

**MODULATION OF THE SEROTONIN TRANSPORTER AND  
RECEPTORS BY ANTIDEPRESSANTS AND ECSTASY**

by

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others

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## Abstract

The serotonergic (5-hydroxytryptamine, 5-HT) system, has been implicated in a variety of pathological conditions including schizophrenia, Parkinson's disease and depression. The 5-HT transporter (SERT) is responsible for the synaptic clearance of 5-HT in the central nervous system and the accumulation of 5-HT by platelets. SERT is a molecular target for clinically effective antidepressants and for the popular recreational drug of abuse, methylenedioxymethamphetamine (MDMA, Ecstasy). Serotonin specific reuptake inhibitors (SSRIs), bind to and inhibit SERT causing an increase in synaptic 5-HT as an initial response to relieving depression. However the nature of the link between the initial actions of these drugs *in vitro* and the weeks of treatment required for clinical improvements remains unresolved. Neuroadaptive changes not only of SERT, but also of the 5-HT receptors known to regulate 5-HT neurotransmission (5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors), may account for this delayed onset of action. Unlike antidepressants, MDMA is a substrate for SERT and can also affect 5-HT neurotransmission. In contrast to antidepressants, MDMA is selectively neurotoxic to serotonergic nerve terminals in both animals and humans.

In this thesis the effects of chronic antidepressant treatments and MDMA sub-chronic treatments on the density and affinity of the 5-HT transporter and 5-HT receptors in rats is investigated, using radioligand binding and immunological techniques. The influence of potentially neuroprotective drugs on the effects of MDMA is also examined.

A [<sup>3</sup>H]citalopram binding assay has been used to label SERT in rat brain, blood platelets and adrenal medulla. The pharmacology of these binding sites are identical. A library of six SERT site directed antibodies have been characterised. A monoclonal antibody which specifically recognises denatured SERT and displays the appropriate immunohistochemical labelling, also immunoprecipitates SERT and specifically recognises the native form from rat neocortex and platelets. SERT protein has also been specifically detected in the rat adrenal medulla using this antibody. A [<sup>3</sup>H]nisoxetine binding assay has been used to label the noradrenaline transporter (NET) in both the rat brain and adrenal medulla revealing an identical pharmacology.

A [<sup>3</sup>H]5-CT binding assay has been developed in the presence of receptor specific drugs to mask particular 5-HT receptor subtypes to reveal a pharmacology consistent with binding to 5-HT<sub>7</sub> receptors. A [<sup>3</sup>H]GR125,743 binding assay has been developed to reveal a pharmacology consistent with binding to a combination of 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors.

After chronic SSRI (citalopram or fluoxetine) and tianeptine (an atypical antidepressant) treatments, adaptive changes of SERT were not observed in rat brain or adrenal medulla. No such changes were also observed for 5-HT<sub>1A</sub> receptors in the brain. However decreases in 5-HT<sub>7</sub>, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptor density were observed in rat frontal neocortex after chronic SSRI (citalopram or fluoxetine) but not tianeptine treatments.

Repeated MDMA administration causes similar depletions in the number of SERT binding sites in both the brain and adrenal gland, measured 2 weeks after the final dose of MDMA. FK506, which has been proposed as a neuroprotective drug, prevents this MDMA induced SERT reduction in the brain, but does not protect adrenal SERT depletion. The MDMA induced depletion of SERT in the brain is still apparent 13 weeks after the final dose of MDMA, but not in the adrenal glands. In the brain, MDMA induced SERT depletion is also prevented by a free radical scavenger.

This thesis shows that chronic dosing with SSRIs involves site specific adaptive changes of 5-HT<sub>1B/1D</sub> nerve terminal autoreceptors and 5-HT<sub>7</sub> receptors suggesting a potential role of these receptors in the mechanism of action of these antidepressant drugs. The sensitivity of adrenal chromaffin cell SERT to MDMA is similar to brain SERT, but may involve a different mechanism and length of action. In the brain this MDMA induced depletion of SERT is likely to involve the formation of free radicals and mechanisms blocked by the immunosuppressive drug FK506. The characterisation of an anti-SERT antibody capable of native SERT recognition will be a useful tool for future studies.

### **Publications arising from the thesis**

Wren, P.B., Sharkey, J., McCarter, J.F.M., Kelly, P.A.T., Kelly, J.S. & Olverman, H.J. (1998). MDMA-induced depletion of 5-HT transporters in the rat adrenal medulla. *Proc.4th IUPHAR Satellite meeting on Serotonin*, PP61.

Wren, P.B., Lawrence, J.A., Kelly, J.S. & Olverman, H.J. (1998). Chronic fluoxetine or tianeptine treatments do not alter the density of the 5-HT transporter. *Eur.J.Neurosci.*, **10**, 351P.

Wren, P.B., Burns, N.J., Kelly, P.A.T., Lawrence, J.A., Olverman H.J. & Williams, B.C. (1997). Pharmacological characterisation of the 5-HT transporter in the rat adrenal medulla. *Br.J.Pharmacol.*, **122**, 209P.

Lawrence, J.A., Butcher, S.B., Wren, P.B., Kelly, J.S. & Olverman H.J. (1997). Effect of chronic tianeptine treatment on the rat 5-HT transporter. *Br.J.Pharmacol.*, **122**, 302P.

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## **CHAPTER 1**

### **INTRODUCTION**

The studies described in this thesis were undertaken to determine the potential modulatory role of clinically used antidepressant drugs and the recreational drug of abuse, MDMA (Ecstasy) on the 5-hydroxytryptamine (5-HT; Serotonin) system, with particular emphasis on the 5-HT transporter (SERT) and the 5-HT<sub>1A</sub>, 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors. This introduction firstly reviews the discovery, biochemistry and anatomy of the 5-HT nervous system. Following a review of 5-HT receptor nomenclature, the structural, operational and pharmacological characteristics of the transporter and individual receptor subtypes are described. The involvement of the 5-HT system in clinical depression is outlined, highlighting the reported characteristics of various classes of antidepressant drugs. In the final section the consequences of MDMA exposure are outlined.

## 1.1 Discovery of 5-HT

The existence of an endogenous serum factor causing vasoconstriction (Brodie, 1900), and the presence of a substance, termed enteramine, which was abundant in the enterochromaffin cells of the gut, and caused smooth muscle contraction (Vialli & Erspamer, 1933) were the first descriptions of 5-HT. The vasoconstrictor substance was purified from serum, named serotonin (Rappaport *et al.*, 1947) and identified as 5-hydroxytryptamine (Rappaport *et al.*, 1948; Rappaport, 1949). Enteramine and 5-HT were subsequently shown to be chemically and biologically identical (Erspamer & Asero, 1952). The presence of 5-HT in the mammalian brain was established (Twarog & Page, 1953; Amin *et al.*, 1954), following the development of a sensitive bioassay in the isolated heart of *Venus mercenaria*, one of several mollusc species where 5-HT potently excites the heart (Erspamer & Ghiretti, 1951). It was recognised that several alkaloids including the ergot derivative, lysergic acid diethylamide (LSD), showed structural similarities to 5-HT, and that these compounds antagonised the contractile effects of 5-HT on vascular smooth muscle in experimental animals (Shaw & Wolley, 1953). Several of the compounds caused 'mental aberrations' in man, including hallucinations and a transient state resembling schizophrenia (Woolley & Shaw, 1954a). Consequently, Woolley & Shaw (1954a & b) suggested that 5-HT may be a chemical mediator in the brain, being involved in

the maintenance of normal mental processes, and that interference with actions of this compound may contribute to the production of some mental disorders.

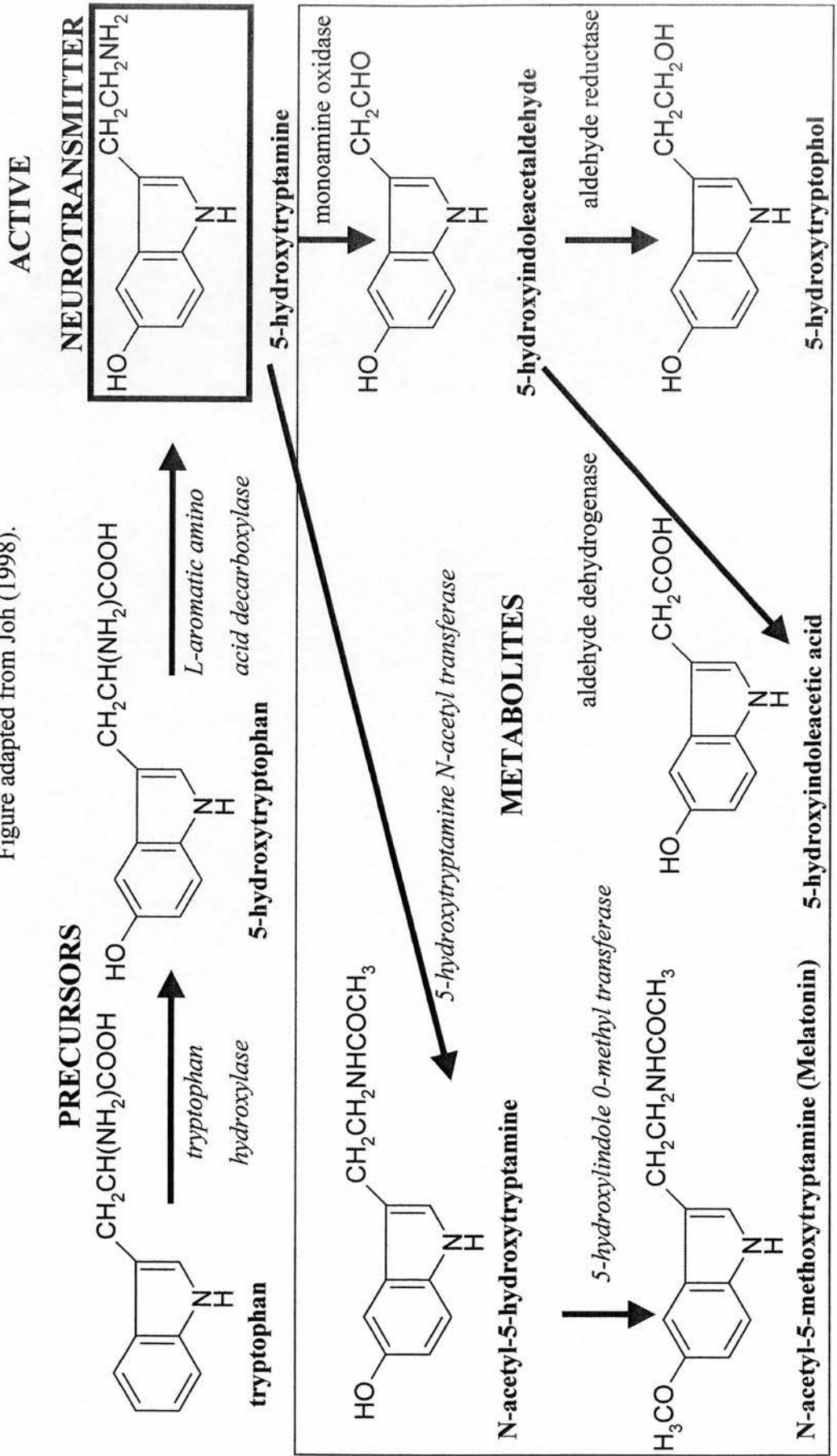
## 1.2 Biosynthesis and Metabolism of 5-HT

The synthesis of 5-HT (Figure 1.1) from the essential dietary amino acid L-tryptophan and its subsequent metabolism is well documented and has been extensively reviewed elsewhere as a two step process (see Bosnin, 1978). Availability of tryptophan is believed to be the rate-limiting step in 5-HT synthesis (Grahame-Smith, 1964). Following neuronal uptake via a neutral amino acid carrier at the blood-brain barrier (Blasberg & Lajtha, 1966), the 5-position of the indole ring of tryptophan is hydroxylated by the cytoplasmic enzyme, tryptophan hydroxylase, using O<sub>2</sub>, reduced pteridin and NADPH as cofactors (Joh *et al.*, 1975). The resulting 5-hydroxytryptophan is rapidly decarboxylated by non-specific L-aromatic amino acid decarboxylase, forming 5-hydroxytryptamine (Clark *et al.*, 1954). Whilst the first reaction is unique to serotonergic neurons, L-aromatic amino acid decarboxylase also decarboxylates L-DOPA in catecholaminergic neurons leading to the synthesis of dopamine and noradrenaline.

