubilisation, which favours the suggestion that the differences between

α_2-adrenoceptors arise from heterogeneities in the molecular architecture of the receptor. In soluble preparations agonists cannot induce high affinity states of the receptor. Therefore this technique argues against heterogeneous binding due to different agonist affinity states and suggests absolute conformational differences may exist between different α_2-adrenoceptor subtypes. McKernan and colleagues (1986) have shown that thiol groups are not involved in the structural differences but that there are some differences in the carbohydrate portion of the receptor, although it is not clear to what extent the carbohydrate portion of the molecule is involved in ligand binding. This is nevertheless interesting because the subdivision of α-adrenoceptors will probably be ultimately based on their primary structure. The possibility exists therefore that α_2-adrenoceptors could differ sufficiently to allow the identification of antagonists which could be targeted to different tissue or different α_2-subtypes.

The figures and tables are presented at the end of the results section.

6.2.1 Receptor binding assays

$5HT_{1A}$ binding assays

[^{3}H]-5HT was found to label a $5HT_{1A}$ binding site on rat cortex membranes (Kd 4.95nM; Bmax 132fmol/mg protein), similarly [^{3}H]-8-OH-DPAT was found to label a $5HT_{1A}$ binding site in the rat cortex (Kd 1.3nM; Bmax 102fmol/mg protein) and rat hippocampus (Kd 1.1nM; Bmax 534fmol/mg protein) see chapter 3. [^{3}H]-Ketanserin was found to label a $5HT_2$ binding site in the rat frontal cortex with high affinity (Kd 0.42nM) and Bmax, 240fmol/mg protein, see chapter 4. These assays were used to determine the 5HT binding affinity of compounds used in the following studies.

α_1-adrenoceptor binding assay

[^{3}H]-Prazosin was found to bind to a single population of high affinity sites (Kd 0.2nM; Bmax 98fmol/mg protein) (fig. 6.1) on the rat cerebral cortex membranes which was characteristic of the α_1-adrenoceptor showing high affinity for WB4101 (0.9nM) and prazosin (0.3nM) and low affinity for yohimbine (398nM) and imiloxan (>10,000nM). This assay was used to determine the α_1-adrenoceptor binding affinities in the following studies.

A more detailed description of the α_2-adrenoceptor binding assay is given below.

6.2.2 A correlation of the affinities of standard compounds for α-adrenoceptor and 5HT binding sites

Rat cortex

Table 6.1 shows the affinity values, determined in the rat cerebral cortex, for a wide variety of compounds for the α_1-adrenergic, α_2-adrenergic, $5HT_{1A}$ and $5HT_2$ binding sites. The compounds represent those available at the start of this thesis. The $5HT_2$ antagonists cyproheptadine, mianserin, methysergide and ketanserin showed moderate to high affinity for the α_1-adrenoceptor binding site. Yohimbine showed moderate affinity for the $5HT_{1A}$ binding site.

Rat hippocampus

Table 6.2 shows the affinity values for selected ligands for the α_2-adrenergic and $5HT_{1A}$ binding sites in the rat hippocampus. Yohimbine, rauwolscine and WY26703 showed moderately high affinity for the $5HT_{1A}$ binding site on rat hippocampal membranes. Idazoxan and imiloxan were inactive at the $5HT_{1A}$ site, as was the buspirone metabolite, MJ 13653. The very selective $5HT_{1A}$ ligand DP-5CT was inactive at the α_2-adrenoceptor binding site as was 5CT. Interestingly the $5HT_{1A}$ ligands buspirone, 8-OH-DPAT methysergide and RU24969, which are all proposed to be agonists or at least partial agonists at the $5HT_{1A}$ site, showed moderate affinity for the α_2-adrenoceptor binding site (pIC_{50} 6.5-7.14). However, all the compounds inhibited [^{3}H]-yohimbine binding with Hill slopes less than unity. Therefore it was decided to further investigate the interaction of these compounds with the α_2-adrenoceptor binding site and examine the possibility of agonist action, multiple [^{3}H]-yohimbine binding sites or an allosteric interaction.

6.2.3 α_2-adrenoceptor binding assays

1. [^{3}H]-Yohimbine binding to rat cerebrocortical membranes

[^{3}H]-Yohimbine was found to bind to a single population of high affinity sites (Kd 5.2±0.9nM; Bmax 121±10fmol/mg protein) over the concentration range of 0.1-15nM. At 1.5nM [^{3}H]-yohimbine specific binding represented 60-65% of the total binding.

Table 6.3 shows the affinity values for inhibition of [^{3}H]-yohimbine binding to rat cortex. Rauwolscine was found to have a significantly higher affinity than yohimbine ($p<0.01$). BDF6143 was the most potent imidazoline tested (0.6nM). Whereas idazoxan showed similar affinity to yohimbine. Prazosin and oxymetazoline produced biphasic displacement curves which could be statistically best described using a two site model. Prazosin showed high affinity (50nM) for approximately 50% of [^{3}H]-yohimbine binding (fig. 6.2). The rank order of potency for the

adrenergic compounds was rauwolscine > yohimbine > oxymetazoline >> prazosin = WB4101 >>> indoramin. Except for prazosin and oxymetazoline all these ligands showed Hill slopes not significantly different from unity.

5HT was shown to be inactive. However, the four $5HT_{1A}$ ligands tested, buspirone, methysergide, RU24969 and 8-OH-DPAT, all showed biphasic displacement curves with a greater than two log order difference in affinity between the two sites. The rank order of potency for the high affinity site was methysergide > RU24969 > buspirone > 8-OH-DPAT. A representative inhibition curve for RU24969 is shown in fig. 6.3, methysergide in fig. 6.4 and 8-OH-DPAT in fig. 6.5. Figure 6.4 also shows that in the presence of $1x10^{-7}$ M prazosin the high affinity site for methysergide is blocked. Similarly, in the presence of $1x10^{-7}$ M prazosin the high affinity component of buspirone, 8-OH-DPAT and RU24969 binding is abolished, which suggest that the site showing high affinity for prazosin and the $5HT_{1A}$ ligands is the same site. This site will now be referred to as site A and it represents approximately 40% of the total binding in the cortex. In contrast in the presence of $1x10^{-7}$ M prazosin oxymetazoline still shows high affinity for [^{3}H]-yohimbine binding.

2. [^{3}H]-Yohimbine binding to rat hippocampal membranes

Similarly, over a concentration range of 0.1-15nM, [^{3}H]-yohimbine was found to bind to a single population of high affinity α_2-adrenoceptor binding sites (Kd 5.8±0.7nM) with a low receptor number (Bmax 72±6fmol/mg protein) on rat hippocampal membranes. At 1.5nM [^{3}H]-yohimbine specific binding represented 55-60% of total binding.

Table 6.4 shows the Ki values for inhibition of [^{3}H]-yohimbine binding to rat hippocampus. Yohimbine and rauwolscine show equal affinity for this site. Whereas idazoxan and imiloxan showed lower affinity than in the cortex. Prazosin produced biphasic displacement curves which could be statistically best fitted to two sites revealing a very high affinity component (7.9nM). Oxymetazoline also showed biphasic displacement curves

(fig. 6.6). The rank order of potency of the adrenergic compounds was prazosin > rauwolscine = yohimbine = oxymetazoline > WB4101 >>> indoramin.

The inhibition by the 5HT compounds was biphasic as seen in the cortex. The order of potency for the high affinity site was RU24969 > methysergide > 8-OH-DPAT. Buspirone showed very high affinity (39.8nM) (fig. 6.7) and selectivity 310 fold siteA/siteB. The ratio of site A to site B is approximately 30:70%in the hippocampus.

3. [^{3}H]-Yohimbine binding to human platelet membranes

[^{3}H]-Yohimbine was found to bind to only one site on human platelet membranes with very high affinity (Kd 0.66±0.07nM; Bmax 152±10fmol/mg protein). At 1.5nM [^{3}H]-yohimbine specific binding represented 80-85% of total binding.

Table 6.5 shows the Ki values for inhibition of [^{3}H]-yohimbine binding to human platelets by a series of α-adrenergic and 5HT compounds. Yohimbine was the most potent compound tested (0.79nM) with a slightly higher affinity than its stereoisomer rauwolscine (1.04nM). Both showed almost a log order higher affinity than in the rat brain. Oxymetazoline showed high affinity (3.9nM) whereas prazosin was relatively weak (871nM); both were best fitted to a one site fit. The rank order of potency for the adrenergic compounds was yohimbine > rauwolscine > oxymetazoline = WB4101 >>> prazosin > indoramin (fig. 6.8).

5HT was inactive at the site. However RU24969 and methysergide showed moderate potency, 76nM and 89nM respectively. 8-OH-DPAT was less active (217nM) and buspirone was relatively weak (603nM) (fig. 6.8). The Hill slopes were determined for each curve and were found to be not significantly different from unity (table 6.5). The values determined for 8-OH-DPAT, RU24969 and methysergide were similar to their high affinity site for [^{3}H]-yohimbine binding in the rat cortex and hippocampus. However, buspirone was less active than would be predicted from its affinity in the rat brain.

6.2.4 A comparison of the binding characteristics of [^{3}H]-yohimbine in the rat cortex, rat hippocampus and human platelet

Saturation curves for rat cortex, rat hippocampus and human platelets membranes labelled by [^{3}H]-yohimbine are shown in fig. 6.9 (also see table 6.9), along with the Scatchard plot for each curve. The experiments were analysed using the non-linear curve fitting programme 'Ligand' (Munson and Rodbard, 1980) and indicated a high affinity site for each tissue over the concentration range 0.1-15nM.

A good correlation can be drawn between the Ki values determined in the rat cortex and rat hippocampus site B (r= 0.724) (fig. 6.10). Similarly a correlation can be drawn between the two tissues using site A (r=0.991). This suggests that the [^{3}H]-yohimbine binding is similar in both tissues. Standard α_2-adrenoceptor antagonists yohimbine, rauwolscine, WY26703 and BDF6143 show approximately equal affinity in the rat cortex and rat hippocampus. Rauwolscine shows higher affinity than yohimbine in the cortex but equal affinity to yohimbine in the hippocampus. However, the imidazoline compounds imiloxan and idazoxan show greater affinity in the cortex than the hippocampus.

Yohimbine and rauwolscine showed a ten fold higher affinity for the platelet α_2-adrenoceptor binding site. as did methysergide, RU24969 and 8-OH-DPAT.

5HT/NA interaction

The $5HT_{1A}$ ligands showed higher affinity for site A in the hippocampus. The anxiolytic buspirone showed markedly higher affinity for the hippocampus site A than for the platelet. Therefore a further study was undertaken to investigate if any indication of an interaction between α_2-adrenoceptors and $5HT_{1A}$ receptors could be found in the rat hippocampus. 5HT has no direct affinity for the binding of [^{3}H]-yohimbine to rat hippocampus at concentrations up to $1x10^{-4}$ M. To investigate if 5HT acts as an allosteric modulator saturation assays were performed as described in section 2.1.6b, in the presence or absence of 1 or 10nM

5HT. No significant change was found in Kd or Bmax (table 6.6).

An alternative explanation for the two site nature of prazosin, oxymetazoline and the 5HT compounds could be that they act as agonists at one or both of the α_2-adrenoceptor sites. Therefore the inhibition curves were repeated in the presence of 0.1mM GTP. While in parallel experiments GTP increased the IC_{50} values of noradrenaline and adrenaline in all three tissues it failed to alter the inhibition curves of prazosin, 8-OH-DPAT, RU24969 and methysergide (table 6.7). However GTP was found to induce a small but significant shift in the IC_{50} value determined for buspirone in the hippocampus (table 6.7). Prazosin and the other compounds, with perhaps the exception of buspirone, are probably antagonists at the α_2-adrenoceptor sites.

Finally to investigate if any structural differences could be determined between the α_2-adrenoceptor binding site in the cortex or hippocampus, a study on the effect of chemical modifying agents was undertaken.

6.2.5 The effect of chemical modifying agents on α_2-adrenoceptor binding sites

In order to further study the heterogeneity revealed by competition studies, the effects of the modifying agents N-ethylmaleimide (NEM; selective for the -SH group of cysteine) and phenylglyoxal (PGL; selective for the guanidino group of arginine) on α_2-adrenoceptors in rat cortex and hippocampus was investigated (fig. 6.11 & 6.12; table 6.8).

Prior incubation with NEM results in alkylation of a thiol group preventing binding of [^{3}H]-yohimbine to the α_2-adrenoceptor in the rat cortex as reported by Mattens et al (1984) and now shown also to occur in the hippocampus. Similarly preincubation with PGL causes a concentration-dependent decrease in receptor number in the hippocampus and cortex.

Scatchard transformations of the binding data in the present of either compound were best fitted to a one site fit suggesting that both [^{3}H]-yohimbine binding sites are

effected. Therefore for both populations of α_2-adrenoceptors there is evidence of arginine and cysteine residues at or near the binding site of [^{3}H]-yohimbine, and there is no evidence to suggest that there is any variation in the position or accessibility of these chemical moieties between the cortex and the hippocampal α_2-adrenoceptor binding sites.

It begins to appear that [^{3}H]-yohimbine labels two distinct populations of binding sites in the rat cortex and hippocampus which can be differentiated by prazosin, oxymetazoline and the 5HT ligands, methysergide, RU24969 and 8-OH-DPAT (which accounts for the low Hill slopes in table 6.2). Different proportions of the two sites exist in different brain regions which probably best explains the differences between the cortex and hippocampus α_2-adrenoceptors. No evidence for an atypical α_2-adrenoceptor in the hippocampus was found. Similarly no evidence for a α_2/5HT link in the hippocampus was seen, except that the unusually high α_2-adrenoceptor affinity for buspirone merits further investigation. However, evidence for α_2-adrenoceptor heterogeneity was found. Therefore a further study of the characteristics of α_2-adrenoceptor binding sites in the rat brain was made using [^{3}H]-idazoxan as radioligand.

6.2.6 A comparison of the binding of [^{3}H]-idazoxan in the rat cortex, rat hippocampus

1. [^{3}H]-Idazoxan binding to rat cerebral cortex membranes

In a Tris EDTA buffer system [^{3}H]-idazoxan over the concentration range 1-12nM bound to a single population of high affinity sites (Kd 4.1±0.9nM; 87±8fmol/mg protein), fig. 6.13. At 2nM [^{3}H]-idazoxan specific binding represented 65-70% of total binding.

2. [^{3}H]-Idazoxan binding to rat hippocampal membranes

Similarly under the same conditions [^{3}H]-idazoxan labels a single population of sites with high affinity (Kd 3.5±0.5nM) and a low receptor number (Bmax 36±6fmol/mg

protein) in the hippocampus (fig. 6.13). At 2nM [^{3}H]-idazoxan specific binding represented 60-65% of total binding.

Competition experiments with [^{3}H]-idazoxan to rat cortex showed the imidazolines were more potent than the yohimbine-like compounds. BDF6143, idazoxan and WY26703 showed slightly higher affinity in the hippocampus than the cortex whereas prazosin showed a 6 fold higher affinity in the hippocampus. The 5HT ligands showed similar affinities in the cortex and hippocampus. Interestingly buspirone was devoid of affinity in both the cortex and hippocampus (pKi <5). The results are presented in table 6.9. The Hill slopes were determined for each curve and were found not to be significantly different from unity (table 6.9). Fig. 6.14 shows the inhibition curves for [^{3}H]-idazoxan binding in the rat hippocampus. Idazoxan itself was found to displace nsb, determined in displacement studies using boiled membranes as described in section 4.2.1d for displacement of [^{3}H]-ketanserin nsb by cold ketanserin. 8-OH-DPAT was also found to displace the nsb unlike in the [^{3}H]-yohimbine binding assays where its biphasic displacement curves could not be explained by the displacement of nsb.

6.2.7 A Comparison of binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan

Significant differences were observed between the maximum number of [^{3}H]-yohimbine and [^{3}H]-idazoxan binding sites in the rat cortex and hippocampus, (table 6.10). The density of α_2-adrenoceptors was significantly higher using [^{3}H]-yohimbine in both tissues. Kd values for each ligand were not however significantly different in either tissue. [^{3}H]-yohimbine showed higher affinity for the human platelet α_2-adrenoceptor.

Affinity values for the displacement of [^{3}H]-yohimbine and [^{3}H]-idazoxan from rat cortex and rat hippocampus membranes are shown in tables 6.3,6.4 and 6.9. Noradrenaline and adrenaline displaced the binding to both ligands with IC_{50} values in the nanomolar range and Hill

coefficents <1. The affinity values were markedly attenuated in the presence of 0.1mM GTP with nH values brought closer to unity (see table 6.7).

Affinity values for yohimbine, rauwolscine and WY26703 determined in the rat cortex and hippocampus for displacement of [^{3}H]-yohimbine were higher than for displacment of [^{3}H]-idazoxan. In contrast the imidazoline structures idazoxan, imiloxan and BDF6143 (except in the cortex) show higher affinities for the site labelled by [^{3}H]-idazoxan. The 5HT compounds show higher affinities for a site labelled by [^{3}H]-yohimbine; buspirone is inactive at the idazoxan site and methysergide is 100 fold weaker.

A good correlation could be made between the binding to [^{3}H]-yohimbine site B and the [^{3}H]-idazoxan binding site, r=0.845;p<0.001, (fig. 6.15). In contrast a poor correlation is made with site A, r=0.456 (fig. 6.16). Similarly in the rat hippocampus a significant correlation could be made between the binding to [^{3}H]-yohimbine site B and [^{3}H]-idazoxan binding site, r=0.803;p<0.001, (fig. 6.17) but not for site A, r=0.304, (fig. 6.18). Therefore these results suggest that [^{3}H]-yohimbine labels two populations of binding sites in rat brain both of which would appear to be α_2-adrenoceptor subtypes, from competition studies with antagonists, from comparison with α_2-adrenoceptors in other tissues and from the use of other α_2-adrenoceptor radioligands. Additional studies were performed using pharmacological tools to selectively block one of the sites in order to determine the affinity of NA for the two sites.

Agonist affinities for the [^{3}H]-yohimbine binding studies

Prazosin at a concentration of $1x10^{-7}$ M (see fig. 6.2) was chosen to block the [^{3}H]-yohimbine binding site A in the rat cortex. This method proved to be highly reproducable. Idazoxan at a concentration of $1x10^{-7}$ M was chosen to block the [^{3}H]-yohimbine site B. However, idazoxan did not prove a reliable tool giving varible specific binding. Nevertheless agonist inhibition curves were generated in the presence of absence of 0.1mM GTP. The results are shown in table 6.11). NA showed higher affinity

for the site B, similar to its affinity for the total binding. However, NA was less potent at site A. UK14304 showed high affinity for the site B but low affinity for site A. 5HT was inactive at both sites. A further investigation of the site A was undertaken. Rauwolscine showed high affinity for [^{3}H]-yohimbine binding to site A (pKi 20nM), although not as high as its affinity value determined for total binding (pKi 7.1nM; table 6.3). The finding indicates that this site is probably also an α_2-adrenoceptor binding site.

6.2.8 Effect of assay buffer constituents on [^{3}H]-yohimbine and [^{3}H]-idazoxan binding

In the above studies, assays were performed under identical conditions. Preliminary studies were undertaken to examine the influence of buffer ions on the specific binding of the two ligands.

In the physiological incubation medium (physiological buffer pH 7.4 at 25^{o}C containing (mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose 5.5; equilibrated with 95% O_2/5% CO_2 at 25^{o}C before use) [^{3}H]-idazoxan over the range 1-12nM bound to a single population of high affinity sites in rat cortex with similar affinity 4.7±0.8nM to assays performed in Tris HCl buffer. A higher, although not significantly higher, number of binding sites (107±12fmol/mg protein) was labelled. No significant difference was found in the Kd or Bmax determined for [^{3}H]-idazoxan binding to rat hippocampus. Similarly in a physiological buffer [^{3}H]-yohimbine was found to label a slightly higher number of binding sites (130±11fmol/mg protein). Competition experiments showed that pKi values determined with [^{3}H]-yohimbine (1.5nM) were not significantly different in Tris HCl or physiological buffer. Antagonist inhibition of [^{3}H]-idazoxan at a fixed concentration of 2nM produced slightly higher affinity values in the physiological buffer.

The influence of divalent ions on the binding of the two ligands was also examined. Assays performed in a Tris HCl

buffer pH 7.4 at 25°C containing 2.6mM Ca^{2+} resulted in a significant decrease in the percentage binding of both ligands, particularly the [^{3}H]-yohimbine. Similarly assays performed in Tris HCl buffer pH 7.4 at 25°C containing 5mM Mg^{2+} caused a decrease in the specific binding of both ligands and a decrease in the affinity values obtained with [^{3}H]-yohimbine.

6.2.9 [^{3}H]-Idazoxan binding to human platelet membranes

Preliminary investigations were undertaken to establish the binding characteristics of [^{3}H]-idazoxan to human platelets which might help investigations of α_2-adrenoceptor subtypes. However, difficulty was encountered in obtaining specific binding, only 30% specific binding at 2nM [^{3}H]-idazoxan. Platelet membranes were prepared as described in methods section 2.1.1a, and incubated with [^{3}H]-idazoxan in a Tris EDTA buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4) for 30min at 25°C. Under these conditions [^{3}H]-idazoxan labelled a small number of binding sites (Bmax 52fmol/mg protein) with high affinity (Kd 6.5nM).

[^{3}H]-PRAZOSIN BINDING TO RAT CEREBROCORTICAL MEMBRANES

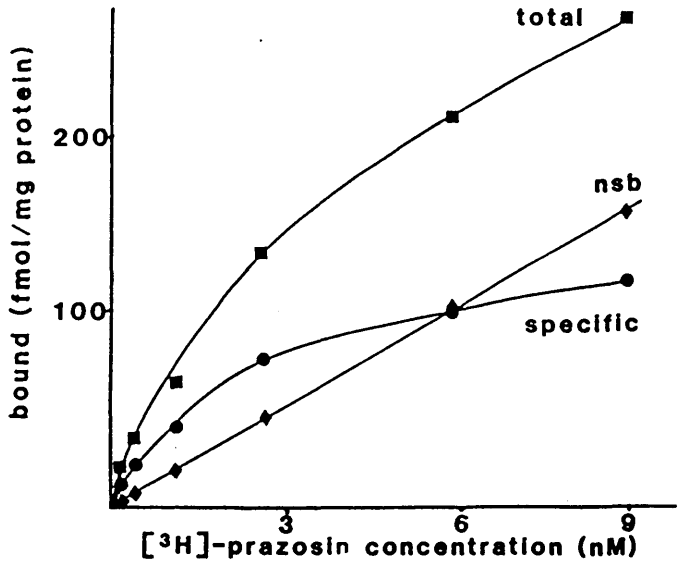


Fig. 6.1 Relationship between total, nsb and specific binding and ligand concentration. [^{3}H]-prazosin concentrations of 0.1-8.9nM were incubated with washed rat cerebrocortical membranes at 25^{o}C for 30min in Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm phentolamine.

PRAZOSIN INHIBITION OF [^{3}H]—YOHIMBINE BINDING TO RAT CEREBROCORTICAL MEMBRANES

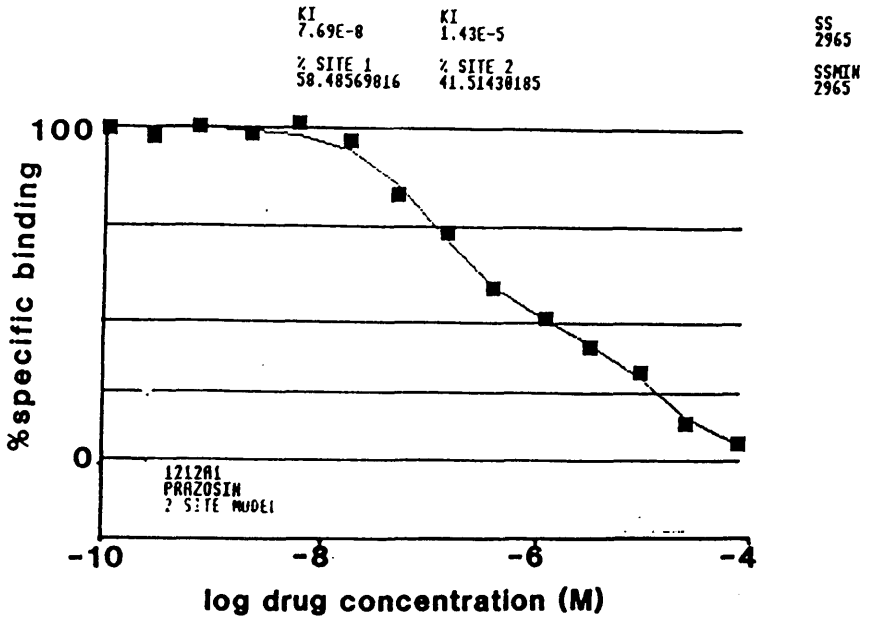


Fig. 6.2 Inhibition studies of [^{3}H]-yohimbine to rat cerebrocortical membranes were conducted over a concentration range of $1x10^{-10}$ -$1x10^{-4}$ M prazosin. Incubations were performed for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm phentolamine. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.001$. (1 site, ssmin 62882, DF 11; 2 site, ssmin 2965, DF 9; F value 91.1)

INHIBITION OF SPECIFIC [^{3}H]-YOHIMBINE BINDING TO RAT CEREBROCORTICAL MEMBRANES RU24969

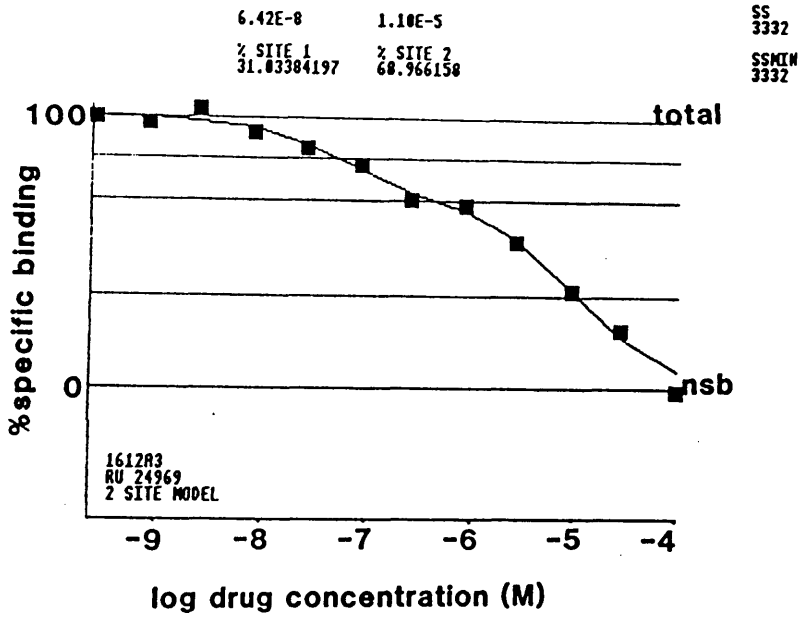


Fig. 6.3 Inhibition studies of [^{3}H]-yohimbine to rat cerebrocortical membranes were conducted over a concentration range of $3x10^{-10}$ -$1x10^{-4}$ M RU24969. Incubations were performed for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm phentolamine. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.001$.