In most cells in the central nervous system (CNS), 5-HT is metabolised by monoamine oxidase (MAO; Weissbach *et al.*, 1961), which is located on the outer membrane of synaptic terminal mitochondria (Schnaitman & Greenawalt, 1968). MAO-A, one of two isoforms of this enzyme is clorgyline sensitive (Johnston, 1968), and oxidatively deaminates 5-HT forming the intermediate 5-hydroxyindole acetaldehyde. This intermediate is further metabolised by NAD<sup>+</sup>-sensitive aldehyde dehydrogenase, forming the major metabolite 5-hydroxyindole acetic acid (5-HIAA; Duncan & Sourkes, 1974). In addition, 5-hydroxyindole acetaldehyde can be metabolised by NADPH-sensitive aldehyde reductase, forming 5-hydroxytryptophanol (Eccleston *et al.*, 1966). However this metabolite represents only 1% of 5-HIAA levels in rat brain (Cheifez & Warsh, 1980). 5-HT in areas such as the pineal gland and in the retina, however, can be further metabolised to form the active substance melatonin via N-acetyl serotonin (Feldstein & Williamson, 1968; Klein & Weller, 1970; Klein *et al.*, 1971).

**Figure 1.1: Biosynthesis and Metabolism of 5-HT.**

Figure adapted from Joh (1998).



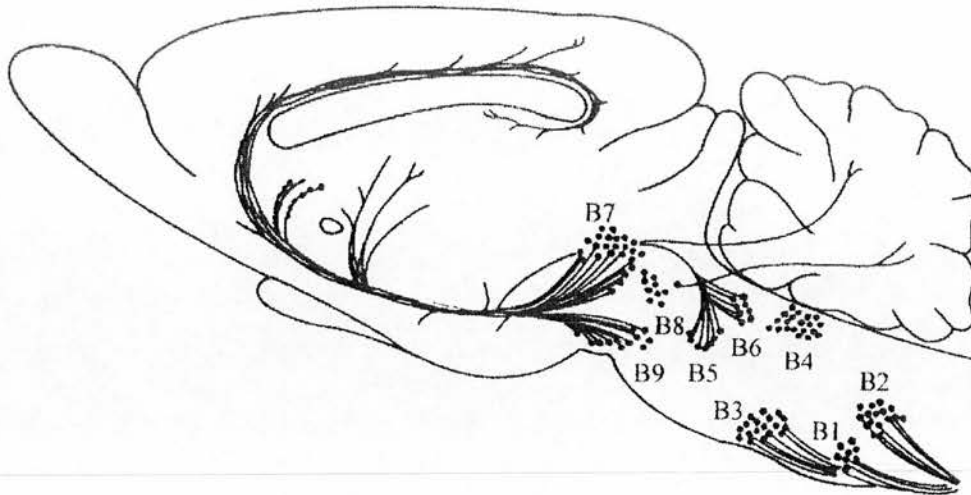


### 1.3 Anatomy of 5-HT Pathways in the CNS

The central or midline (raphé) location of the large neurons in the brain stem has attracted anatomists since the time of Ramon y Cajal (1911), who described these cells as large multipolar neurons with uncertain projections. The discrete localisation of 5-HT in the CNS was achieved using a modified method of the histochemical formaldehyde-induced fluorescence technique described by Falck *et al.*, (1962). Using this technique, the localisation of the cell bodies and axon terminals of rat brain neurons containing 5-HT were identified (Dahlstrom & Fuxe, 1964 & 1965; Fuxe, 1965). The original classification designated the central 5-HT-containing cell body fluorescent clusters into nine distinct cell groups (B1-B9; Dahlstrom & Fuxe, 1964) predominantly in the raphé region of the midbrain (Figure 1.2). In further work, these same workers localised the 5-HT-containing nerve terminals (Dahlstrom & Fuxe, 1965; Fuxe, 1965), thus delineating the basic anatomy of the 5-HT neuronal system. To enable the path of 5-HT neurones to be traced in serial sections of rat brain, mechanical hemisections and electrocoagulations were carried out in order to increase the amount of neurotransmitter caudal to the lesion, enabling 5-HT axonal visualisation (Ungerstedt, 1971). This work showed that the 5-HT system consisted of two distinct subdivisions, with the most caudal 5-HT cell groups in the medulla (B1-B3) giving rise to axons which descend to innervate the ventral and dorsal horns and the lateral column of the spinal cord, whilst axons arising from cell bodies in the dorsal and median raphé nuclei project to the forebrain (Figure 1.2). Neuronal 5-HT has been more precisely localised by autoradiography (Aghajanian & Bloom, 1967; Descarries *et al.*, 1975; Azmitia & Segal, 1978). The detailed mapping of the serotonergic system was finally established immunohistochemically, using a highly specific polyclonal antiserum directed against 5-HT (Steinbusch *et al.*, 1978; Steinbusch, 1981).

#### **Ascending (Rostral) System**

The rostral part of the 5-HT system has cell bodies in the caudal linear nucleus and the dorsal and the median raphé nuclei (Törk, 1990), corresponding to cell groups B6-B9 (Dahlstrom & Fuxe, 1964). These ascending projections are extensive and innervate diverse regions of the cerebral cortex, limbic system, basal



**Figure 1.2: Schematic Illustration of Major 5-HT Pathways.**

The cell groups B1-B9 represent those described by Dahlstrom & Fuxe (1964) and correspond to the raphé nuclei (B1-nucleus raphé pallidus; B2-nucleus raphé obscurus; B3-nucleus raphé magnus; B4-nucleus raphé obscurus (dorsolateral); B5-median raphé nucleus (caudal); B6-dorsal raphé nucleus (caudal); B7-dorsal raphé nucleus (rostral); B8-caudal linear nucleus and nucleus raphé pontis; B9-nucleus raphé pontis and suprallemniscal region). Projections from B1-B3 descend to innervate the spinal cord, whilst B5-B9 innervate the forebrain. (Adapted from Cooper *et al.*, *The Biochemical Basis of Neuropharmacology*, 1982).

ganglia and diencephalon (Steinbusch, 1981). In rats there is evidence for two distinct populations of fibres in the cortex (Kohler *et al.*, 1980), which differ in their morphology, nucleus of origin and distribution (Kosofsky & Molliver, 1987). The D fibres arising from the dorsal raphé nucleus have very fine axons (<1µm diameter) with minute irregularly spaced varicosities that branch frequently (Kosofsky & Molliver, 1987; Molliver, 1987; Mamounas & Molliver, 1988; Mulligan & Törk, 1988). These axons project to associative and limbic cortical areas concentrated within layers III-IV, and to subcortical forebrain nuclei including the basal ganglia, amygdala, septum and in periventricular areas of the hypothalamus and thalamus (Azmitia & Segal, 1978; Jacobs & Azmitia, 1992; Baumgarten & Grozdanovic, 1994). The M fibres arising from the median raphé nucleus have large non-varicose axons (up to 2µm in diameter) giving rise to infrequent branches of fine fibres with large spherical varicosities and a beaded appearance (Kosofsky & Molliver, 1987; Molliver, 1987; Mamounas & Molliver, 1988; Mulligan & Törk, 1988). These fibres project to the basal olfactory forebrain areas, septal complex, hypothalamic nuclei, hippocampal formation and many neo- and allocortical areas (predominantly layers I and II; Azmitia & Segal, 1978; Jacobs & Azmitia, 1992; Baumgarten & Grozdanovic, 1994). The difference in morphology between the two populations of fibres in the ascending serotonergic system has been implicated in their relative susceptibility to chemical destruction, whereby the fine dorsal raphé fibres are more prone to destruction by drugs such as *p*-chloroamphetamine (Mamounas & Molliver, 1988) and MDMA (Ecstasy; O'Hearn *et al.*, 1988).

### **Descending (Caudal) System**

Dahlstrom & Fuxe (1965) described the projection of axons from 5-HT cell bodies in the medulla (B1-B3; Dahlstrom & Fuxe, 1964) via a ventromedial pathway to the ventral horn and a lateral pathway to the central grey area of spinal cord as reported in monkeys (Azmitia & Gannon, 1986). 5-HT like immunoreactivity has been demonstrated throughout the spinal grey area, with highest densities in laminae I-IIa of the dorsal horn, laminae VIII and IX of the ventral horn and in the intermediolateral column of the thoracic cord (Bowker *et al.*, 1982). Combining this immunohistochemical method with the retrograde cell marker horseradish peroxidase, the major serotonergic input to the spinal cord was identified as being

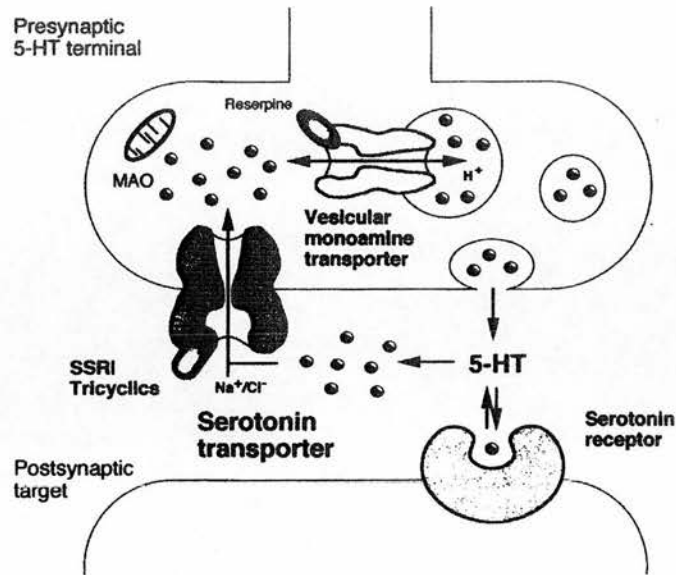
from cell groups B1-3, with groups B5, B7 and B9 having a smaller input to cervical regions of the cord (Bowker *et al.*, 1982). Serotonergic endings from the nucleus raphé magnus make direct contact with the neurons in the dorsal horn that give rise to the spinothalamic tract (Ruda *et al.*, 1982; Hylden *et al.*, 1986).

#### 1.4 5-HT Neurotransmission

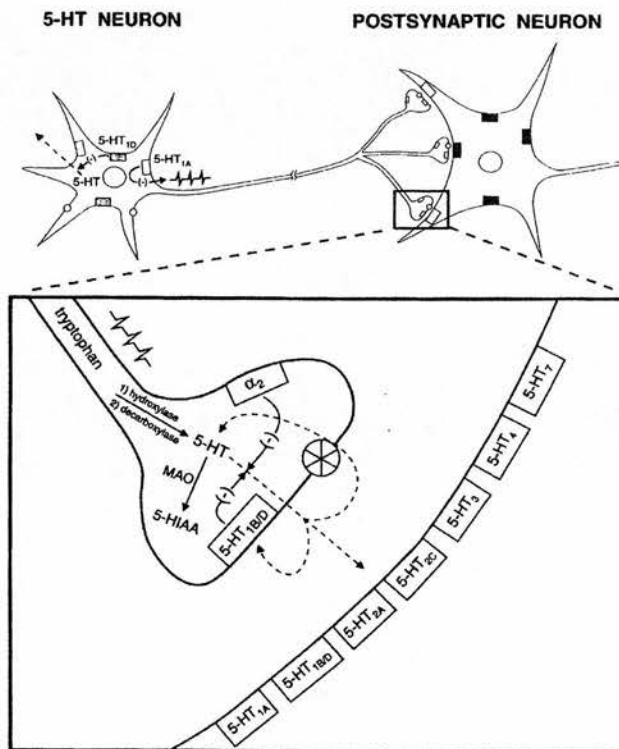
Figure 1.3a depicts a typical serotonergic synapse showing the 5-HT nerve terminal in close proximity to the post-synaptic target neuron. Following the neuronal synthesis of the neurotransmitter, 5-HT is taken up by the vesicular monoamine transporter and stored in nerve terminal vesicles with a specific 5-HT binding protein (Tamir & Gershon, 1990). Upon arrival of an action potential the resultant nerve terminal depolarisation causes the opening of voltage-sensitive calcium channels and subsequent  $\text{Ca}^{2+}$  entry. Secretory vesicles already docked to the plasma membrane release their contents by exocytosis initiated by this increase in intracellular  $\text{Ca}^{2+}$ . For those secretory granules that are fixed to the cytoskeleton,  $\text{Ca}^{2+}$ -calmodulin/cAMP-dependent phosphorylation of  $\text{Ca}^{2+}$ -sensitive vesicle membrane proteins, such as synapsin I, allows the docking of the granules to the plasma membrane and release of the neurotransmitter (see Burgoyne & Cheek, 1995). The mechanism of exocytosis is not however the only mechanism that can account for neurotransmitter release. One other postulated mechanism for the release of 5-HT by 5-HT releasing drugs such as addictive amphetamines, is a carrier mediated-release process. Unlike exocytosis, carrier mediated-release is  $\text{Na}^+$ , but not  $\text{Ca}^{2+}$  dependent, does not rely on a vesicular but on a cytoplasmic pool of neurotransmitter, is not modulated by pre-synaptic receptors, and is blocked by uptake inhibitors (Rudnick & Wall, 1992; Levi & Raiteri, 1993).

The main autoregulatory effect of 5-HT on its own release is inhibitory. In Figure 1.3b, 5-HT released from both the terminal and the somatodendritic area has a profound influence on its own neurotransmission such that the amount of neurotransmitter released is subject to receptor-dependent regulation. 5-HT autoreceptors located on the cell bodies and dendrites of serotonergic neurons, i.e. the somatodendritic 5-HT autoreceptors, can be activated by 5-HT released from the somatodendritic area or recurrent branches of the serotonergic axon. This may be

(a)



(b)



**Figure 1.3: Schematic Representations of 5-HT Neurotransmission**

(a) represents a serotonergic synapse from Lesch, 1998. (b) represents pre-synaptic and post-synaptic factors regulating the effectiveness of 5-HT neurotransmission from Blier & De Montigny, 1998.

of the same neuron or from axons of other serotonergic neurons innervating the cell bodies and dendrites of the neuron under consideration. According to the location of these receptors, their stimulation induces a decrease in neuronal firing (5-HT<sub>1A</sub> receptors; see section 1.5.2) or in somatodendritic release (5-HT<sub>1D</sub> receptors; see section 1.5.2). 5-HT<sub>1B/1D</sub> autoreceptors are also known to be located on serotonergic nerve terminals, whose stimulation results in the inhibition of release of 5-HT from the nerve terminal (see section 1.5.2). 5-HT<sub>1A</sub> receptor stimulation is known to reduce a high threshold Ca<sup>2+</sup> current (Penington & Kelly, 1990; Penington *et al.*, 1991; Penington & Fox, 1994) and to increase conductance to potassium ions (Aghajanian & Lakoski, 1984). On the otherhand, stimulation of 5-HT<sub>1B/1D</sub> receptors is known to inhibit adenylate cyclase with subsequent inhibition of cAMP. Apart from these 5-HT autoreceptors,  $\alpha_2$ -adrenergic heteroreceptors located on 5-HT nerve terminals also inhibit 5-HT release (Figure 1.3b; Maura *et al.*, 1982). All these factors can contribute to a net decrease in 5-HT neurotransmission.

Once in the synaptic cleft, 5-HT interacts with a number of post-synaptic receptors causing a cascade of events through their second messenger coupling resulting in either excitatory or inhibitory post-synaptic potentials. For example, intracellular studies in brain slices have demonstrated directly that 5-HT can be both inhibitory by 5-HT<sub>1A</sub> receptor activation and excitatory via 5-HT<sub>2A</sub> receptor activation upon the same cortical pyramidal cells (see Araneda & Andrade, 1991). 5-HT neurotransmission is terminated prior to the next round of action potentials, by active clearance of the neurotransmitter back into the pre-synaptic neuron by a high-affinity 5-HT transporter located on the pre-synaptic membrane (Figure 1.3; see section 1.6.2). 5-HT is then either accumulated into the synaptic vesicles by the vesicular monoamine transporter (see section 1.6.2) or is inactivated by monoamine oxidase (MAO). MAO exists as two isoforms (MAO-A and MAO-B; Benedetti & Dostert, 1992). Dopamine and tyramine are non-specific substrates for both forms. 5-HT and noradrenaline are preferentially metabolised by MAO-A, whereas the B form has a substrate preference for phenylethylamine and benzylamine. Within the nerve terminal, MAO-A regulates the concentration of free 5-HT by metabolising 5-HT to its inactive metabolite 5-HIAA.

Taken as a whole, 5-HT neurotransmission is therefore highly regulated. It can be influenced by not only drugs which affect the activation of various autoreceptors and heteroreceptors on serotonergic neurons, but also by drugs that block 5-HT re-uptake (serotonin specific re-uptake inhibitors, SSRIs), vesicular transport (e.g. reserpine) or monoamine oxidase activity.

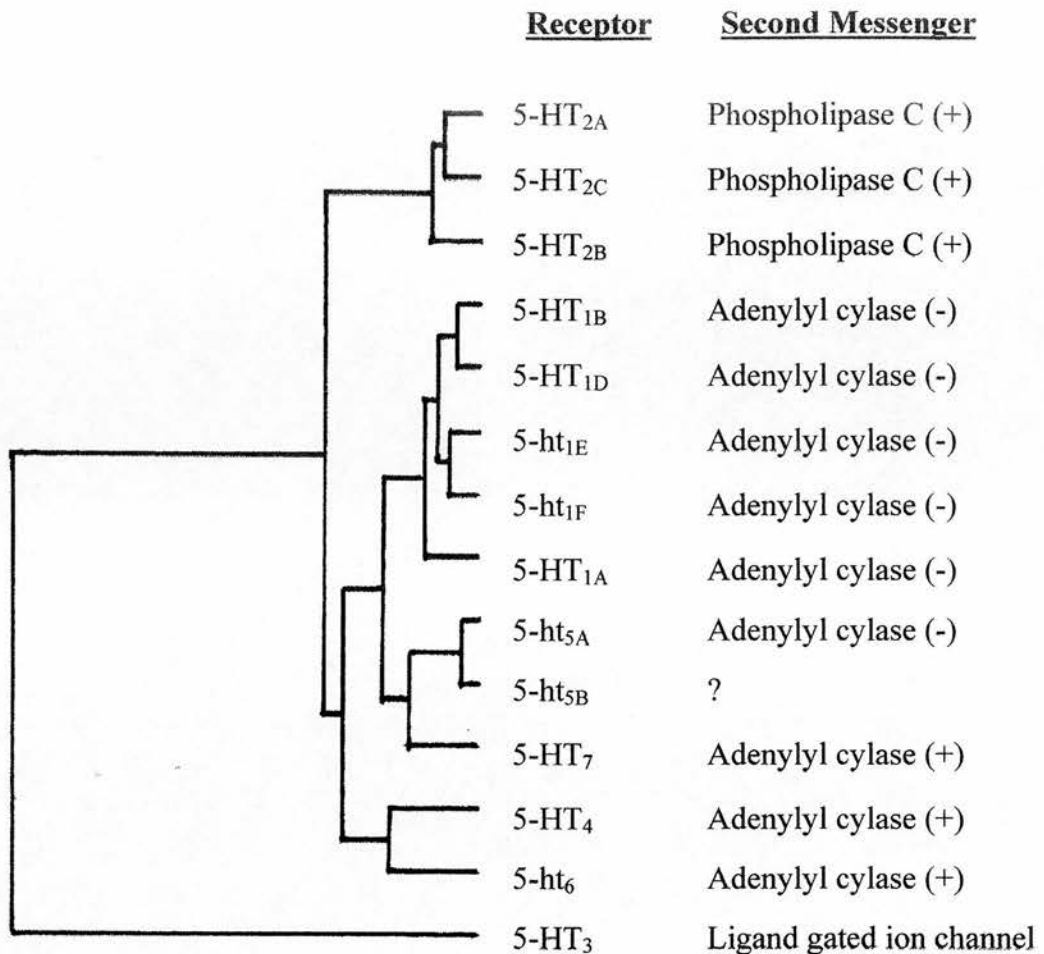
## **1.5 Classification of 5-HT Receptors**

5-HT exerts its actions via specific interactions with cell surface receptors. Currently seven 5-HT receptor gene families giving rise to a total of 14 receptor subtypes have been identified using molecular cloning techniques. Analysis of primary amino acid sequences has enabled receptor subtypes to be grouped based on the degree of amino acid sequence homology (Figure 1.4), whereby each member of the same receptor gene family share common structural, operational and transductional characteristics (Hoyer & Martin, 1997). At the molecular level it has been established, mainly using recombinant receptor models and hydropathy profiles, that 5-HT receptors are seven putative transmembrane spanning G-protein coupled metabotropic receptors (Figure 1.5). However an exception to this is the 5-HT<sub>3</sub> receptor which is a ligand-gated ion channel.

### **1.5.1 A Historical Perspective**

The current system of 5-HT receptor classification and nomenclature is summarised in Table 1.1 (Hoyer & Martin, 1997) and is in accordance with the guidelines outlined by the International Union of Pharmacology (IUPHAR) receptor nomenclature committee (Vanhoutte *et al.*, 1996; Humphrey & Barnard, 1998). This section deals with a historical perspective leading up to this current classification.