METHYSERGIDE INHIBITION OF [^{3}H]-YOHIMBINE BINDING TO RAT CEREBROCORTICAL MEMBRANES

a control

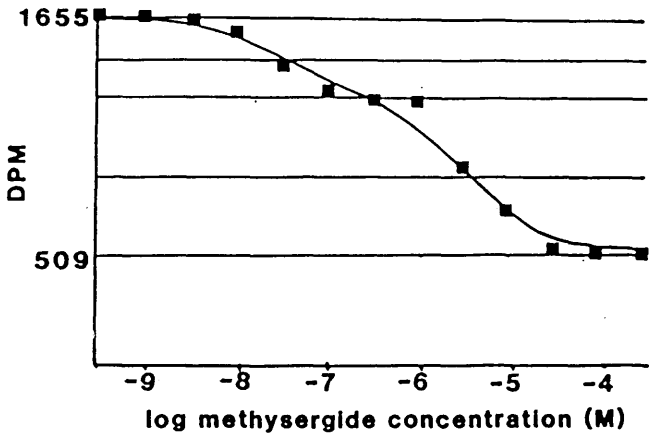


Fig. 6.4a Inhibition studies of [^{3}H]-yohimbine to rat cerebrocortical membranes were conducted over a concentration range of $3x10^{-10}$ -$3x10^{-4}$ M methysergide. Incubations were performed for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm phentolamine. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.01$.

b in the presence of 10^{-7} M prazosin

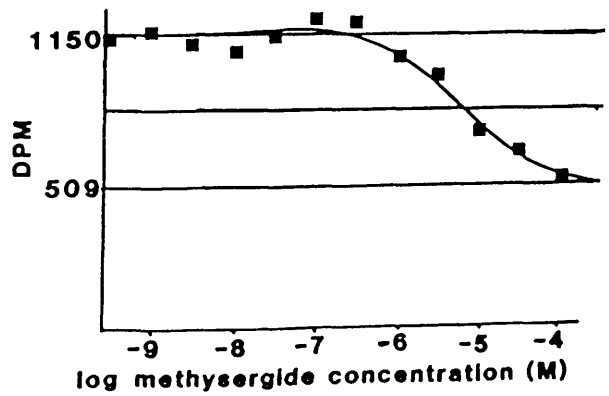


Fig. 6.4b shows the inhibition of [^{3}H]-yohimbine binding in the presence of $1x10^{-7}$ M prazosin which blocks 44% of the specifically bound [^{3}H]-yohimbine.

8-OH-DPAT INHIBITION OF [^{3}H]-YOHIMBINE BINDING TO RAT HIPPOCAMPAL MEMBRANES

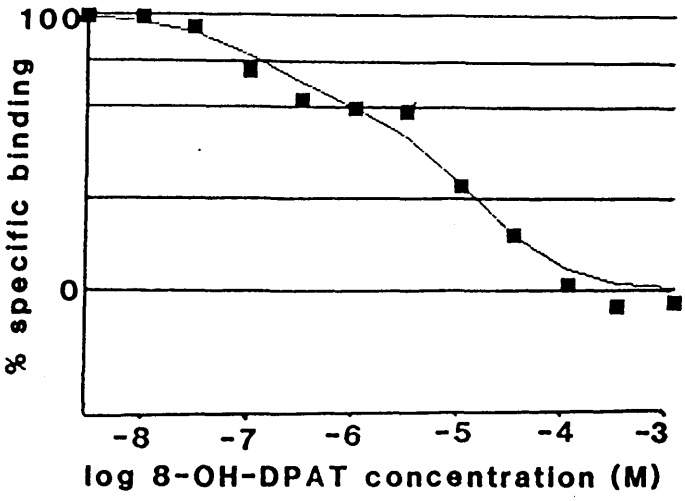


Fig. 6.5 Inhibition studies of [^{3}H]-yohimbine to rat cerebrocortical membranes were conducted over a concentration range of $3x10^{-9}$ -$1x10^{-3}$ M 8-OH-DPAT. Incubations were performed for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm phentolamine. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.01$.

OXYMETAZOLINE INHIBITION OF [^{3}H]-YOHIMBINE BINDING TO RAT HIPPOCAMPAL MEMBRANES

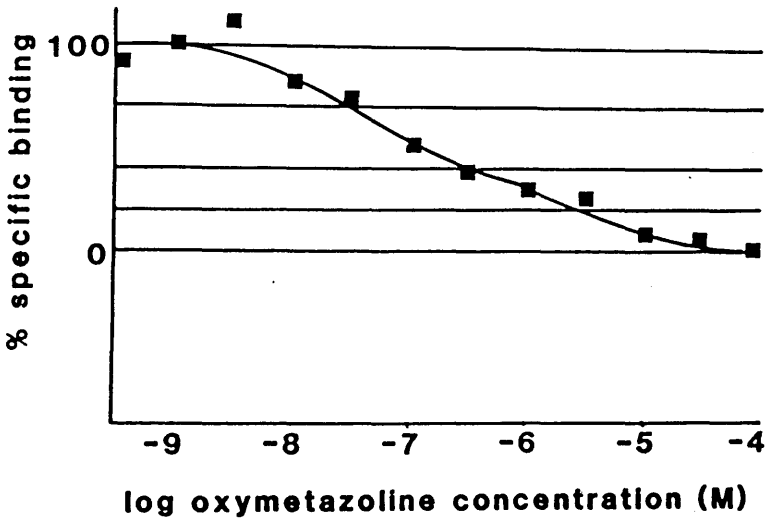


Fig. 6.6 Inhibition studies of [^{3}H]-yohimbine to rat hippocampal membranes were conducted over a concentration range of $3x10^{-10}$ $-1x10^{-4}$ M oxymetazoline. Incubations were performed for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10μm phentolamine. The data describes a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.001$.

BUSPIRONE INHIBITION OF [^{3}H]-YOHIMBINE BINDING TO RAT HIPPOCAMPAL MEMBRANES

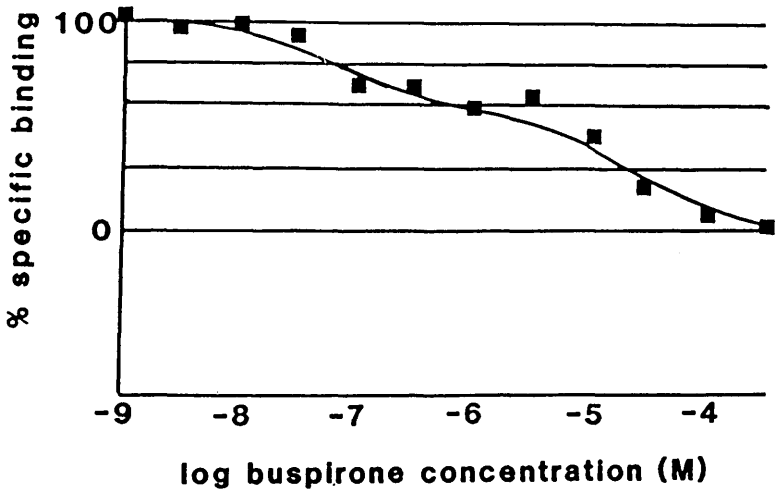


Fig. 6.7 Inhibition studies of [^{3}H]-yohimbine to rat hippocampal membranes were conducted over a concentration range of $1x10^{-9}$ -$3x10^{-4}$ M buspirone. Incubations were performed for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH7.4). Non-specific binding was determined in the presence of 10μm phentolamine. The data represents a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit was significantly better than a one site fit, $p<0.01$.

INHIBITION OF SPECIFIC [^{3}H]-YOHIMBINE BINDING

TO HUMAN PLATELET MEMBRANES

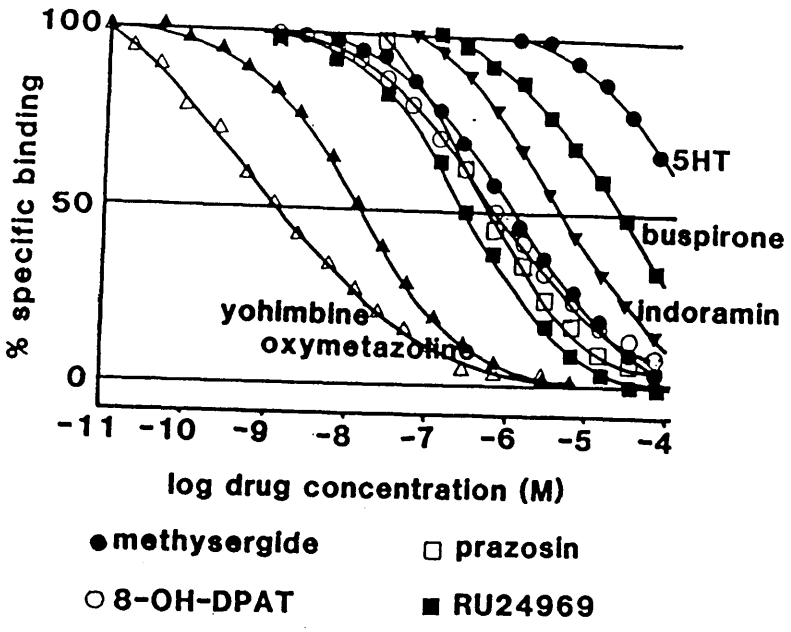


Fig. 6.8 Inhibition of specific bound [^{3}H]-yohimbine to human platelet membranes by yohimbine, oxymetazoline, methysergide, 8-OH-DPAT, RU24969, prazosin, indoramin, buspirone and 5HT. Incubations were performed at approximately 1.5nM [^{3}H]-yohimbine for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4).

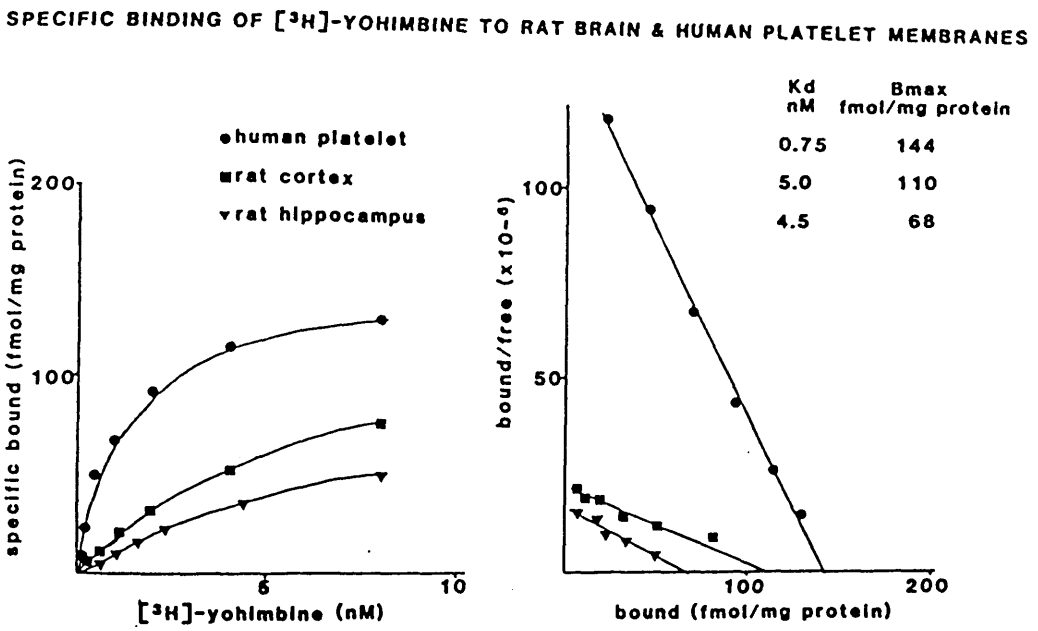


Fig. 6.9 Specific binding of [^{3}H]-yohimbine to rat cerebral cortex, rat hippocampus and human platelet membranes. Specific binding is defined as that binding displaced by 10μm phentolamine, and the data describes a single experiment performed in duplicate in each tissue.