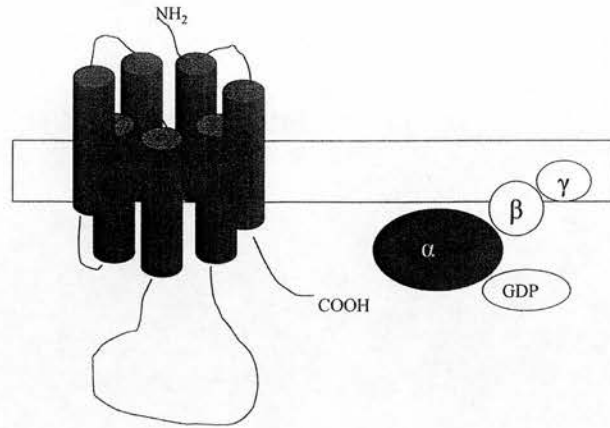
Multiple 5-HT receptor subtypes were first evident after two receptors involved in the 5-HT-induced contractile response in guinea-pig ileum were described (Gaddum & Picarelli, 1957). One of these receptors could be antagonised by morphine, termed the M receptor. The other receptor could be antagonised by dibenzylamine (phenoxybenzamine) termed the D receptor. The development of radioligand binding techniques provided the main tool to show the existence of multiple 5-HT receptor subtypes. Initial experiments demonstrated that [<sup>3</sup>H]spiroperidol (spiperone) labelled 5-HT binding sites (Leysen *et al.*, 1978). It was



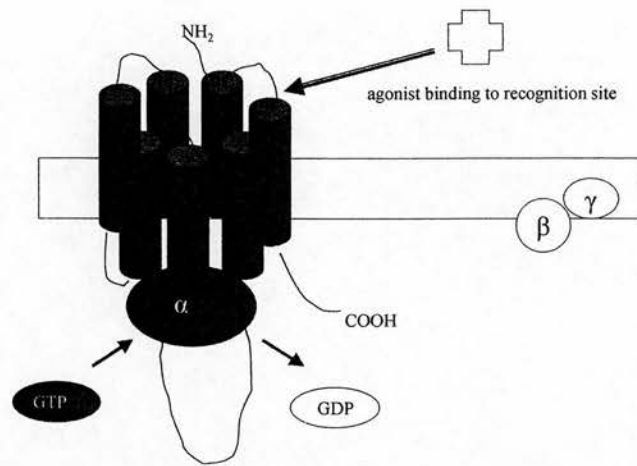
**Figure 1.4: Dendrogram Showing the Evolutionary Relationship and Second Messenger Systems Between Human 5-HT Receptor Subtypes**

5-ht<sub>5A</sub> and 5-ht<sub>5B</sub> represent 5-HT receptors which are murine in origin. (+) represents stimulation whereas (-) represents inhibition and ? represents unknown signal transduction pathway. The lower case appellation for receptors lacking a well defined physiological role is denoted for the appropriate receptor subtypes according to recent nomenclature guidelines (Hoyer & Martin, 1997). Figure taken and adapted from Barnes & Sharp (1999).





(a) Resting State



(b) Activated State

### Figure 1.5: Schematic Representation of G-Protein Coupling

The G-protein consists of three subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). In the resting state the G-protein exists as an unattached  $\alpha\beta\gamma$  trimer, with GDP occupying the site on the  $\alpha$  subunit. On receptor occupation by an agonist ligand, the receptor acquires high affinity for the trimer through conformational changes in the cytoplasmic regions (mainly large loop structures between transmembrane domains) of the seven transmembrane receptor structure. This causes the bound GDP to dissociate and to be replaced by GTP which causes dissociation of  $\alpha$  GTP from the  $\beta\gamma$  subunits. This  $\alpha$  GTP diffuses in the membrane to activate a variety of second messenger systems (targets) and is controlled by the existence of a variety of G-proteins determined by variations in the  $\alpha$  subunit. The process is terminated when the hydrolysis of GTP to GDP occurs through the GTPase activity of the  $\alpha$  subunit. The resulting  $\alpha$  GDP dissociates from the effector, and reunites with the  $\beta\gamma$  subunits, completing the cycle. G protein coupling and its regulation is reviewed in Birnbaumer *et al.*, (1990), Simon *et al.*, (1991) and Birnbaumer (1992).

Current Nomenclature	Former Nomenclature	Reference
5-HT <sub>1A</sub>	5-HT <sub>1A</sub> 5-HT <sub>1α</sub>	Pedigo <i>et al.</i> , 1981 Hartig, 1989
5-HT <sub>1B</sub>	Human 5-HT <sub>1Dβ</sub> Rat 5-HT <sub>1B</sub> Human 5-HT <sub>1B</sub> Rat 5-HT <sub>1B</sub>	Weinshank <i>et al.</i> , 1992 Pedigo <i>et al.</i> , 1981 Hartig <i>et al.</i> , 1996 Hartig <i>et al.</i> , 1996
5-HT <sub>1D</sub>	5-HT <sub>1Dα</sub> Human 5-HT <sub>1D</sub> Rat 5-HT <sub>1D</sub>	Weinshank <i>et al.</i> , 1992 Hartig <i>et al.</i> , 1996 Hartig <i>et al.</i> , 1996
5-ht <sub>1E</sub>	5-HT <sub>1E</sub>	Leonhardt <i>et al.</i> , 1989
5-ht <sub>1F</sub>	5-HT <sub>1Eβ</sub> 5-HT <sub>1F</sub>	Amlaiky <i>et al.</i> , 1992 Adham <i>et al.</i> , 1993a & b
5-HT <sub>2A</sub>	D S <sub>2</sub> 5-HT <sub>2</sub> 5-HT <sub>2</sub> 5-HT <sub>2α</sub>	Gaddum & Picarelli, 1957 Leysen & Laduron, 1977 Peroutka & Snyder, 1979 Bradley <i>et al.</i> , 1986 Hartig, 1989
5-HT <sub>2B</sub>	5-HT <sub>2F</sub>	Kursar <i>et al.</i> , 1992
5-HT <sub>2C</sub>	5-HT <sub>1C</sub> 5-HT <sub>2β</sub>	Pazos <i>et al.</i> , 1984 Hartig <i>et al.</i> , 1996
5-HT <sub>3</sub>	M	Gaddum & Picarelli, 1957
5-HT <sub>4</sub>	R <sub>L</sub>	Shenker <i>et al.</i> , 1987
5-ht <sub>5A</sub>	5-HT <sub>5α</sub> 5-HT <sub>5A</sub>	Erlander <i>et al.</i> , 1993 Plassat <i>et al.</i> , 1993
5-ht <sub>5B</sub>	5-HT <sub>5β</sub> 5-HT <sub>5B</sub>	Erlander <i>et al.</i> , 1993 Matthes <i>et al.</i> , 1993
5-ht <sub>6</sub>	5-HT <sub>6</sub>	Monsma <i>et al.</i> , 1993
5-HT <sub>7</sub>	R <sub>H</sub> 5-HT <sub>x</sub>	Shenker <i>et al.</i> , 1987 Plassat <i>et al.</i> , 1993

**Table 1.1: Summary of Current 5-HT Receptor Nomenclature.**

Current nomenclature is according to the IUHPAR Receptor Nomenclature Committee for 5-HT receptors by Hoyer & Martin, (1997).

later shown that [<sup>3</sup>H]5-HT and [<sup>3</sup>H]spiperone labelled two distinct non-interconverting population of binding sites in rat cortex, termed 5-HT<sub>1</sub> and 5-HT<sub>2</sub> respectively (Peroutka & Snyder, 1979). It was also observed that displacement of [<sup>3</sup>H]5-HT binding by spiperone was not monophasic, suggesting heterogeneity of the 5-HT<sub>1</sub> binding site (Peroutka & Snyder, 1979). Subsequently two components of [<sup>3</sup>H]5-HT binding with nanomolar (5-HT<sub>1A</sub>) and micromolar (5-HT<sub>1B</sub>) affinity was confirmed for spiperone displaceable binding (Pedigo *et al.*, 1981). Furthermore a third 5-HT<sub>1</sub> binding site was described using [<sup>3</sup>H]5-HT autoradiography in porcine choroid plexus and named 5-HT<sub>1C</sub> (Pazos *et al.*, 1984).

The first attempt at 5-HT receptor classification involved the correlation of ligand binding studies with functional responses (Bradley *et al.*, 1986). Three classes of receptors were described: 5-HT<sub>1</sub>-like, 5-HT<sub>2</sub> and 5-HT<sub>3</sub>. The 5-HT<sub>1</sub>-like receptors represented a heterogeneous group at which 5-HT has high affinity. At these sites, the actions of 5-HT were mimicked by 5-CT and antagonised by methysergide and methiothepin, but not by 5-HT<sub>2</sub> or 5-HT<sub>3</sub> antagonists. The 5-HT<sub>2</sub> receptor was analogous to the D receptor described by Gaddum & Picarelli (1957) and the 5-HT<sub>2</sub> receptor of Peroutka & Snyder (1979). The 5-HT<sub>2</sub> receptor was distinguished by the high affinity of the antagonists, ketanserin, methysergide and spiperone. The 5-HT<sub>3</sub> receptor was analogous to the M receptor described by Gaddum & Picarelli (1957). This receptor site was characterised by the high affinity of 2-methyl 5-HT (agonist), antagonism by cocaine and its derivatives (MDL 72222 & ICS 205 930), and the lack of affinity of 5-HT<sub>1</sub>-like or 5-HT<sub>2</sub> antagonists.

This classification was revised when additional [<sup>3</sup>H]5-HT binding sites were shown to remain after displacement with 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1C</sub> and 5-HT<sub>2</sub> compounds and were pharmacologically identified as 5-HT<sub>1D</sub> and 5-HT<sub>1E</sub> binding sites (Heuring & Peroutka, 1987; Leonhardt *et al.*, 1989). The molecular cloning of these subtypes prompted a new nomenclature to be proposed based not only on pharmacological characteristics, but also on molecular structure and second messenger linkage (Hartig, 1989). This modified scheme described the 5-HT<sub>1</sub> and 5-HT<sub>2</sub> families as G-protein coupled receptors (linked to adenylyl cyclase & phospholipase C respectively) and the 5-HT<sub>3</sub> receptor as a ligand-gated ion channel. Following the observation of the similarity of sequences and functional properties of

the 5-HT<sub>2</sub> and 5-HT<sub>1C</sub> receptor subtypes (Peroutka, 1990a), the latter was renamed as the 5-HT<sub>2C</sub> receptor and included in the 5-HT<sub>2</sub> receptor family. The molecular cloning of the 5-HT<sub>2F</sub> receptor (Kursar *et al.*, 1992) was renamed the 5-HT<sub>2B</sub> receptor and included in a revised nomenclature of 5-HT receptor subtypes (Humphrey *et al.*, 1993). This newly proposed nomenclature for 5-HT receptors also included the pharmacologically identified 5-HT<sub>1D</sub> and 5-HT<sub>1E</sub> receptors (Heuring & Peroutka, 1987; Leonhardt *et al.*, 1989) and the cloned 5-HT<sub>1F</sub> subtype (Amlaiky *et al.*, 1992) as members of the 5-HT<sub>1</sub> family. It also recognised the new 5-HT<sub>4</sub> receptor family positively coupled to adenylyl cyclase (Dumuis *et al.*, 1988a & b). The IUPHAR approved nomenclature system integrated the operational (pharmacological), transductional (functional) and structural receptor characteristics employed by Humphrey *et al.*, (1993) to generate a more rigorous review for the classification of receptors for 5-HT (Hoyer *et al.*, 1994). This included new subtypes following the cloning of 5-ht<sub>5A</sub>, 5-ht<sub>5B</sub>, 5-ht<sub>6</sub> and 5-ht<sub>7</sub> receptors (Hoyer *et al.*, 1994; Martin & Humphrey, 1994). These receptors were given lower case appellation as they lacked well-defined functions.