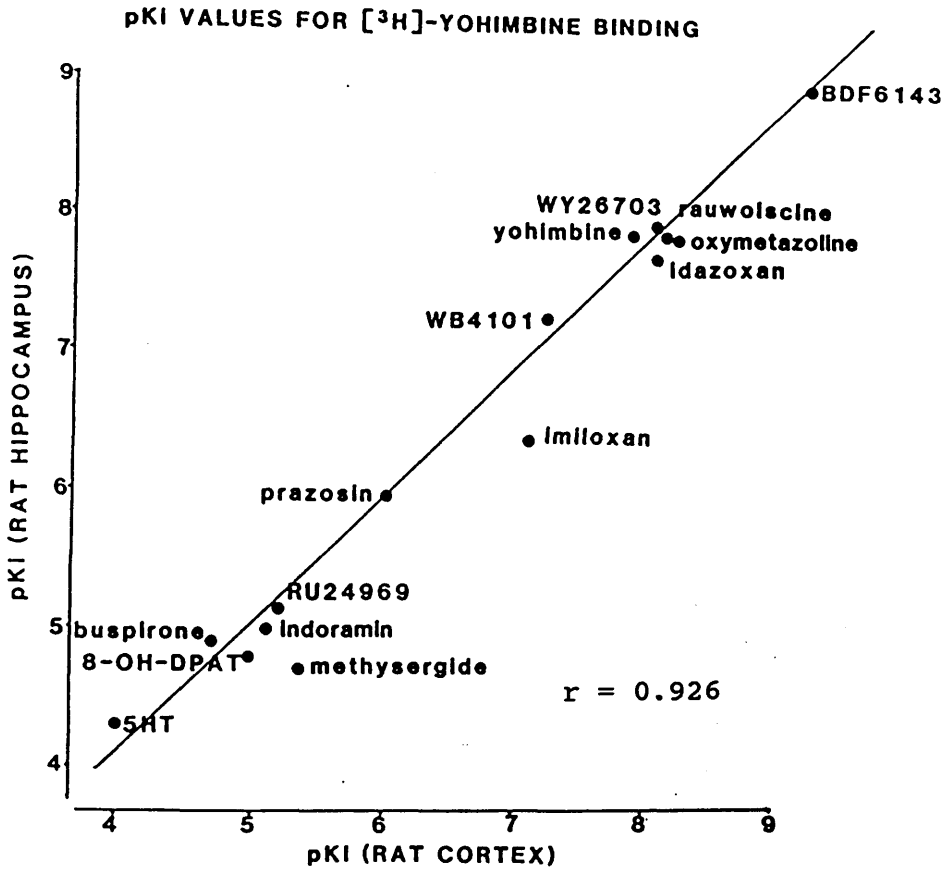


Fig. 6.10 Correlation between binding affinities of compounds for [^{3}H]-yohimbine binding to rat cortex and rat hippocampal membranes, (site B).

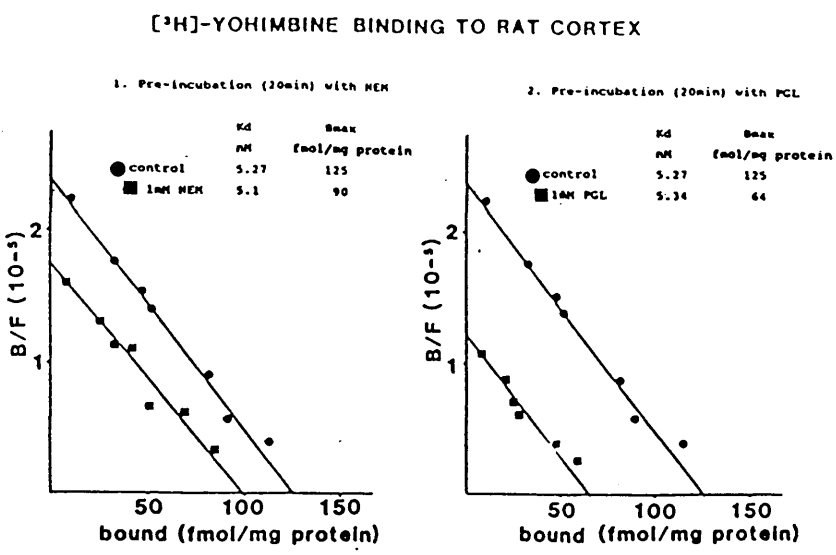


Fig. 6.11 Scatchard plots of [^{3}H]-yohimbine saturation binding to rat cerebrocortical membranes: ● control; ■ pretreated for 20min at 37°C with 1 or 10mM NEM or 20min with 1mM PGL.

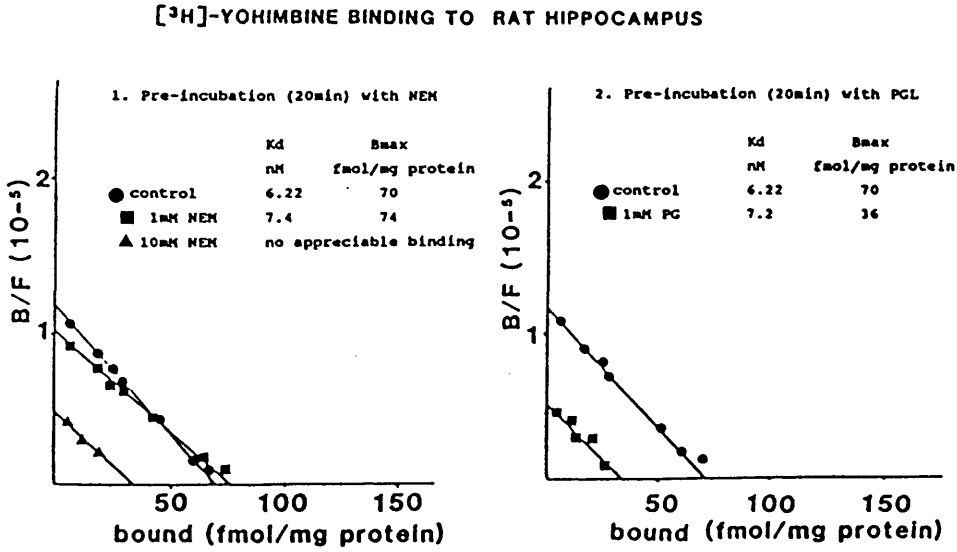


Fig. 6.12 Scatchard plots of [^{3}H]-yohimbine saturation binding to rat hippocampal membranes: ● control; ■ pretreated for 20min at 37°C with 1 or 10mM NEM or 1mM PGL.

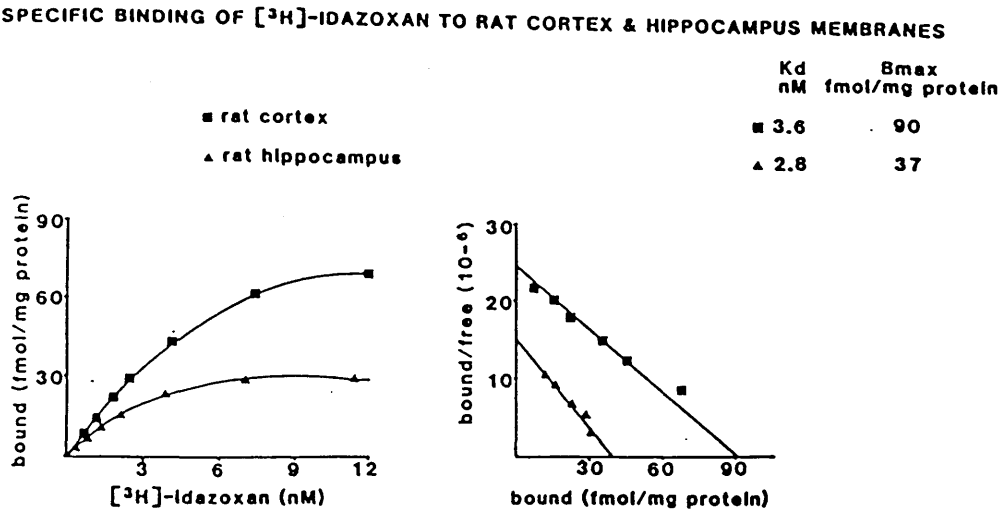


Fig. 6.13 Specific binding of [^{3}H]-idazoxan to rat cerebral cortex and rat hippocampus. Specific binding is defined as that binding displaced by 3μM phentolamine, and the data describes a single experiment performed in duplicate in each tissue.

INHIBITION OF SPECIFIC [^{3}H]-IDAZOXAN BINDING TO RAT HIPPOCAMPAL MEMBRANES

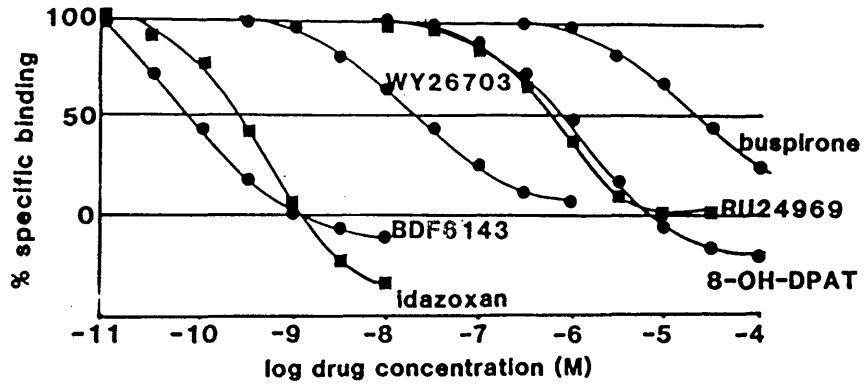


Fig. 6.14 Inhibition of specific binding of [^{3}H]-idazoxan binding to rat hippocampal membranes by idazoxan, BDF6143, WY26703, 8-OH-DPAT, RU24969 and buspirone. Incubations were performed at approximately 1nM [^{3}H]-idazoxan for 30min at 25^{o}C in a Tris HCl buffer (50mM Tris HCl, 0.5mM EDTA; pH 7.4). Non-specific binding was defined using 3μM phentolamine.

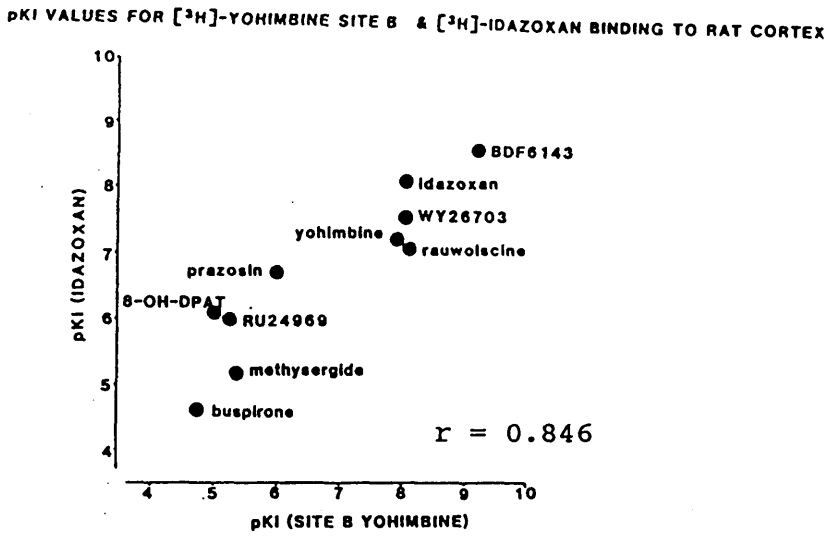


Fig. 6.15 Correlation between binding affinities of compounds for [^{3}H]-yohimbine site B and [^{3}H]-idazoxan binding to rat cerebrocortical membranes.

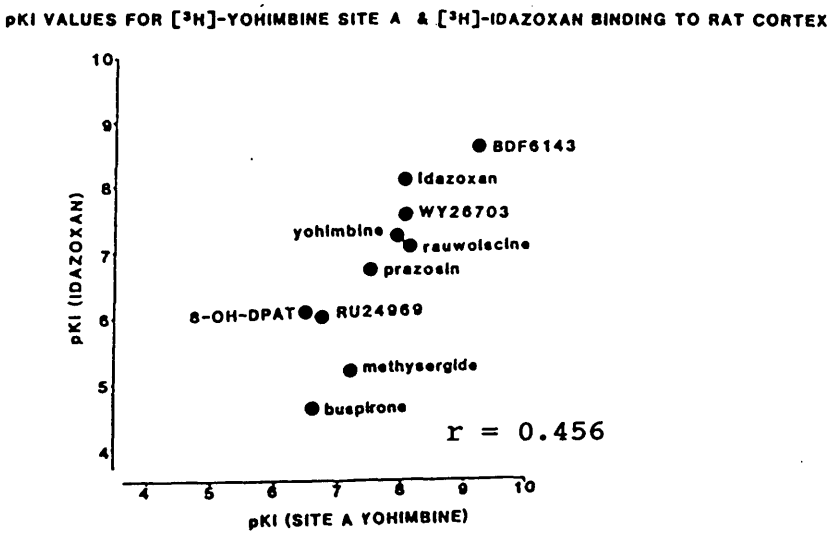


Fig. 6.16 Correlation between binding affinities of compounds for [^{3}H]-yohimbine site A and [^{3}H]-idazoxan binding to rat cerebrocortical membranes.

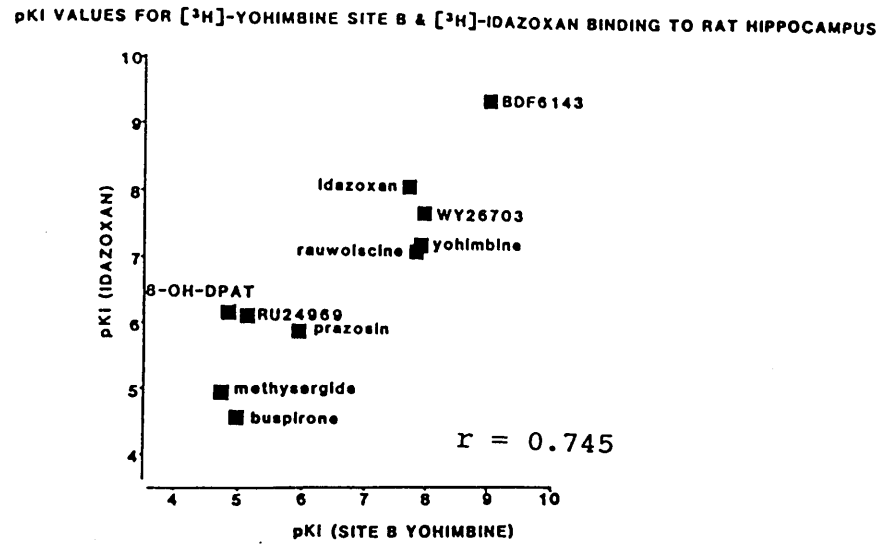


Fig. 6.17 Correlation between binding affinities of compounds for [^{3}H]-yohimbine site B and [^{3}H]-idazoxan binding to rat hippocampal membranes.

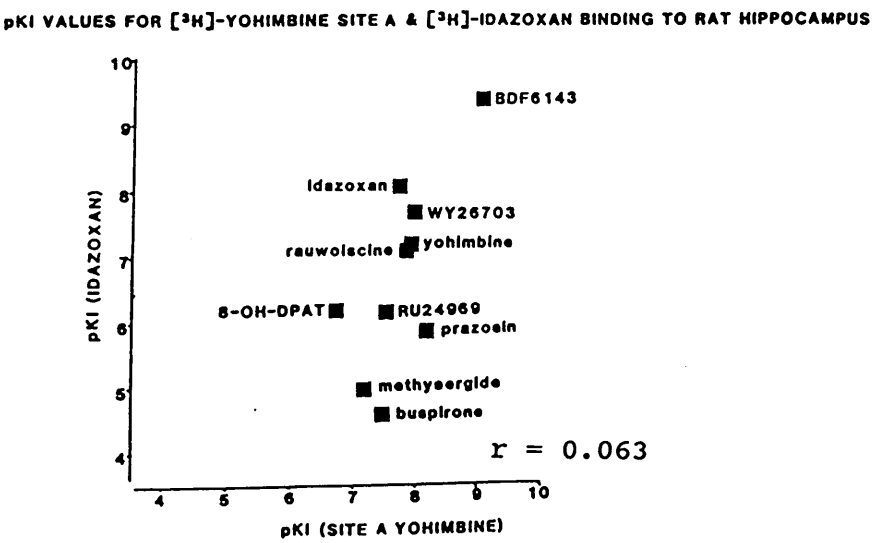


Fig. 6.18 Correlation between binding affinities of compounds for [^{3}H]-yohimbine site A and [^{3}H]-idazoxan binding to rat hippocampal membranes.

Table 6.1 Affinity values for alpha-adrenergic and 5HT binding sites on rat cortex

	α_1	α_2	$5HT_{1A}$	$5HT_2$
		Ki (nM)		
5HT antagonists				
ketanserin	18 ± 1.3	1259 ± 105	900 ± 60	2 ± 0.9
methysergide	186 ± 35	447 ± 76	125 ± 16	10 ± 0.7
cyproheptadine	175 ± 42	501 ± 77	1000 ± 75	6 ± 0.7
mianserin	89 ± 14	63 ± 9	1995 ± 115	11 ± 0.9
Dopamine antagonists				
spiperone	35 ± 10	355 ± 51	180 ± 30	1.6 ± 1
α-Adrenoceptor antagonists				
prazosin	0.6 ± 0.08	389 ± 45*	>10000	1259 ± 85
phentolamine	7 ± 1.1	3 ± 0.8	>10000	1000 ± 80
yohimbine	398 ± 15	13 ± 1	125 ± 20	200 ± 25
imiloxan	>10000	79 ± 19	>10000	>10000
Agonists		**IC_{50} (nM)**		
noradrenaline	8912 ± 980*	758 ± 83*	>10000	>10000
adrenaline	4467 ± 340*	178 ± 46*	>10000	>10000
5HT	>10000	>10000	20 ± 2	501 ± 36
dopamine	>10000	>10000	>10000	>10000
histamine	>10000	>10000	>10000	>10000

The affinity values were obtained for specific binding of [^{3}H]-prazosin to rat cerebral cortex (alpha$_1$), [^{3}H]-yohimbine binding to rat cerebral cortex (alpha$_2$), [^{3}H]-5HT binding to rat cerebral cortex ($5HT_{1A}$) and [^{3}H]-ketanserin binding to rat frontal cortex ($5HT_2$). The concentration of drug to inhibit binding by 50% was obtained graphically and converted to a Ki value when the hill slopes were not significantly different from unity by the Cheng Prusoff equation. Values shown are the mean ± s.e.m. of at least three experiments performed in duplicate.

*nH<1

Table 6.2 Affinity values for α_2-adrenergic and $5HT_{1A}$ binding sites in rat hippocampus

compound	pIC_{50} α_2	pIC_{50} $5HT_{1A}$
yohimbine	7.9 ± 0.06	6.9 ± 0.2
rauwolscine	7.9 ± 0.09	7.1 ± 0.09
WY26703	8.05 ± 0.07	6.5 ± 0.2
idazoxan	7.65 ± 0.1	5.5 ± 0.3
imiloxan	7.20 ± 0.1	<5
MJ 13653	7.0 ± 0.2	<5
buspirone	6.85* ± 0.09	7.95 ± 0.1
8-OH-DPAT	6.80* ± 0.09	8.52 ± 0.07
TFMPP	6.5* ± 0.2	6.90 ± 0.2
methysergide	7.02* ± 0.1	7.15 ± 0.1
RU24969	7.14* ± 0.1	8.01 ± 0.08
5CT	5.8	9.13 ± 0.1
DP-5CT	<5	9.25 ± 0.1

The affinity values were obtained for specific binding of [^{3}H]-yohimbine ($alpha_2$) and [^{3}H]-8-OH-DPAT ($5HT_{1A}$) to rat hippocampal membranes. The results are presented as IC_{50} values corrected of free ligand concentration as some of the compounds showed Hill slopes <1 (*). Values shown are the mean ±s.e.m. of at least three experiments performed in duplicate.

Table 6.3 Inhibition of [^{3}H]-yohimbine binding to rat cortex

	Ki (nM)		selectivity ratio A/B	% A:B
	site A	site B		
Yohimbine	12.6 ± 0.9			
Rauwolscine	7.1 ± 1.3			
WY26703	9.1 ± 1.6			
Idazoxan	9.1 ± 0.9			
Imiloxan	79.4 ± 19			
BDF6143	0.6 ± 0.1			
Prazosin	50 ± 14	999 ± 320	20	50:50
WB4101	57.5 ± 11			
Indoramin	8128 ± 540			
Oxymetazoline	120 ± 40	5.6 ± 5.3	0.05	40:60
5HT	>100,000			
Buspirone	269 ± 43	19498 ± 1200	72	40:60
8-OH-DPAT	371 ± 34	10715 ± 898	29	40:60
RU24969	199 ± 26	6309 ± 721	32	40:60
Methysergide	67.6 ± 14	4467 ± 1203	66	40:60

The affinity values were obtained for specific [^{3}H]-yohimbine binding to rat cortex. Experiments were performed using 1-2nM [^{3}H]-yohimbine and the concentration of drug that inhibited specific binding by 50% (IC_{50}) was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. Values shown are the mean Ki ±s.e.m. of at least three experiment performed in duplicate. Results were evaluated by computer-assisted curve fitting procedures described under methods. A two site fit was accepted only if it was statistically better than a one site fit. Determination of the affinity for site B was also carried out in the presence of $1x10^{-7}$ M prazosin to distinguish site B from site A.

Table 6.4 Inhibition of [^{3}H]-yohimbine binding to rat hippocampus

	Ki (nM) site A	Ki (nM) site B	selectivity ratio A/B	% A:B
Yohimbine	13.4 ± 2.3			
Rauwolscine	13.8 ± 1.8			
WY26703	11.5 ± 1.9			
Idazoxan	21.4 ± 3.1			
Imiloxan	457 ± 56			
BDF6143	1.1 ± 0.1			
Prazosin	7.9 ± 12	1148 ± 221	145	50:50
WB4101	57.5 ± 11			
Indoramin	10233 ± 2100			
Oxymetazoline	1380 ± 173	13.7 ± 3.9	0.1	31:69
5HT	51286			
Buspirone	39.8 ± 7	12303 ± 1360	309	30:70
8-OH-DPAT	209 ± 42	16281 ± 2500	78	30:70
RU24969	31.6 ± 11	7413 ± 1250	234	40:60
Methysergide	70.7 ± 8	20417 ± 1555	288	40:60

The affinity values were obtained for specific [^{3}H]-yohimbine binding to rat hippocampus. Experiments were performed using 1-2nM [^{3}H]-yohimbine and the concentration of drug that inhibited specific binding by 50% (IC_{50}) was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. Values shown are the mean Ki ±s.e.m. of at least three experiment performed in duplicate. Results were evaluated by computer-assisted curve fitting procedures described in methods. A two site fit was accepted only if it was significantly better than a one site fit. Determination of the affinity for site B was also carried out in the presence of $1x10^{-7}$ M prazosin to distinguish site B from site A.

Table 6.5 Inhibition of [^{3}H]-Yohimbine binding to Human platelets

	Ki (nM)	nH
Yohimbine	0.79 ± 0.14	1.03
Rauwolscine	1.04 ± 0.07	1.08
Idazoxan	4.98 ± 0.10	0.94
Imiloxan	366 ± 36	0.99
BDF6143	1.09 ± 0.15	1.00
Prazosin	871 ± 145	0.93
WB4101	4.0 ± 0.4	1.01
Indoramin	1259 ± 89	1.11
Oxymetazoline	3.9 ± 0.3	1.03
5HT	>50000	-
Buspirone	603 ± 93	0.94
8-OH-DPAT	217 ± 32	0.97
RU24969	76 ± 16	0.97
Methysergide	89 ± 23	0.95

The affinity values were obtained for specific [^{3}H]-yohimbine binding to human platelets. Experiments were performed using 1-2nM [^{3}H]-yohimbine and the concentration of drug that inhibited specific binding by 50% (IC_{50}) was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] represents the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. Values shown are the mean Ki ±s.e.m. of at least three experiment performed in duplicate. The hill slopes were also determined and were not significantly different from unity.