Nomenclature for 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors has a complex history. 5-HT<sub>1B</sub> receptors were first described pharmacologically in the rat brain, representing the component of [<sup>3</sup>H]5-HT binding with low (micromolar) affinity for spiperone (Pedigo *et al.*, 1981). However a corresponding binding site could not be detected in the human brain with 5-HT<sub>1B</sub> ligands (Hoyer *et al.*, 1986a). The identification of a pharmacologically distinct receptor class in non-rodents was termed 5-HT<sub>1D</sub> (Heuring & Peroutka, 1987; Hoyer & Middlemiss, 1989). Initially the expression of these receptors was thought to be species specific. 5-HT<sub>1B</sub> receptors were found in rat, mouse, cat, hamster and opossum, whereas 5-HT<sub>1D</sub> receptors were found in other species including man, calf, dog, and guinea pig. (Middlemiss *et al.*, 1988; Schipper & Tulp, 1988; Schlicker *et al.*, 1989; Hoyer *et al.*, 1990). However it was noted that these two sites had a common anatomical distribution (Pazos & Palacios, 1985a; Waeber *et al.*, 1988). At this early stage it was speculated that the two receptors were species equivalents which displayed different pharmacology (Hoyer & Middlemiss, 1989). This initial idea became complicated by the discovery of two related human receptor genes which were isolated on the basis of their sequence homology with an

orphan receptor (dog RDC4) with 5-HT<sub>1</sub> receptor-characteristics. These two genes demonstrated a 77% sequence homology in the transmembrane domain and when expressed displayed a pharmacological profile identical to that of the originally described 5-HT<sub>1D</sub> site and not that of the rodent 5-HT<sub>1B</sub> site (Weinshank *et al.*, 1992; Hamblin *et al.*, 1992b). These two receptors were designated 5-HT<sub>1D $\alpha$</sub>  and 5-HT<sub>1D $\beta$</sub>  to reflect their identical binding profiles and similar structural characteristics (Hartig *et al.*, 1992). Using a homology screening approach, the rat 5-HT<sub>1B</sub> receptor was cloned and shown to be a protein of 386 amino acids (Vioigt *et al.*, 1991; Adham *et al.*, 1992; Hamblin *et al.*, 1992a). This rat 5-HT<sub>1B</sub> receptor shared 96% transmembrane sequence homology with the human 5-HT<sub>1D $\beta$</sub>  receptor (Adham *et al.*, 1992; Hartig *et al.*, 1992; Jin *et al.*, 1992;). Site-directed mutagenesis of the human 5-HT<sub>1D $\beta$</sub>  receptor demonstrated that substitution of a single amino acid residue (threonine for asparagine at position 355) dramatically altered the pharmacology of the receptor. The resultant binding profile was virtually identical to that of the rat 5-HT<sub>1B</sub> receptor (Metcalf *et al.*, 1992; Oskenberg *et al.*, 1992; Parker *et al.*, 1993). These findings, together with the discovery of a rat gene homologous to the human 5-HT<sub>1D $\alpha$</sub>  receptor and which encoded a receptor with a 5-HT<sub>1D</sub> binding site profile (Hamblin *et al.*, 1992a & b), lead to a recent reassessment of the nomenclature for the 5-HT<sub>1B/D</sub> receptors (Hartig *et al.*, 1996; Table 1.2). This nomenclature change recognised that despite differing pharmacology, the human 5-HT<sub>1D $\beta$</sub>  receptor is a species equivalent of the rodent 5-HT<sub>1B</sub> receptor. Therefore the 5-HT<sub>1D $\beta$</sub>  receptor was realigned to the 5-HT<sub>1B</sub> receptor classification (Table 1.2). To take account of the fact that the pharmacology of the 5-HT<sub>1B</sub> receptor shows significant differences across species, prefixes are now used to denote species specific 5-HT<sub>1B</sub> receptors: the rat becomes r5-HT<sub>1B</sub> and the human becomes h5-HT<sub>1B</sub> (Table 1.2). Likewise the 5-HT<sub>1D $\alpha$</sub>  receptor expressed in the rat and human, and other species, became the equivalent r or h5-HT<sub>1D</sub> receptor (Table 1.2).

This re-classification of the 5-HT<sub>1B/D</sub> receptors is currently used in the most recent of 5-HT receptor nomenclature publications (Table 1.1; Hoyer & Martin, 1997). This classification system includes the 5-HT<sub>7</sub> receptor losing its lower case appellation due to the discovery of firm evidence that this receptor subtype is endogenous and fulfils a physiological role (Carter *et al.*, 1995; Ullmer *et al.*, 1995).

Receptor	Species homologue		
	Human	Rat	Dog
5-HT <sub>1D</sub>	h5-HT <sub>1D</sub> (5-HT <sub>1Dα</sub> )	r5-HT <sub>1D</sub>	ca5-HT <sub>1D</sub> (RDC4)
5-HT <sub>1B</sub> <sup>a</sup>	h5-HT <sub>1B</sub> (5-HT <sub>1Dβ</sub> )	r5-HT <sub>1B</sub>	

**Table 1.2: Receptor Nomenclature for 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> Receptors.**

Previous receptor names are shown in parentheses. Species abbreviations (ca, dog; h, human; r, rat) differentiate species-specific gene and receptor proteins. <sup>a</sup>Denotes species-specific pharmacology (see Hartig *et al.*, 1996 where figure taken from).

Throughout this thesis those receptors that still lack a well-defined function are given a lower case appellation (i.e. 5-ht<sub>1E</sub>, 5-ht<sub>1F</sub>, 5-ht<sub>5A</sub>, 5-ht<sub>5B</sub> and 5-ht<sub>6</sub> receptors) consistent with the IUPHAR guidelines for naming receptors (Vanhoutte *et al.*, 1996; Humphrey & Barnard, 1998).

The chromosomal location of the gene and the number of amino acids of encoded gene of the human 5-HT receptor subtypes and 5-HT transporter are summarised in Table 1.3. Such localisations have allowed association studies to potentially link the 5-HT system with disease states such as depression and schizophrenia (Ogilvie *et al.*, 1996; Busatto & Kerwin, 1997). Table 1.4 summarises the main pharmacological tools currently available to label these receptor subtypes for radioligand binding and/or for autoradiographic distribution studies. The physiological functions of these 5-HT receptor subtypes in terms of behavioural, neurochemical and neuroendocrine responses to specific agonists and/or antagonists are summarised in Table 1.5.

The structure, distribution and pharmacological characteristics of each of the mammalian 5-HT receptor subtypes are briefly outlined below. More comprehensive reviews are available (Zifa & Fillion, 1992; Boess & Martin, 1994; Hoyer *et al.*, 1994; Martin & Humphrey, 1994; Hoyer & Martin, 1997; Gerhardt & Van Heerikhuizen, 1997; Barnes & Sharp, 1999). The presence of 5-HT receptors has also been reported in a variety of invertebrates, although currently no guidelines for nomenclature have been recommended from the IUPHAR Serotonin Receptor Nomenclature Committee. These include three 5-HT<sub>1</sub> like receptors (Sadou *et al.*, 1992; Sugamori *et al.*, 1993), two 5-HT<sub>2</sub> like receptors (Colas *et al.*, 1995; Gerhardt *et al.*, 1996), a 5-HT<sub>7</sub> like receptor (Witz *et al.*, 1990) and two 5-HT receptors cloned from *Aplysia* (Li *et al.*, 1995). These are reviewed by Sadou & Hen, (1994) and Gerhardt & Van Heerikhuizen (1997).

### **1.5.2 The 5-HT<sub>1</sub> Receptor Family**

The 5-HT<sub>1</sub> receptor family consists of five receptor subtypes (5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-ht<sub>1E</sub> and 5-ht<sub>1F</sub>) defined by their high degree of amino acid sequence homology (>60% in the seven transmembrane regions; Hoyer *et al.*, 1994). These receptor subtypes contain between 365 and 422 amino acids, and unlike other

Subtype	Chromosomal Location	Amino Acids
5-HT <sub>1A</sub>	5q11.2-q13 <i>Kobilka et al., 1987</i>	422 <i>Chanda et al., 1993</i>
5-HT <sub>1B</sub>	6q13 <i>Jin et al., 1992</i>	390 <i>Weinshank et al., 1992</i>
5-HT <sub>1D</sub>	1p34.3-36.3 <i>Libert et al., 1991</i>	377 <i>Hamblin &amp; Metcalf, 1991</i>
5-ht <sub>1E</sub>	6q14-15 <i>Levy et al., 1992</i>	365 <i>McAllister et al., 1992</i>
5-ht <sub>1F</sub>	13p13-p14.1 <i>Erdmann et al., 1997</i>	366 <i>Lovenberg et al., 1993b</i>
5-HT <sub>2A</sub>	13q14-q21 <i>Sparkes et al., 1991</i>	471 <i>Saltzmann et al., 1991</i>
5-HT <sub>2B</sub>	2q36.3-37.1 <i>Le Coniat et al., 1996</i>	481 <i>Kursar et al., 1994</i>
5-HT <sub>2C</sub>	Xq24 <i>Milatovich et al., 1992; Stam et al., 1994</i>	458 <i>Saltzman et al., 1991</i>
5-HT <sub>3</sub>	Chromosome 11 A detailed mapping of the 5-HT <sub>3</sub> gene yet to be reported ( <i>Uetz et al., 1994; Miyake et al., 1995</i> ).	487
5-HT <sub>4</sub>	5q31-q33 <i>Claeyssen et al., 1997</i>	388 <i>Van den Wyngaert et al., 1991</i>
5-ht <sub>5A</sub>	7q35-36 <i>Matthes et al., 1993</i>	357 <i>Rees et al., 1994</i>
5-ht <sub>5B</sub>	2q11-13 <i>Matthes et al., 1993</i>	370 <i>Matthes et al., 1993</i>
5-ht <sub>6</sub>	1p35-36 <i>Kohen et al., 1996</i>	440 <i>Kohen et al., 1996</i>
5-HT <sub>7</sub>	10q23.3-24.3 <i>Gelertner et al., 1995</i>	5-HT <sub>7(a)</sub> 445 5-HT <sub>7(b)</sub> 432 5-HT <sub>7(d)</sub> 479 <i>Heidmann et al., 1997</i>
5-HT Transporter	17q11.1-17q12 <i>Ramamoorthy et al., 1993a</i>	630 <i>Lesch et al., 1994</i>

**Table 1.3: Summary of the Chromosomal Location and Primary Structure of the Human 5-HT Receptors and Transporter.**



Receptor	Radioligand	Selective antagonists	Selective agonists	G protein coupling
5-HT <sub>1A</sub>	[ <sup>3</sup> H]8-OH DPAT [ <sup>3</sup> H]WAY100635	WAY100635 NAD 299	-	G <sub>i/o</sub>
5-HT <sub>1B</sub>	[ <sup>125</sup> I]GTI [ <sup>3</sup> H]CP 93129 [ <sup>3</sup> H]CP 96501 [ <sup>3</sup> H]GR125,743	SB216 641 SB224 289	CP 93129	G <sub>i/o</sub>
5-HT <sub>1D</sub>	[ <sup>3</sup> H]sumatriptan [ <sup>125</sup> I]GTI [ <sup>3</sup> H]GR125,743	BRL 15572	-	G <sub>i/o</sub>
5-HT <sub>1E</sub>	[ <sup>3</sup> H]5-HT	-	-	G <sub>i/o</sub>
5-HT <sub>1F</sub>	[ <sup>3</sup> H]5-HT [ <sup>3</sup> H]sumatriptan [ <sup>3</sup> H]LSD	-	LY344864 LY334370	G <sub>i/o</sub>
5-HT <sub>2A</sub>	[ <sup>3</sup> H]Ketanserin [ <sup>3</sup> H]MDL100907	MDL100907	-	G <sub>q/11</sub>
5-HT <sub>2B</sub>	[ <sup>3</sup> H]5-HT	SB200646 SB204741	BW 723C86	G <sub>q/11</sub>
5-HT <sub>2C</sub>	[ <sup>3</sup> H]mesulergine	SB242084 RS-102221	$\alpha$ -Me-5-HT	G <sub>q/11</sub>
5-HT <sub>3</sub>	[ <sup>3</sup> H]zacopride [ <sup>125</sup> I]zacopride	granisetron ondansteron tropisetron MDL72222	2-Me-5-HT m-chlorophenyl- biguanide	Cation channel
5-HT <sub>4</sub>	[ <sup>3</sup> H]GR113808 [ <sup>125</sup> I]SB207710	GR113808 SB207710 SDZ 205557	BIMU 1 + 8 RS67506 ML10302	G <sub>s</sub>
5-HT <sub>5A</sub>	[ <sup>3</sup> H]5-CT [ <sup>3</sup> H]LSD	-	-	G <sub>s</sub> ?
5-HT <sub>5B</sub>	[ <sup>3</sup> H]5-CT [ <sup>3</sup> H]LSD	-	-	G <sub>s</sub> ?
5-HT <sub>6</sub>	[ <sup>3</sup> H]5-CT [ <sup>3</sup> H]LSD	Ro 04-6790 Ro 63-0563	-	G <sub>s</sub>
5-HT <sub>7</sub>	[ <sup>3</sup> H]5-CT [ <sup>3</sup> H]LSD; [ <sup>3</sup> H]5-HT	SB258719	-	G <sub>s</sub>

**Table 1.4: Summary of Pharmacological Tools Available to Identify 5-HT Receptors.** Adapted from Hoyer & Martin, (1997).