Table 6.6 Effect of 5HT in vitro on rat hippocampal α_2-adrenoceptor binding sites

	Kd nM	Bmax fmol/mg protein
control	5.8 ± 0.7	72 ± 6
+ 1nM 5HT	5.6 ± 0.8	75 ± 8
+ 10nM 5HT	5.4 ± 1.0	73 ± 10

Scatchard analysis were performed as described under methods, in the absence or presence of 1nM or 10nM 5HT. The results represent the mean ± s.e.m of at least three experiments performed in duplicate. No significant difference was found between the groups

Table 6.7 Efect of GTP on affinity values for α_2-adrenoceptor binding sites in the rat hippocampus

	-GTP	+GTP
adrenaline	7.12 ± 0.05	5.83 ± 0.06**
noradrenaline	6.65 ± 0.07	5.60 ± 0.05**
prazosin	6.9 ± 0.1	6.85 ± 0.1
8-OH-DPAT	6.8 ± 0.09	6.7 ± 0.08
RU24969	7.14 ± 0.1	7.22 ± 0.06
methysergide	7.02 ± 0.1	6.9 ± 0.1
buspirone	6.85 ± 0.1	6.26 ± 0.06*

The affinity values were obtained for specific [^{3}H]-yohimbine binding to rat hippocampus in the presence or absence of 0.1mM GTP. The results are presented as IC_{50} values corrected of free ligand concentration as some of the compounds showed Hill slopes <1 (*). Values shown are the mean ±s.e.m. of at least three experiments performed in duplicate.
Statistical significance (*p<0.01; **P<0.001) from the corresponding value determined in the absence of GTP.

Table 6.8 The effect of pretreatment with NEM or PGL on α_2-adrenoceptor binding sites

	Kd nM	Bmax fmol/mg protein
RAT CORTEX		
control	5.2 ± 0.9	121 ± 10
+ 1 mM NEM	5.1 ± 0.7	90 ± 8
+ 10 mM NEM	No appreciable binding	
+ 1 mM PGL	5.3 ± 0.7	64 ± 10*
RAT HIPPOCAMPUS		
control	5.8 ± 0.7	72 ± 6
+ 1 mM NEM	6.4 ± 1.0	74 ± 5
+ 10 mM NEM	7.1 ± 1.2	35 ± 5*
+ 1 mM PGL	6.7 ± 1.0	36 ± 10*

Scatchard analysis were performed as described under methods, with membranes for 20min at 37°C in the absence or presence of N-ethylmaleimide (NEM) or phenylglyoxal (PGL). The results represent the mean ± s.e.m of at least three experiments performed in duplicate.

**Statistical significance ($p<0.05$) from the corresponding value determined in the absence of NEM or PGL.

Table 6.9 Inhibition of [^{3}H]-Idazoxan binding

compound	Ki (nM) Cortex	Hippocampus
Yohimbine	56.2 ± 10	51.7 ± 8
Rauwolscine	81.3 ± 13	69.5 ± 12
WY26703	29.5 ± 0.8	19.9 ± 1.7
Idazoxan	7.6 ± 0.8	2.0 ± 0.2
BDF6143	2.2 ± 0.1	0.3 ± 0.05
Prazosin	195 ± 32	1259 ± 354
Buspirone	25119 ± 900	19952 ± 260
8-OH-DPAT	851 ± 165	931 ± 98
RU24969	955 ± 81	903 ± 150
Methysergide	7413 ± 890	11000 ± 700

The affinity values were obtained for specific [^{3}H]-idazoxan binding to rat cortex and hippocampus. Experiments were performed using approximately 2nM [^{3}H]-idazoxan and the concentration of drug that inhibited specific binding by 50% (IC_{50}) was obtained graphically and converted to a Ki value using the relationship $Ki = IC_{50}/(1+[L]/Kd)$, where [L] representes the free ligand concentration and Kd = the equilibrium dissociation constant of the radioligand. Values shown are the mean Ki ±s.e.m. of at least three experiment performed in duplicate. The hill slopes were also determined and were not significantly different from unity.

Table 6.10 Specific binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan to human platelets, rat cerebral cortex and rat hippocampus membranes

	[^{3}H]-yohimbine		[^{3}H]-idazoxan	
	Kd	Bmax	Kd	Bmax
Platelets	0.66 ± 0.07	152 ± 10	-	-
Cortex	5.2 ± 0.9	121 ± 10	4.1 ± 0.9	87 ± 8
Hippocampus	5.8 ± 0.7	72 ± 6	3.5 ± 0.5	36 ± 6

Specific binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan to human platelets, rat cortex and rat hippocampus membranes. Values shown were obtained using the non-linear itterative curve programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.m. of at least three experiments.

Table 6.11 Agonist affinity values for [^{3}H]-yohimbine binding sites

	pIC$_{50}$ (nH)					
	Control		1x10^{-7} M idazoxan (site A)		1x10^{-7} M prazosin (site B)	
	-GTP	+GTP	-GTP	+GTP	-GTP	+GTP
NA	6.81 (0.47)	6.12(1.02)	5.73	<5	6.58(0.8)	5.95(1.2)
UK*	7.63 (0.82)	7.53(0.97)	<5	<5	7.89(1.14)	7.85(1.1)
5HT	<5	<5	<5	<5	<5	<5

Affinity values for agonists were determined in the presence or absence of 0.1mM GTP for [^{3}H]-yohimbine binding. Assays were performed in the absence or presence of 1x10^{-7} idazoxan and 1x10^{-7} prazosin to distinquish the two populations of [^{3}H]-yohimbine binding sites. Values shown are the pIC$_{50}$ (nH) of at least 3 experiments performed in duplicate. All standard errors fall within 5% of the mean.

*UK represents UK 14304

6.3 Discussion

These results provide further evidence for heterogeneity of α_2-adrenoceptor binding sites. Differences were observed in the pharmacological profile of α_2-adrenoceptors in the rat brain and human platelet, which is consistent with a previous report suggesting a possible heterogeneity of α_2-adrenoceptors binding sites in the human platelet and rat cortex (Cheung et al, 1982). The non-rodent and rodent α_2-adrenoceptors have been classified as α-2A and α-2B respectively (α-2A yohombine=rauwolscine >>> prazosin; α-2B rauwolscine>yohimbine>prazosin, Nahorski et al, 1985). Bylund, (1985) extended this classification to include oxymetazoline which shows higher affinity for the rodent than the non-rodent subtype. However, a comparison of rat brain and human platelet α_2-adrenceptors must now take into account the possible heterogenous nature of α_2-adrenoceptors in the rat cortex and hippocampus as shown by the biphasic curves for prazosin, oxymetazoline (Bylund, 1985; this study) and 8-OH-DPAT, RU24969 and methysergide (this study). The human platelet appears to consist of a single population of α_2-adrenoceptors (Cheung et al, 1982; this study) whereas the human brain has been shown to consist of a heterogeneous population of α_2-adrenoceptors (Petrash and Bylund, 1986).

[^{3}H]-Yohimbine labels more than one α_2-adrenoceptor subtype in the rat brain. However, neither subtype can be immediately classified as an α-2A subtype similar to that seen in the human platelet since the affinity of yohimbine and rauwolscine at both of the sites is 10 fold less than for the platelet α-2A subtype. It is also difficult to use selectivity of yohimbine/rauwolscine as an index for classification of the two subtypes, as in the original classification (Nahorski et al, 1985), because a less than 10 fold difference in affinity for the two sites is not sufficient to significantly resolve the data from saturation or competition assays.

Prazosin and oxymetazoline were found to inhibit the binding of [^{3}H]-yohimbine in a biphasic manner (site A:B) in the cortex (40:60) and hippocampus (30:70). Furthermore the affinities of prazosin for its lower affinity site (site B) was similar to that found in the human platelet, i.e. the α-2A subtype. Similar affinity values have been reported for prazosin at the α-2A subtype described in rat cortex (Bylund, 1985) human brain (Petrash and Bylund, 1986) and human platelet (Cheung et al, 1982).

In this study a number of other compounds, methysergide, RU24969, 8-OH-DPAT and buspirone were found to inhibit [^{3}H]-yohimbine binding in a biphasic manner which could be statistically best fitted to a two site model. However, in this case the higher affinity site (site A) for these compounds was found to be similar to the platelet α-2A subtype.

An alternative explanation for the two site nature of these compounds is that they act as agonists at one or both of the α_2-adrenoceptor binding sites. However 0.1mM GTP failed to alter the inhibition curves of these compounds, except for buspirone, while in parallel experiments, GTP decreased the affinity of noradrenaline by 5 fold for site A and 2 fold for site B. These results indicate that agonist activity of these compounds does not account for the two site inhibition data and therefore supports the conclusion of receptor subtypes. Bylund, (1985) concluded that the biphasic inhibition shown by prazosin in the cortex and hippocampus could not be explained by negative cooperativity or different affinity states of a single receptor.

The pharmacological profile of both sites indicates that it is most unlikely (under these assay conditions) that there is a significant interaction of [^{3}H]-yohimbine with other receptor systems. Broadhurst and Wyllie, (1986) suggested that [^{3}H]-rauwolscine labels a saturable, low affinity, spiperone-sensitive site on rat cortical membranes which they postulate is a $5HT_2$ binding site. A similar low affinity component has been suggested for [^{3}H]-yohimbine binding (Michel and Whiting, 1984). They

concluded that it most resembled a low affinity α_2-adrenoceptor binding site. However, the later study was carried out using concentrations in excess of 20nM to label the low affinity site. In the present study competition experiments were carried out using 1.5nM [^{3}H]-yohimbine, therefore it is unlikely that any of the binding was to the low affinity site.

The site showing high affinity for 8-OH-DPAT, RU24969, methysergide and buspirone is not related to the 5HT$_{1A}$ receptor binding site since all these compounds have >10 fold higher affinity for the 5HT$_{1A}$ binding site. 5HT, 5CT and DP-5CT were inactive at this site, whereas all show nanomolar affinity for the 5HT$_{1A}$ binding site. Similarly Petrash and Bylund, (1986) showed that the α_2-adrenoceptor subtype with very high affinity for prazosin is not related to the α_1-adrenoceptor which has 250 times higher affinity for prazosin.

[^{3}H]-Yohimbine did not label a 5HT binding site, at concentrations up to 15nM in the rat brain. Interestingly yohimbine has been shown to have high affinity (50nM) for the 5HT$_{1D}$ binding site of human and bovine brain membranes (Hoyer et al, 1987a; Heuring and Peroutka, 1987), and it is possible to speculate that low affinity [^{3}H]-yohimbine binding, especially in these tissues may be related to a 5HT binding site (see chapter 3). However, no direct labelling of a 5HT$_1$ binding site was found in the present study and no evidence of a 5HT$_{1A}$/α_2-adrenergic receptor/receptor interaction was found in the hippocampus where both are present. 5HT had no modulatory effect on the α_2-adrenoceptor binding in the hippocampus, although the unusually high affinity of buspirone for a binding site labelled by [^{3}H]-yohimbine in the hippocampus, which was significantly higher than in the cortex, merits further investigation. NA has been shown to act as a modulator of 5HT release in the rat hippocampus (Feuerstein et al, 1985).

No evidence of an atypical α_2-adrenoceptor in the hippocampus was found, except perhaps the high affinity of buspirone. No variation in the position or accessibility of

the chemical moieties, arginine and cysteine, between the calf cortex, human platlet and human uterus (Matten et al, 1984), or rat cortex and rat hippocampus (this study) was seen. Jacobs et al, (1982) showed that α_2-adrenoceptors in the human platelet are not sensitive to dithiothreitol (DTT), which suggests the lack of essential disulphide bonds in the binding unlike the β-adrenoceptor which does contain an essential disulphide bond (Guellaen and Hanoune, 1979). Similarly Mckernan et al (1986) showed DTT to be ineffective against solubilized rabbit spleen and human spleen α_2-adrenoceptors. The effect of DTT in the rat brain should therefore be investigated. The same group were unable to show any differences between human and rabbit spleen α_2-adrenoceptors using sulfhydryl agents. However they did find some differences in the carbohydrate portion of the receptor. Therefore the study of physiochemical properties of the receptors could be a useful method for investigating possible receptor heterogeneity.

Further investigations of α_2-adrenoceptor heterogeneity were undertaken in this study using [^{3}H]-idazoxan. Initial studies in rat cortex and hippocampus membranes showed that the Ki values for displacing [^{3}H]-yohimbine or [^{3}H]-idazoxan binding differed between the yohimbine-like and imidazoline α_2-adrenoceptor antagonists. The yohimbine-like compounds showed higher affinity for [^{3}H]-yohimbine binding whereas the imidazolines showed higher affinity for [^{3}H]-idazoxan binding which suggests differences in the α_2-adrenoceptor populations labelled by the two radioligands.

The density of α_2-adrenoceptor binding sites in the rat cortex reported in the literature is wide (90-242fmol/mg protein) as a result of different incubation media and of membrane preparations being purified to different extents. For comparison of the binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan in this thesis identical methodology was used for preparation of tissue membranes and binding assays. Under the present conditions (crude membrane preparation and Tris HCl buffer system: 50mM Tris HCl, 0.5mM EDTA; pH

7.4 at 25°C) the specific binding of the α_2-adrenoceptor antagonists [^{3}H]-yohimbine (1-15nM) and [^{3}H]-idazoxan (1-12nM) to membranes from rat cerebrum and human platelets was rapid, reversible, saturable and of high affinity. Extensive washing of the membrane preparation with EDTA was used to remove endogenous Mg^{2+} and other divalent cations which can reduce the affinity of the radioligand and complicate analysis of displacement curves generated by competing drugs (Cheung et al, 1982).