<b>Behavioural</b>		<b>Neurochemical</b>
5-HT syndrome	1A	1A(-), 1B(-), 1D(-), 3(+), 4(+)
Hypothermia	1A, 1B, 2A	1A(+), 1B(-), 2A(-); 2C(-), 3(-)
Hyperphagia	1A	1A(+), 1B(-), 2A(-), 3(-), 4(+)
Anxiolysis	1A, 2C, 3(antag.), 4 (antag.)	1A(-), 1D(-)
Sexual behaviour	1A, 2A	2A(-), 2C(-), 3(+), 4(+)
Discriminative stimulus	1A, 2A	2C(+)
Hypophagia	1B, 2C	2A(-), 2C(-), 3(+)
Locomotion	1B(+); 2C(-), 3(- antag.)	3(+)
Myoclonic jerks	1B	3(-)
Penile erection	1B, 2C	
Aggression	1B(-)	
Pain	1A (+), 2(+), 1B(-), 2(-), 3(-)	
Head Twitch	2A	1A(+), 2A(+), 2C(+)
Wet dog shake (rat)	2A	1A(+), 1B(+), 2A(+), 2C(+)
Sleep	2A, 2C	1B(+), 2A(+), 2C(+)
Migraine	1B(-), 1D(-), 1F(-), 2C(+)	1A(+),
Cognition	3(+ antag.), 4(+)	2A(+)
Reward	3(- antag.)	2A(+)

**Table 1.5: Functional Consequences of 5-HT Receptor Subtype Activation**

5-HT receptors involved in the behavioural, neurochemical and neuroendocrine functions are given according to their subtype (i.e. 1A = 5-HT<sub>1A</sub> receptor). Inhibitory (-) or excitatory (+) effects on the functional responses are given in brackets. Such responses are also noted in the presence of antagonists (antag.). Functional responses for 5-HT<sub>1E/1F/5A/5B</sub> and 5-HT<sub>6</sub> receptors are absent as definite physiological roles have not been defined. The 5-HT<sub>7</sub> receptor does have identified functional responses (see text) but none that can be currently assigned to the common functions of the 5-HT<sub>1-4</sub> receptors shown above. Table adapted from Barnes & Sharp (1999).

5-HT receptor subtype families lack introns within the coding regions of their genes. The 5-HT<sub>1</sub> receptors are all believed to couple negatively to adenylate cyclase via pertussis toxin-sensitive G proteins.

### **The 5-HT<sub>1A</sub> Receptor**

The 5-HT<sub>1A</sub> receptor was the first 5-HT receptor to be fully sequenced identified by screening a genomic library for homologous sequences to the β<sub>2</sub>-adrenoreceptor (Kobilka *et al.*, 1987; Fargin *et al.*, 1988; Albert *et al.*, 1990). The rat and human 5-HT<sub>1A</sub> receptor nucleic acid sequences are 88% homologous to each other (Kobilka *et al.*, 1987; Fargin *et al.*, 1988; Albert *et al.*, 1990). The 5-HT<sub>1A</sub> receptor displays significantly less homology with other members of the family of G-protein coupled 5-HT receptors such as 5-HT<sub>2A</sub> (19%) and 5-HT<sub>2C</sub> (18%) receptors and the 5-HT<sub>1D</sub> receptors (43%) (Fujiwara *et al.*, 1993). [<sup>3</sup>H]8-OH DPAT provided the first pharmacological profile of the 5-HT<sub>1A</sub> binding site (Gozlan *et al.*, 1983). The agonists 5-CT, DP-5-CT, 5-HT and 8-OH DPAT have high affinity for the recombinant 5-HT<sub>1A</sub> receptor. However it is now apparent that 8-OH DPAT has moderate affinity at the cloned 5-HT<sub>7</sub> receptor (Shen *et al.*, 1993; Lovenberg *et al.*, 1993a). The antagonist spiperone, and the β-adrenoreceptor antagonists such as pindolol and propranolol also display high affinity for the 5-HT<sub>1A</sub> receptor (Fargin *et al.*, 1988; Newman-Tancredi *et al.*, 1992). Mutagenesis studies have established that a single amino acid residue in the 7<sup>th</sup> transmembrane (Asp 385) is responsible for the high affinity stereoselectivity of the receptor for certain β-adrenoreceptor ligands such as pindolol (Guan *et al.*, 1992). A number of potent and specific 5-HT<sub>1A</sub> receptor antagonists have since been developed, WAY100635 being the most potent, although NAD-299 appears to be somewhat more selective (Fletcher *et al.*, 1996; Johansson *et al.*, 1997). These ligands have been used extensively to study the distribution of 5-HT<sub>1A</sub> binding sites in the rat brain (Hoyer *et al.*, 1985a; Pazos *et al.*, 1987a; Pompeiano *et al.*, 1992; Hume *et al.*, 1994; Khwaja, 1995). The density of 5-HT<sub>1A</sub> binding sites is highest in limbic brain areas, notably hippocampus, lateral septum, cortical areas (particularly cingulate & entorhinal cortex), and also the dorsal raphé nuclei, with lower levels in the median raphé and some hypothalamic and thalamic nuclei. In contrast, levels of 5-HT<sub>1A</sub> binding sites in the extrapyramidal areas such as the caudate-putamen, globus pallidus, substantia nigra and cerebellum

were barely detectable. *In vivo* labelling of the 5-HT<sub>1A</sub> receptor has been achieved in both mouse (Laporte *et al.*, 1994) and the human living brain (Pike *et al.*, 1995). The distribution of mRNA encoding the 5-HT<sub>1A</sub> receptor and the localisation pattern of receptor protein using site specific antibodies is almost identical to the 5-HT<sub>1A</sub> binding site (Chalmers & Watson, 1991; Hamon *et al.*, 1991; Gozlan *et al.*, 1993; Burnet *et al.*, 1995; Kia *et al.*, 1996a). 5-HT<sub>1A</sub> receptors are predominantly somatodendritic (Pompeiano *et al.*, 1992; Burnet *et al.*, 1994; Kia *et al.*, 1996b), existing on non-5-HT cell bodies in neuronal 5-HT projection areas, mainly post-synaptically in 5-HT neuronal projection areas and as autoreceptors on 5-HT perikarya in the raphé nuclei. The reduction of raphé 5-HT<sub>1A</sub> binding sites observed following 5,7-DHT lesions confirms the cell body autoreceptor status of this subtype at this particular location (Vergé *et al.*, 1986). In neurons, 5-HT<sub>1A</sub> receptors inhibit adenylyl cyclase (De Vivo & Maayani, 1986; Weiss *et al.*, 1986). However in hippocampal tissue, there are reports of positive coupling to adenylate cyclase stimulating cAMP formation (Shenker *et al.*, 1983; Markstein *et al.*, 1986), which may be attributable to the pharmacologically similar 5-HT<sub>7</sub> receptor. Interestingly the high density of dorsal raphé 5-HT<sub>1A</sub> receptors does not appear to couple to the inhibition of adenylate cyclase (Clarke *et al.*, 1996). The 5-HT<sub>1A</sub> receptor is also linked via a pertussis toxin-sensitive G protein to K<sup>+</sup> channel opening in rat hippocampal membranes, leading to hyperpolarisation due to increased K<sup>+</sup> conductance and decreased neuronal firing (Andrade *et al.*, 1986). In isolated dorsal raphé nucleus neurones, activation of somatodendritic 5-HT<sub>1A</sub> autoreceptors induces activation of an inward rectifying K<sup>+</sup> current (Williams *et al.*, 1988; Penington *et al.*, 1993) and an inhibition of voltage-activated high threshold Ca<sup>2+</sup> currents (Penington & Kelly, 1990; Penington & Fox, 1994 & 1995). In both cases, the response to 5-HT is G-protein mediated via a direct interaction between G proteins and the respective ion channel (Innis & Aghajanian, 1987; Penington *et al.*, 1991; Penington *et al.*, 1993; Penington & Fox, 1994). This two fold mechanism induces membrane hyperpolarisation which inhibits neuronal firing (Vandermaelen *et al.*, 1986; Blier & De Montigny, 1987; Godbout *et al.*, 1990; Schechter *et al.*, 1990; Hadrava *et al.*, 1995). This effect is also produced indirectly by serotonin specific re-uptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs) which induce an

activation of 5-HT<sub>1A</sub> autoreceptors due to an increase in extracellular 5-HT at the somatodendritic level (Blier & De Montigny, 1983 & 1985; Chaput *et al.*, 1986; Artigas, 1993).

### **The 5-HT<sub>1B</sub> Receptor**

There are a large number of available ligands with high affinity for the rat 5-HT<sub>1B</sub> receptor but most are not selective. The most potent agonists include RU 24969, 5-CT and CP93129, with methiothepin, cyanopindolol & pindolol being potent but less specific antagonists (see Hoyer *et al.*, 1994). These compounds have high affinity for other 5-HT receptor subtypes particularly 5-HT<sub>1A</sub> receptors. The low affinity for 5-HT<sub>1B</sub> sites of other drugs such as 8-OH DPAT, WAY100635, ritanserin and tropisetron helps to identify the 5-HT<sub>1B</sub> receptor. However the compound GR 127935 has high selectivity for 5-HT<sub>1B/1D</sub> versus other 5-HT receptors and is a potent antagonist in functional models (Skingle *et al.*, 1995). In order to radiolabel 5-HT<sub>1B</sub> receptors in rat brain tissue, a variety of radioligands have been used. These include [<sup>3</sup>H]5-HT binding in the presence of blocking concentrations of 5-HT<sub>1A</sub> and 5-HT<sub>1C</sub> (now 5-HT<sub>2C</sub>) receptor ligands (Peroutka, 1986), or [<sup>125</sup>I]iodocyanopindolol in the presence of 30µM isoprenaline and 100nM 8-OH DPAT to avoid β-adrenoceptor and 5-HT<sub>1A</sub> binding (Hoyer *et al.*, 1985a & b). Other radioligands used include the non-selective [<sup>3</sup>H]Dihydroergotamine ([<sup>3</sup>H]DHE; Hamblin *et al.*, 1987) and the more selective ligands [<sup>125</sup>I]GTI (serotonin-5-O-carboxymethyl-glycyl-[<sup>125</sup>I]tyrosinamide; Boulenguez *et al.*, 1992) or [<sup>3</sup>H]CP 93129 / CP 96501 (Koe *et al.*, 1992a & b). [<sup>125</sup>I]GTI also however labels a low density of 5-HT<sub>1D</sub> receptors in rat brain (Bruinvels *et al.*, 1993a & b). Despite their high sequence homology and similar brain distribution, the rat and mouse 5-HT<sub>1B</sub> receptors are pharmacologically distinct from the human (Hamblin *et al.*, 1992a, b). The most striking difference is that certain β-adrenoceptor antagonists including cyanopindolol, SDZ 21009, isamoltane, pindolol and propranolol have higher affinity for the 5-HT<sub>1B</sub> receptor in the rodent than human (see Boess & Martin, 1994). This difference can be accounted for by a single amino acid difference in the putative 7<sup>th</sup> transmembrane region at position 355 (asparagine in rat, threonine in human; Metcalf *et al.*, 1992; Oksenberg *et al.*, 1992; Parker *et al.*, 1993). Despite the reported similarity of human 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptor pharmacologies (Weinshank *et al.*, 1992), it is now apparent

that certain drugs may distinguish these two receptor subtypes. For example the 5-HT<sub>2</sub> receptor antagonists, ketanserin and ritanserin, show some selectivity (15-30 fold) for the human 5-HT<sub>1D</sub> versus 5-HT<sub>1B</sub> receptor (Kaumann *et al.*, 1994; Pauwels *et al.*, 1996). More recently, the first antagonists with selectivity (at least 25-fold) for the human 5-HT<sub>1B</sub> (SB 216641 & SB 224289) and human 5-HT<sub>1D</sub> (BRL155172) receptors have been developed (Price *et al.*, 1997; Schlicker *et al.*, 1997). An analogue of GR127,935 with higher potency at human 5-HT<sub>1B/1D</sub> receptors, namely GR125,743 has become available as a radioligand (Audinot *et al.*, 1997; Doménech *et al.*, 1997) but as yet no published data are available concerning its potential labelling of these receptors in the rat. Autoradiographic studies in the rat have been hindered by the need to use relatively non-selective ligands in the presence of masking agents to identify 5-HT<sub>1B</sub> binding sites as described above. However a high density of 5-HT<sub>1B</sub> binding sites have been identified in many brain areas but particularly in the substantia nigra, globus pallidus, ventral pallidum and entopeduncular nucleus of the rat basal ganglia (Pazos & Palacios, 1985; Vergé *et al.*, 1986; Bruinvels *et al.*, 1993a & b). The availability of the newer 5-HT<sub>1B/1D</sub> selective radioligand, [<sup>3</sup>H]GR125, 743 may help verify these earlier studies. Specific 5-HT<sub>1B</sub> receptor antibodies have also been used to show similar high 5-HT<sub>1B</sub> receptor densities in the globus pallidus, substantia nigra and dorsal subiculum, with lower levels in the caudate-putamen, cerebral cortex, central grey and superior colliculus (Langlois *et al.*, 1995; Sari *et al.*, 1997 & 1999). Some forebrain areas (e.g. striatum) also express high levels of 5-HT<sub>1B</sub> binding sites and 5-HT<sub>1B</sub> receptor mRNA (Voigt *et al.*, 1991; Boschert *et al.*, 1994; Bruinvels *et al.*, 1994a & b; Doucet *et al.*, 1995). However, *in situ* hybridisation studies have revealed that 5-HT<sub>1B</sub> mRNA does not correlate with its binding sites in all brain areas (Bruinvels *et al.*, 1994a & b). For example, despite the high density of 5-HT<sub>1B</sub> binding sites in many areas of the rat basal ganglia there is little 5-HT<sub>1B</sub> mRNA. *In situ* hybridisation studies have also shown high 5-HT<sub>1B</sub> receptor mRNA signals in the dorsal and median raphé nuclei (Voigt *et al.*, 1991; Boschert *et al.*, 1994; Bruinvels *et al.*, 1994a & b) which are markedly reduced by 5-HT neuronal destruction (Doucet *et al.*, 1995). In the raphé nuclei however there is a very low density of 5-HT<sub>1B</sub> binding sites (Vergé *et al.*, 1986; Voigt *et al.*, 1991; Boschert *et al.*, 1994; Bruinvels *et al.*, 1993a & 1994a & b).

Mismatches between 5-HT receptor protein and mRNA previously observed in mice have been explained by assuming that 5-HT<sub>1B</sub> receptors are transported along fibres far from somas where they are synthesised (Boschert *et al.*, 1994). Together these data suggest that 5-HT<sub>1B</sub> receptors are both presynaptic and postsynaptic. There is now convincing evidence that 5-HT<sub>1B</sub> receptors function as 5-HT autoreceptors at the 5-HT terminal modulating 5-HT release (Middlemiss & Hutson, 1990; Fink *et al.*, 1995; Bühlen *et al.*, 1996). There is also evidence that 5-HT<sub>1B</sub> receptors are located on non-5-HT terminals, thereby acting as heteroreceptors (Maura & Raiteri, 1986; Cassel *et al.*, 1995). A novel endogenous modulator of the 5-HT<sub>1B</sub> receptor subtype has also been identified and termed 5-HT moduline (Rouselle *et al.*, 1996; Fillion *et al.*, 1996). This tetrapeptide (Leu-Ser-Ala-Leu) has similar binding properties and an autoradiographic distribution to that of the 5-HT<sub>1B</sub> receptor, and has been proposed as a novel neurotransmitter (for review, see Massot *et al.*, 1998). In the rat and calf substantia nigra, 5-HT<sub>1B</sub> receptors negatively couple to adenylate cyclase (Bouhelal *et al.*, 1988; Schoeffer & Hoyer, 1989).

### **The 5-HT<sub>1D</sub> Receptor**

It has been difficult to determine the distribution of the 5-HT<sub>1D</sub> receptor in the rat because levels appear to be low and there is a lack of available specific radioligands. However autoradiographic studies with [<sup>125</sup>I]GTI in the presence of 100nM CP93129 to mask binding to 5-HT<sub>1B</sub> binding sites have revealed 5-HT<sub>1D</sub> binding sites in the basal ganglia and even lower levels in the hippocampus and cortex (Bruinvels *et al.*, 1993a & b). Studies in humans have also revealed a similar distribution as well as in specific regions of the midbrain and spinal cord using the ketanserin sensitive component of [<sup>3</sup>H]sumatriptan binding (Castro *et al.*, 1997a). Like the 5-HT<sub>1B</sub> receptor, 5-HT<sub>1D</sub> mRNA has been detected in lower abundance than the protein in these areas (Hamblin *et al.*, 1992a & b; Bruinvels *et al.*, 1994 a & b). The presence of a 5-HT<sub>1D</sub> autoreceptor on 5-HT nerve terminals has been proposed on the basis of *in vitro* evidence of the persistence of 5-HT agonist-induced inhibition of 5-HT release in the cortex and hippocampus of 5-HT<sub>1B</sub> knock-out mice (Piñeyro *et al.*, 1995a). In the rat dorsal raphe nucleus there are high levels of 5-HT<sub>1D</sub> mRNA (Hamblin *et al.*, 1992a & b; Bruinvels *et al.*, 1994 a & b). Indeed the presence of 5-HT<sub>1D</sub> receptors in the dorsal raphe nucleus has also been shown by the

continued 5-HT agonist-induced inhibition of 5-HT release in 5-HT<sub>1B</sub> knockout mice (Piñeyro *et al.*, 1995a). This group concluded that the pharmacology of the 5-HT agonist induced inhibition of [<sup>3</sup>H]5-HT from slices of the rat mesencephalon indicated the presence of a 5-HT<sub>1D</sub> (but not 5-HT<sub>1B</sub>) autoreceptor in this preparation (Piñeyro *et al.*, 1995b). Indeed the 5-HT<sub>1B</sub> agonist CP93129 did not modify release in the dorsal raphe nucleus (Piñeyro *et al.*, 1996). 5-HT<sub>1D</sub> receptors also modulate 5-HT release in the same brain region of the guinea pig brain (El Mansari & Blier, 1996). Like the 5-HT<sub>1B</sub> receptor, the 5-HT<sub>1D</sub> receptor has been shown to act as an auto and heteroreceptor regulating transmitter release, albeit by interpreting the actions of non-selective drugs (Harel-Dupas *et al.*, 1991; Davidson & Stamford, 1995; Feuerstein *et al.*, 1996; Maura & Raiteri, 1996; Piñeyro *et al.*, 1996; Maura *et al.*, 1998). In humans and guinea pig, 5-HT autoreceptors in the cortex appear to be of the 5-HT<sub>1B</sub> type rather than the 5-HT<sub>1D</sub> type based on the blocking ability of the respective selective antagonists, SB 216641 and BRL 15572 (Schlicker *et al.*, 1997). Therefore it is apparent that although the possibility of 5-HT<sub>1D</sub> auto/hetero receptors exist, they may be restricted to specific brain areas and or species. Use of the more recently developed selective drugs should be able to address these questions. The currently understanding of rat 5-HT<sub>1B/1D</sub> receptors, as outlined by Piñeyro & Blier (1999), is that the somatodendritic receptors regulating cell body 5-HT release, independent of neuronal firing are of the 5-HT<sub>1D</sub> receptor subtype whereas those at the nerve terminal are predominantly of the 5-HT<sub>1B</sub> subtype. Currently no second messenger response or *in vivo* functional response can be safely attributed to the activation of CNS 5-HT<sub>1D</sub> receptors due to lack of discriminative brain penetrating drugs. Indeed although it was originally thought that the 5-HT<sub>1D</sub> receptor coupled negatively to adenylate cyclase in the substantia nigra of the calf and guinea pig (Schoeffter & Hoyer, 1989; Waeber *et al.*, 1990), it now seems clear that the receptor detected in these early studies was actually the species equivalent of the 5-HT<sub>1B</sub> receptor.

### **The 5-h<sub>1E</sub> Receptor**

The 5-h<sub>1E</sub> receptor was detected in radioligand binding studies using [<sup>3</sup>H]5-HT in the presence of compounds that blocked 5-HT<sub>1A/1B/1D/2C</sub> receptors. A biphasic 5-HT inhibition curve was detected in cortex and caudate membranes of humans as



well as other species, e.g. guinea pig, dog and rabbit (Leonhardt *et al.*, 1989; Beer *et al.*, 1992). The high affinity site represented binding to 5-HT<sub>1D</sub> receptors, whereas the low affinity site represented a novel receptor with novel pharmacology and was termed 5-HT<sub>1E</sub> (Leonhardt *et al.*, 1989). This receptor is characterised by its high affinity for 5-HT and lower affinity for 5-CT, sumatriptan and ergotamine compared with the 5-HT<sub>1D</sub> site (McAllister *et al.*, 1992). Using the same binding approach, autoradiographic distribution studies have detected high levels in cortex (particularly entorhinal cortex), caudate putamen with lower levels in hippocampus and amygdala in human, rat, mouse and guinea pig brain (Miller & Teitler, 1992; Barone *et al.*, 1993; Bruinvels *et al.*, 1994b). 5-ht<sub>1E</sub> mRNA has been detected in these regions (Bruinvels *et al.*, 1994a) and the receptor is believed to lie postsynaptically (Barone *et al.*, 1993). However the lack of any selective ligands has hindered investigations into its functional role and effect on neurons.