The α_2-adrenoceptor has been shown to exist in different affinity states modulated by the presence of mono- and divalent cations and guanine nucleotides. ^{3}H-Agonist radioligands are affected whereas ^{3}H-antagonist binding is generally little affected by these modulators. Lane et al (1983) suggested that the binding of the antagonist [^{3}H]-idazoxan is markedly increased in the presence of a high concentration of sodium ions (physiological buffer containing 119.2mM Na^+). However, they used a crude synaptosomal P2-fraction prepared in sucrose followed by resuspension in physiological buffer. Apparent maximal [^{3}H]-yohimbine binding to cortex membranes prepared in a similar manner is also increased in the presence of Na^+ ions (Woodcock and Murley, 1982). Further investigation of this membrane preparation (Cheung et al, 1984) revealed it to be comprised of a high proportion of intact synaptosomes containing endogenous NA, which could interfere with the subsequent α_2-adrenoceptor binding assay; these effects could be reversed by Na^+ ions. This is not as marked with membranes prepared in hypotonic buffers.

In this study [^{3}H]-idazoxan binding showed a slight but not significant increase when assays were performed using a physiological buffer, it is tempting to speculate that a high Na^+ concentration favours binding to an imidazoline binding site (see below).

Early models of α_2-adrenoceptor binding suggested the antagonists yohimbine and rauwolscine selectively bind to the free receptor, thus destabilizing receptor-guanine

nucleotide protein complexes; this is based on the modulatory effects of buffer ions and GTP on ^{3}H-antagonist binding (Bylund and U'Prichard, 1983; Woodcock and Murley, 1982; Salama et al, 1982). In terms of binding kinetics this would predict that there are two affinity components of [^{3}H]-yohimbine and [^{3}H]-rauwolscine binding. However, as described above, this is most probably related to the insufficiently washed membrane preparation. Further evidence against this hypothesis has come from studies of solubilised α2-adrenoceptor preparations, which by definition must contain the free receptor protein, the affinity values for yohimbine and rauwolscine are equal to their affinity for membrane preparations (Kawahara and Bylund, 1985). Therefore the two binding sites labelled by [^{3}H]-yohimbine in this study shown by the biphasis curves to prazosin, methysergide, 8-OH-DPAT and RU24969, is unlikely to be due to different affinity states. It remains to be seen if they act at an allosteric binding site.

Under the assay conditions described for this study, Ki values for [^{3}H]-idazoxan binding to the rat cortex and hippocampus were consistent with binding to an α_2-adrenoceptor binding site and were similar to those described for a rat P2 cerebral membrane (Lane et al, 1983). All the competing antagonists showed monophasic inhibition with Hill slopes not significantly different from unity, including prazosin, oxymetazoline, methysergide, 8-OH-DPAT and RU24969, which all showed low affinity. Saturation studies showed that [^{3}H]-idazoxan labelled fewer binding sites in the rat cortex and hippocampus. Since neither of these two compounds have been reported to have α_2-adrenoceptor agonist activity, the total binding (Bmax) provides evidence of a difference in the population of α_2-adrenoceptors labelled by the two ligands. As discussed above a number of compounds showed biphasic inhibition curves for [^{3}H]-yohimbine binding. The affinities of these compounds for inhibition of [^{3}H]-idazoxan binding showed a good correlation with [^{3}H]-yohimbine site B. Similarly in the rabbit cerebral

cortex [^{3}H]-yohimbine has been reported to label almost twice as many binding sites as does [^{3}H]-idazoxan, and both ligands are reported to label an α_2-adrenoceptor binding site (Hamilton et al, 1988).

In contrast recent autoradiographical and pharmacological evidence has been presented suggesting that [^{3}H]-idazoxan labels two distinct α_2-adrenoceptor populations in the rat brain with high affinity, only one of which is labelled by [^{3}H]-rauwolscine (Boyajian et al, 1987; Boyajian and Leslie, 1987). These studies were carried out using a Na^+/K^+ buffer system. One possibility which could account for the the large percentage of [^{3}H]-idazoxan binding is that, under these conditions, idazoxan binds preferentially to the proposed imidazoline site (Ernsberger et al, 1987). There is evidence from functional studies that many central actions of imidazoline α_2-adrenoceptor agonists, such as clonidine, may be mediated by an imidazoline receptor (Bousquet and Feldman, 1987). There is even some evidence of an endogenous imidazoline agonist, a clonidine displacement substance (Atlas et al, 1987).

The α_2-adrenoceptor binding site on human platelets labelled by [^{3}H]-yohimbine appeared to be a hybrid of the sites A and B described for rat brain membranes. Unfortunately binding of [^{3}H]-idazoxan to the human platelet was not able to resolve this because of the failure to achieve sufficient specific binding. This may be related to the procedures used resulting in degradation of either the ligand or the α_2-adrenoceptor binding site. However, this is unlikely since under identical conditions [^{3}H]-yohimbine binding was entirely consistent with binding to an α_2-adrenoceptor. McLaughlin et al, (1987) reported specific binding of [^{3}H]-idazoxan to human platelets to be saturable and to a single population of high affinity sites (Kd 1.43nM; Bmax 240fmol/mg protein). The assays were performed under similar conditions, platelet membranes were incubated with [^{3}H]-idazoxan (0.25-8nM) for 20min at 22^{o}C in a Tris EDTA buffer system (50mM Tris HCl, 0.5mM EDTA) at

pH 7.2. However, they do not indicate the level of nsb which was determined in the presence of 10μM idazoxan. In the present study idazoxan was found to displace nsb at this concentration.

In conclusion, evidence suggests that α_2-adrenoceptor binding sites are not homogeneous. [^{3}H]-Yohimbine labels two sites in the rat brain both of which have the overall profile of an α_2-adrenoceptor, although a number of significant differences between the two sites have arisen. Site B correlates with the binding of [^{3}H]-idazoxan, a similar pharmacological profile is seen for both sites; moderately high affinity of NA, high affinity for the α_2-adrenoceptor antagonists, yohimbine, rauwolscine and the imidazolines, idazoxan and imiloxan but low affinity for prazosin, 8-OH-DPAT, RU24969 and methysergide.

The second binding site labelled by [^{3}H]-yohimbine, site A, shows lower affinity for NA although this is still a higher affinity than for any other agonist tested. It also shows high affinity for α_2-adrenoceptor antagonists, yohimbine and rauwolscine. It is distinguished by high affinity of prazosin, 8-OH-DPAT, RU24969 and methysergide. The latter four compounds also show moderately high affinity for the α_2-adrenoceptor binding site on human platelets. Recently 8-OH-DPAT has been shown to be a post-synaptic α_2-adrenoceptor antagonist (Crist and Surprenant, 1987; PA2 6.9-7.2). Therefore this site also appears to be an α_2-adrenoceptor binding site.

In view of the differences in [^{3}H]-yohimbine and [^{3}H]-idazoxan binding it seems neither ligand should be considered the ligand of choice for the study of α_2-adrenoceptors.

Further investigations of the properties of α_2-adrenoceptor subtypes in both membrane and soluble populations are needed. It will also be important to establish whether the above differences in α_2-adrenoceptors are reflected by appropriate functional responses.

Chapter 7: GENERAL DISCUSSION

7. GENERAL DISCUSSION

In each of the preceding chapters a detailed discussion of the experimental results has been provided, and it is therefore the purpose of the general discussion to concentrate on the significance of the results and prospects for future research.

In this study a number of high affinity 5HT and α_2-adrenoceptor binding sites have been described, in different tissues and species. However, before claiming new receptor types two important questions have to be answered:- 1. When are high affinity binding sites sufficently different to suggest heterogeneity? 2. When can a high affinity binding site be called a receptor?

Classification of receptors on a pharmacological basis requires reliable methods for the quantitative assessment of agonist and antagonist affinities for putative receptor subtypes (Furchgott 1972, Kenakin, 1984). In radioligand binding studies the affinity constants of antagonists provide the critical evidence for receptor classification. However, binding studies on their own cannot be taken as the sole criterion for receptor classification. High affinity binding sites are usually considered to be receptors when it has been shown that the occupation of the site by an agonist elicits a pharmacological response, (e.g. contraction of a tissue) or biochemical response (e.g. increase in adenylate cyclase), and the pharmacological characteristics of the binding site relate to those of the response. However, if by definition a receptor is coupled to a response, then it is difficult to classify binding sites which represent 'receptor' sites that have become uncoupled from the response, either during development or evolution, but have the same properties as those coupled to a functional response.

Therefore one of the major difficulties in the early stages of any binding study is to know which binding sites actually represent receptors which lead to known effects on the cell. The use of a selective and high affinity

radioligand is the most important tool for ligand binding studies. Evidence of 5HT receptor subtypes in this study is drawn from a high degree of uniformity across many different tissues and species with respect to the binding affinity of the individual 5HT subtype radioligands. A number of 5HT receptors were studied, $5HT_{1A}$, $5HT_{1B}$, $5HT_{1C}$, ($5HT_{1D}$) and $5HT_2$ (where binding and functional studies were performed in the same tissue). For each of these there is literature evidence of second messengers and functional responses. In addition there is functional and preliminary binding evidence for a $5HT_3$ receptor, which was not studied in the thesis.

Although a receptor requires a response a fully characterised binding site showing a pharmacologically distinct profile, possibly with its own selective ligands, should not be ignored. Central $5HT_1$ binding sites were all defined before a functional correlate was available. Many potentially useful pharmacological systems with specific binding/receptor sites may await identification and therapeutic exploitation.

Evidence for heterogeneity must be carefully assessed as receptors which mediate the same response in the same way and respond similarly to known antagonists, may still be different in different cells or different tissues, in different environments or biochemical or pathological states, in different species or different individuals at different stages of development. After all, receptors are proteins made up of a number of subunits. Haemoglobin, used by A.J.Clark (1937) in his brilliant discussion of the reaction between proteins and drugs, has now been shown to consist of at least 7 tetramers as well as the normal adult α_2/β_2 haemoglobin. Under certain conditions some tetramers behave like models of functional receptors. Under other conditions they may bind a ligand without giving a response. Although haemoglobin is not a typical protein it suggests the possibility that all pharmacological receptors (proteins) of a particular 'type' may not be identical.

Molecular biology techniques which are increasingly

being used to study receptor heterogeneity, have shown that nicotinic receptors have different molecular weights and different, but homologous, amino acid sequences in different tissues and species (Conti-Tronconi et al, 1985; Momoi and Lennon, 1986).

In this study [^{3}H]-Yohimbine was found to label two binding sites in rat cortex and hippocampus with high affinity, both of which appear to be α_2-adrenoceptor-like showing high affinity for α_2-adrenoceptor antagonists (site A showed some affinity for NA whereas other endogenous agonists tested were inactive). A number of compounds (prazosin, methysergide, oxymetazoline, 8-OH-DPAT and RU24969) showed selectivity for the two sites. The heterogeneity was unlikely to be due to different affinity states as yohimbine has no agonist activity and GTP had no effect on the affinity of the selective compounds or other α_2-adrenoceptor antagonists. It remains to be seen if site A (high affinity for prazosin) is an allosteric binding site.

Further characterisation of the two sites must await the development of selective ligands. However, there are some leads. For example Site B correlates well with the [^{3}H]-idazoxan binding site on the rat cortex and rat hippocampus, since both of these ligands, yohimbine and idazoxan, are antagonists at the α_2-adrenoceptor autoreceptor, which may be a functional correlate for this site. However, one drawback of binding to crude membrane preparations, as used in these studies, is the diversity of the possible binding site locations and most of the binding in crude membrane preparations is to post-synaptic α_2-adrenoceptors as shown in lesioning studies (see Bylund and U'Prichard, 1983). A functional correlate for site A awaits further study. It is interesting to speculate that site A may be an α_2-heteroceptor perhaps on 5HT neurons, where an α_2-adrenoceptor mediating 5HT release has been proposed (Raiteri et al, 1983) or on other non-adrenergic neurons. The answers to these questions await the results of selective lesioning studies and release studies.

The strength of ligand binding lies in the ability to study the receptor directly, not only to determine the affinity of compounds but also to study the influence of modulators, e.g. nucleotides and metal ions, which have been implicated in functional studies as participants in the agonist-receptor interactions and in the subsequent reactions which couple the receptors to effector responses.

The binding of [^{3}H]-5HT was found to be complex and influenced by GTP and mono/divalent ions. However, the complex binding curves produced were best described by $5HT_1$ subtypes. [^{3}H]-8-OH-DPAT binding was also influenced by GTP and divalent ions; but the confirmation of multiple $5HT_{1A}$ affinity states must await the introduction of antagonist ligands. The reason for the complex interactions of agonist binding with the $5HT_2$ binding site are at present unknown. There is however evidence from binding studies to implicate the involvement of a guanine nucleotide binding protein in agonist interactions with the $5HT_2$ binding site in the rat brain. The involvement of a guanine nucleotide binding protein in the coupling of the $5HT_2$ binding site to its effector systems has been proposed (Berridge and Irvine 1984). Antagonist binding to $5HT_2$ binding site or α_2-adrenoceptor binding sites labelled by [^{3}H]-yohimbine was not influenced by GTP. In contrast guanine nucleotides have been shown to increase the affinity of some antagonists at D_2 receptors (Wreggett and De Lean, 1984) and β-adrenoceptor sites (Lang and Lemmer, 1985), which suggests that the affinity of certain antagonists can be influenced by receptors interacting with G proteins.

A useful technique for further studies of 5HT and α_2-adrenoceptor subtypes would be to perform binding assays on intact cells. Internalisation and other regulatory processes can also be studied using this technique. However, the use of intact systems in direct binding studies is not without its problems as has been highlighted in studies on the β-adrenoceptor (Pittman and Molinoff,

1980). In these studies a considerable degree of cellular accumulation of lipophilic radioligands, together with the identification of internalised or sequestered binding sites has been reported. While the resolution of these problems in studies on the β-adrenoceptor has been greatly facilitated by the availability of a suitable high affinity, hydrophilic radioligand, [^{3}H]-CGP-12177 (Staehelin and Hertal, 1983), the low affinity (particularly of α_2-radioligands) and high degree of lipophilicity (5HT and α_2-adrenoceptor radioligands) may necessitate the development of antagonist ligands of higher affinity and reduced lipophilicity before accurate studies on these receptors in intact cells can be performed successfully.

The current classification of 5HT receptors and α_2-adrenoceptors was both verified and extended in this thesis, by the study of different tissues and species. Information on receptor subclassification has been useful in the development of novel drugs which will in turn be useful in the treatment of a number of psychiatric disorders in which 5HT has been implicated, such as schizophrenia, anxiety and depression, where it now appears that rather than being independent of the NA, the two systems are probably linked. In addition these studies also showed that 5HT may have a role to play in cerebral ischaemia.

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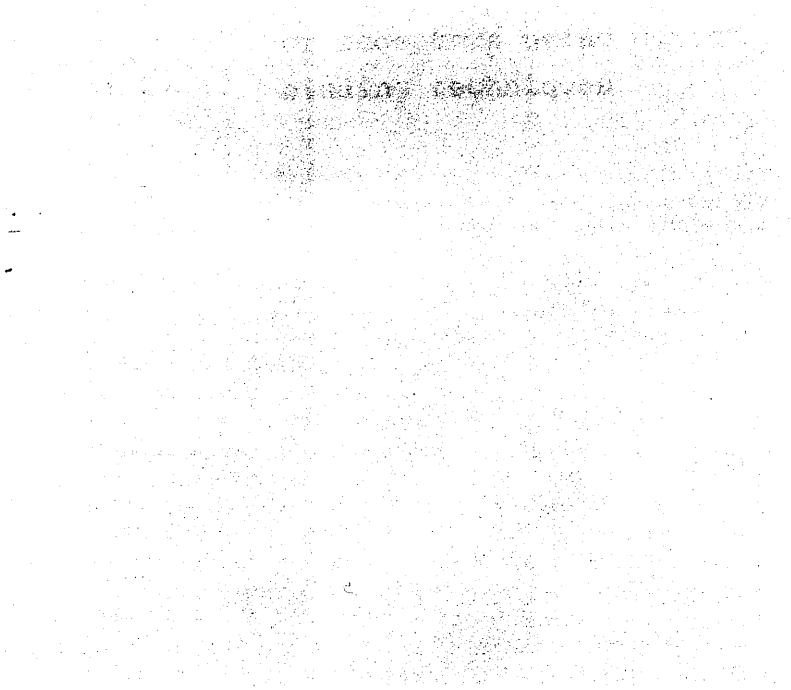


Appendix 1: Theoretical basis for identification of receptors using direct radioligand binding techniques

Appendix 1

Theoretical basis for identification of receptors using direct radioligand binding techniques

Ligand-receptor interactions may be expressed in terms of the following generalised reaction for the simplest case of a single ligand interacting with a homogeneous receptor population.

$$[R] + [L] \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} [RL] \qquad \text{equation 1}$$

Where [R], [L] and [RL] represent the equilibrium concentration of free receptor, free ligand and receptor ligand complex, respectively. K_1 and K_{-1} are rate constants describing the opposing association and dissociation reactions. The equilibrium binding constant of the radioligand for the receptor can be defined as either the association binding constant (Ka) or the dissociation binding constant (Kd).

$$\text{Association constant (Ka)} = \frac{K_1}{K_{-1}} = \frac{[RL]}{[R][L]} \qquad \text{equation 2}$$

$$\text{Dissociation constant (Kd)} = \frac{K_{-1}}{K_1} = \frac{[R][L]}{[RL]} \qquad \text{equation 3}$$

The Kd may be calculated either directly from equilibrium saturation binding experiments or indirectly by determination of the rate constants K_1 and K_{-1}.

Direct measurement of Kd and receptor number (Bmax)

Another property of ligand-receptor interactions is saturability. The maximun number of specific receptor sites (Bmax) is represented by the equation:-

$$[RL] + [R] = Bmax \qquad \text{equation 4}$$

multiple by [L]

$$[RL][L] + [R][L] = Bmax[L]$$

$$[RL][L] + \frac{[RL]}{[RL]}[R][L] = Bmax[L]$$

from equation 3

$$[RL]([L] + Kd) = Bmax[L]$$

$$[RL] = \frac{Bmax[L]}{[L] + Kd} \qquad \text{equation 5}$$

Define:- [L] = free ligand concentration, F

[RL] = concentration of bound ligand, B

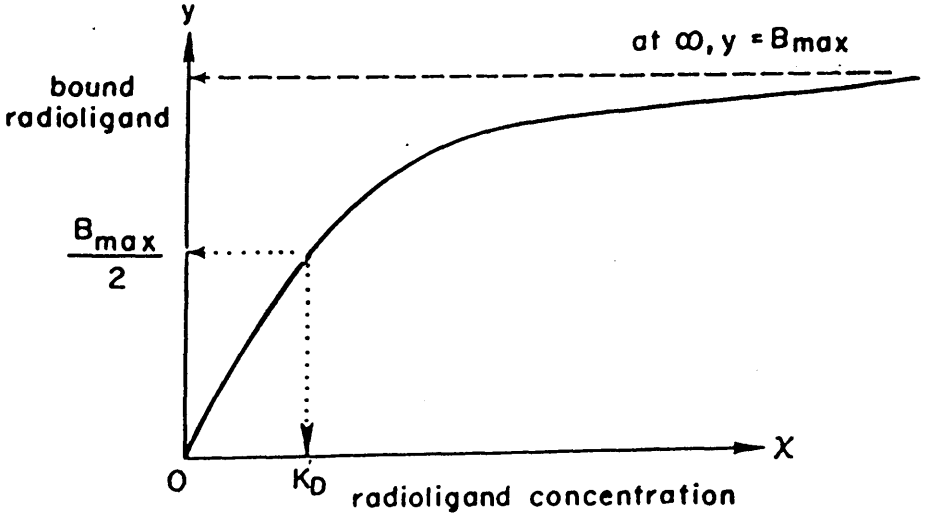
then equation 5 can be written as

$$B = \frac{BmaxF}{F + Kd} \qquad \text{equation 6}$$

This is the hyperbolic relationship of equation 1

$$y = \frac{ax}{b + x}$$

shown graphically:-



Kd = [L] leading to [B] = $\frac{Bmax}{2}$

Bmax is attained only at infinite [F]

The form of the Scatchard plot which is most frequently applied to the analysis of radioligand binding data is the plot of B/F on the ordinate versus B on the abscissa.
From equation 6

$$BF + BKd = BmaxF$$

Divide by F

$$B + B/FKd = Bmax$$

or

$$B/F = \frac{Bmax - B}{Kd} \qquad \text{equation 7}$$

$y = mx + c$ $\qquad B/F = -1/KdxB + Bmax/Kd$

The Hill plot is another transformation of saturation binding data, which is often used to determine the nature of more complex ligand-receptor interactions. The Hill equation (equation 6) can be transformed into a logarithmic form for convenience in plotting.

$$BxKd + B[F]^n = Bmax[F]^n$$

$$BxKd = (Bmax - B)[F]^n$$

$$\frac{B}{(Bmax-B)}Kd = [F]^n$$

$$\frac{B}{(Bmax-B)} = \frac{[F]^n}{Kd}$$

Taking log of both sides

$$\log B/(Bmax-B) = n\log[F] - \log Kd \qquad \text{equation 8}$$

A Hill plot is constructed where the y axis is $\log B/(Bmax-B)$ and the x axis is log [F]. The abscissa value when $\log B/(Bmax-B)=0$ is the affinity. This only equals the Kd value when the slope of the Hill plot, nH, is equal to 1. $nH>1$ indicates positive cooperativity. $nH<1$ indicates, negative cooperativity, multiple, non-interacting binding site or multiple interconvertible affinity states.

As shown above, when bound radioactivity represents binding to a saturable receptor population, pocessing a single affinity, Kd, for the ligand, the plot of bound versus free will yield a rectangular hyperbola. Consequently the determination of the binding parameters, Kd and Bmax, is complex. Numerous methods have been developed for analysis of experimental data. They fall into three main categories, graphical, linear regression analyses of transformed data and direct analysis using non-linear regression analyses.

Each of these methods has both its advantages and disadvantages. The graphical approach is highly predictive since most binding models are described in terms of their graphical appearance. However, this method of analysis is purely subjective with best fits been adjusted by eye and has no statistical basis. A second pseudo-graphical method is often employed in which data are transformed using a linearising transformation (eg Scatchard, 1949) thereby enabling a regression analysis by least squares to be performed, from which estimates of slope and intercept are easily obtained. This method also introduces a degree of statistical validity. The major disadvantage of these linear regression analyses on transformed data is that the transforms used are often prone to magnify errors (eg at very low concentrations of radioligand where accurate determination of bound is difficult). Insuffient saturation concentrations of free radioligand may lead to inaccuate extrapolation to determine Bmax. Because of the inequalities in variance of the measured varibles complicated weighting functions often have to be introduced. For a linear transformation to mainfest a single slope a single ligand must be interacting in a fully reversible manner with a homogeneous population of non-interacting sites. Deviations from linearity occur when complex models of binding operate.

The use of non-linear regression analysis has proved highly sucessful when applied to the analysis of ligand binding data, having widespread applicability. This method can be used to analyse binding of one or more ligands to

several species of interacting or non-interacting sites.

Affinity values for the receptor can also be determined in competition studies. A plot of bound on the y axis versus log [drug] on the x axis (a log scale is used as saturability is easier to obtain for cold drugs), can be used to assess the the concentration of drug inhibiting 50% of the specific binding (IC_{50}). As described above the nH coefficient indicates the complexity of the reaction. The equilibrium dissociation constant for the competitor can be determined from the IC_{50} using the Cheng Prusoff equation, if nH=1. (see data analysis section 2.1.1k).

Determination of association rate constant

From equation 1 it can be seen that the association of a ligand with a receptor is a second order (bimolecular) reaction. The simple rate equation for bimolecular association is

$$\frac{d[RL]}{dt} = Ka[L][R]$$

when intergrated

$$Ka = \frac{2.303}{t[L-R]} \log \frac{R[L-x]}{L[R-x]} \qquad \text{equation 9}$$

with R and L equal to the unoccupied receptor and free ligand respectively, at time t_o, and x is the amount of consumed L and R at time t. Theoretically equation 9 enables the calculation of Ka for any bimolecular reaction when L, R, and x are known. In practice bound ligand is assayed at various time intervals up to the steady-state level (B_{eq}). $Ln[B_{eq}/(B_{eq}-Bt)]$ is plotted versus time, where B_t is the amount of specifically bound ligand at time t. The slope of the line (K_{obs}) is calculated, from which K_1 can be derived using equation 10.

$$K_1 = \frac{K_{obs} - K_{-1}}{[L]} \qquad \text{equation 10}$$

However, estimation of K_1 by this method requires knowledge of the dissociation rate constant.

Determination of dissociation rate constant

The half life ($t_{1/2}$) for loss of specifically bound radioligand is determined from a plot of $\log[B]/[B]_o$ versus time, where [B] is the bound at any time t and $[B]_o$ is bound at time =0. The K_{-1} is calculated as $0.693/t_{1/2}$. Alternatively, the slope of log[B] versus time multiplied by -2.303 yields K_{-1}.

When binding of L to R represents binding to a single class of receptors binding L in a reversible fashion with a fixed affinity, then it is expected that the Kd determined from saturation experiments should be equivalent to the Kd calculated from kinetic data.

$$Kd = \frac{K_{-1}\ min^{-1}}{K_1\ min^{-1}\ M^{-1}} = Molar$$