### **The 5-ht<sub>1F</sub> Receptor**

Using a homology cloning approach with probes derived from the 5-HT<sub>1B</sub> gene, a mouse receptor, having 61% transmembrane sequence homology to the 5-ht<sub>1E</sub> receptor, and termed 5-HT<sub>1EB</sub>, was identified (Amlaiky *et al.*, 1992). A homologous human receptor was subsequently identified and named 5-ht<sub>1F</sub> on the basis of its unique pharmacological profile (Adham *et al.*, 1993 a & b). 5-HT, sumatriptan, methysergide and ergotamine all have relatively high affinity at this site, whilst 5-CT has relatively low affinity (Adham *et al.*, 1993a & b; Lovenberg *et al.*, 1993b). Its high affinity for sumatriptan therefore distinguishes it from the 5-ht<sub>1E</sub> receptor. Two novel and selective 5-ht<sub>1F</sub> receptor agonists, LY344864 and LY334370 have recently become available (Overshiner *et al.*, 1996; Johnson *et al.*, 1997; Phebus *et al.*, 1997). Autoradiographic studies to investigate the distribution of this 5-HT receptor subtype, using the 5-CT-insensitive portion of [<sup>3</sup>H]sumatriptan binding, have revealed a high density of 5-ht<sub>1F</sub> binding sites in the claustrum, with lower levels in some thalamic and amygdala nuclei, cerebral cortex and striatum corresponding to 5-ht<sub>1F</sub> mRNA distribution in the guinea pig brain (Bruinvels *et al.*, 1994a). A similar distribution is apparent in guinea pig, rat and human brain (Waeber & Moskowitz, 1995a & b; Pascual *et al.*, 1996; Castro *et al.*, 1997a;). However the lack of any selective ligands has hindered investigations into its functional role and

effect on neurons, though the more recently identified selective agonists above may help to elucidate the function of this receptor subtype. Indeed initial reports using LY334370 suggest that it does not induce alterations of behavioural effects or monoamine levels in rats (Overshiner *et al.*, 1996) but may be important in the treatment of migraine (Johnson *et al.*, 1997; Phebus *et al.*, 1997).

### 1.5.3 The 5-HT<sub>2</sub> Receptor Family

The 5-HT<sub>2</sub> receptor family consists of three subtypes (5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub>) that have very similar molecular structures and signal transduction pathways. These receptor subtypes contain between 458 and 504 amino acids and members of the 5-HT<sub>2</sub> receptor gene family either have two (5-HT<sub>2A</sub> and 5-HT<sub>2B</sub> receptors) or three introns (5-HT<sub>2C</sub> receptors) in their coding sequence (Yu *et al.*, 1991; Chen *et al.*, 1992; Stam *et al.*, 1994). They all stimulate phospholipase C activity, with subsequent production of inositol phosphates (IP<sub>3</sub>; which increases intracellular calcium) and diacylglycerol (DAG; which increases protein kinase C).

#### The 5-HT<sub>2A</sub> Receptor

The 5-HT<sub>2A</sub> receptor represents the classical 5-HT<sub>2</sub> binding site described by Peroutka & Snyder (1979) as having high affinity for [<sup>3</sup>H]spiperone and [<sup>3</sup>H]LSD and low affinity for [<sup>3</sup>H]5-HT. A rat 5-HT<sub>2A</sub> cDNA sequence was isolated by homology with the rat 5-HT<sub>2C</sub> receptor (Pritchett *et al.*, 1988; Julius *et al.*, 1990). Subsequently a human 5-HT<sub>2A</sub> receptor cDNA clone was isolated with 80% transmembrane homology (Boess & Martin, 1994). The pharmacological profile of the 5-HT<sub>2A</sub> binding site, labelled by [<sup>3</sup>H]spiperone and [<sup>3</sup>H]LSD can be displaced by spiperone and ketanserin with >10 and >1000 fold selectivity over 5-HT<sub>2C</sub> and 5-HT<sub>2B</sub> receptors (Pritchett *et al.*, 1988; Julius *et al.*, 1990; Wainscott *et al.*, 1993). [<sup>3</sup>H]ketanserin was the first selective 5-HT<sub>2A</sub> radioligand reported (Leysen *et al.*, 1982) and is still used today. MDL100907 is a more recently identified potent and selective antagonist of the 5-HT<sub>2A</sub> receptor (Sorenson *et al.*, 1993; Kehne *et al.*, 1996). The agonists DOM, DOI and DOB also bind with high affinity to 5-HT<sub>2A</sub> receptors whereas 5-HT binds with low affinity (Glennon *et al.*, 1992). Receptor autoradiography studies locate this receptor in many forebrain regions, particularly cortical areas, caudate nucleus, nucleus accumbens, olfactory tubercle and

hippocampus of all species studied (Pazos *et al.*, 1985b & 1987b; López-Giménez *et al.*, 1997). These binding sites are closely correlated with 5-HT<sub>2</sub> mRNA (Mengod *et al.*, 1990; Morilak *et al.*, 1993 & 1994; Pompeiano *et al.*, 1994; Burnet *et al.*, 1995). MDL 100907 is currently under development as a PET ligand to study *in vivo* 5-HT<sub>2A</sub> receptors in the living human brain (Lundkvist *et al.*, 1996; Ito *et al.*, 1998).

5-HT<sub>2A</sub> receptor activation results in excitatory responses, whereby 5-HT induced neuronal depolarisations are associated with a reduction of potassium conductances (Aghajanian, 1995), although it is not currently understood as to whether the phosphoinositide signalling pathway has a role in this effect. These excitatory responses have been observed in brain slices from cortex (Araneda & Andrade, 1991; Aghajanian & Marek, 1997) and dentate gyrus of the hippocampus (Piguet & Galvan, 1994)

### **The 5-HT<sub>2B</sub> Receptor**

This receptor was first described in the stomach fundus preparation, where the contractile effect of 5-HT was used as a sensitive bioassay for 5-HT (Vane, 1957). The mouse and rat fundus receptor genes were isolated by low stringency screening for sequences homologous to the 5-HT<sub>2C</sub> receptor (Fouget *et al.*, 1992a & b; Kursar *et al.*, 1992). Subsequently the human 5-HT<sub>2B</sub> receptor, which shares >70% transmembrane homology with the human 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors, was cloned from SH SY5Y neuroblastoma cells and from uterine tissue (Schmuck *et al.*, 1994; Kursar *et al.*, 1994). There is a close pharmacological characterisation between cloned 5-HT<sub>2B</sub> receptors and the rat stomach fundus receptor, with methysergide and metergoline having high affinity, whereas ketanserin and cinanserin show low affinity (Wainscott *et al.*, 1993; Baxter *et al.*, 1994). Yohimbine has higher affinity at 5-HT<sub>2B</sub> receptors than 5-HT<sub>2A</sub> or 5-HT<sub>2C</sub> receptors, and ketanserin, spiperone and mianserin have lower affinity at 5-HT<sub>2B</sub> compared to other 5-HT<sub>2</sub> receptor subtypes (Wainscott *et al.*, 1993; Kennett, 1993; Bohanus *et al.*, 1995). More selective ligands are now available including the 5-HT<sub>2B</sub> antagonist SB 204 741 which shows 20-60 fold selectivity over 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors and also the agonist BW 723C86 which has about 10 fold selectivity for the 5-HT<sub>2B</sub> receptor over the 5-HT<sub>2A/2C</sub> receptors (Baxter *et al.*, 1995; Bonhanus *et al.*, 1995; Baxter, 1996). The detection of 5-HT<sub>2B</sub> receptor protein in the brain has been limited by the lack of selective

radioligands. 5-HT<sub>2B</sub> transcripts have been found in human brain and in rat peripheral tissues, though initially no mRNA was detected in the rat CNS (Kursar *et al.*, 1992, 1994; Pompeiano *et al.*, 1994). Since then low levels have been reported (Flanigan *et al.*, 1995) and site directed antibodies have revealed a distribution to a few brain areas. These regions include the lateral septum, dorsal hypothalamus, medial amygdala and Purkinje cells of the cerebellum (Duxon *et al.*, 1997a). The use of the more selective ligands now available will help not only to confirm its distribution in the CNS but also its function, which as yet has been limited to a possible involvement in anxiety (Kennett *et al.*, 1996a & b; Duxon *et al.*, 1997b).

### **The 5-HT<sub>2C</sub> Receptor**

A [<sup>3</sup>H]5-HT binding site in the choroid plexus of a variety of species was identified that could also be labelled by [<sup>3</sup>H]mesulergine and [<sup>3</sup>H]LSD but not by [<sup>3</sup>H]ketanserin and was termed 5-HT<sub>1C</sub> (Pazos *et al.*, 1984). This was subsequently renamed 5-HT<sub>2C</sub> (Humphrey *et al.*, 1993). Partial cloning of the mouse 5-HT<sub>2C</sub> receptor (Lubbert *et al.*, 1987) was followed by the sequencing of the full length clone in, initially the rat (Julius *et al.*, 1988), and then the mouse (Yu *et al.*, 1991) and human (Saltzman *et al.*, 1991). The 5-HT<sub>2C</sub> receptor gene has three introns and may encode a protein product with eight rather than seven transmembrane domains, which if proven would be unusual for G-protein coupled receptors (Yu *et al.*, 1991). The 5-HT<sub>2B/2C</sub> receptors can be distinguished from the 5-HT<sub>2A</sub> receptor by their high affinity for SB 200646A and SB 206553, and their lower affinity for the antagonists MDL 100907, ketanserin and spiperone (Barnes & Sharp, 1999). The 5-HT<sub>2C</sub> receptor antagonists, SB242084 and RS-102221 show at least two orders of magnitude in selectivity for 5-HT<sub>2C</sub> versus 5-HT<sub>2B</sub>, 5-HT<sub>2A</sub> and other binding sites (Bonhaus *et al.*, 1997; Kennett *et al.*, 1997). 5-HT<sub>2C</sub> receptor transcripts and binding sites (particularly using [<sup>3</sup>H]mesulergine autoradiography) are extensively distributed throughout the brain irrespective of species, being especially abundant in the choroid plexus, anterior olfactory nucleus, piriform cortex, amygdala, CA3 region of the hippocampus and in some areas of the basal ganglia. Binding sites were also observed in several thalamic and hypothalamic nuclei, septum, layers III-IV of the cerebral cortex, and with moderate levels in midbrain and brainstem (Pazos *et al.*, 1985b; Hoffman & Mezey, 1989; Mengod *et al.*, 1990; Pompeiano *et al.*, 1994). This

distribution profile corresponds well with the immunohistochemical (Abramowski *et al.*, 1995; Sharma *et al.*, 1997) and mRNA detection of 5-HT<sub>2C</sub> receptors (Pasqualetti *et al.*, 1999). There is evidence for the 5-HT<sub>2C</sub> receptor-mediated excitation of neurones in several brain areas. For example motoneurons of the facial nucleus *in vitro* and *in vivo* are activated by 5-HT or 5-HT<sub>2</sub> receptor agonists and this effect is mediated by the 5-HT<sub>2C</sub> receptor (Larkman & Kelly, 1991; Aghajanian, 1995).

#### 1.5.4 The 5-HT<sub>3</sub> Receptor

The existence of the 5-HT<sub>3</sub> receptor was first reported by Gaddum & Picarelli (1957) who described a contractile M receptor in guinea pig ileum that was antagonised by morphine. At the molecular level, unlike the other 5-HT receptor subtypes the 5-HT<sub>3</sub> receptor is a ligand-gated ion channel (Derkach *et al.*, 1989; Maricq *et al.*, 1991). The structure of the 5-HT<sub>3</sub> receptor is similar to that of the nicotinic acetylcholine receptor since it consists of five subunits forming a pentameric ion channel complex (Cooper *et al.*, 1991; Boess & Martin, 1994). Since the cloning of one of the subunits of this receptor (5-HT<sub>3A</sub>; 487 amino acids) from a mouse neuroblastoma hamster brain (NCB-20) library (Maricq *et al.*, 1991) extensive electrophysiological properties have been characterised and are reviewed elsewhere (Peters *et al.*, 1994; Jackson & Yakel, 1995). Very briefly this ion channel is cation selective and prone to rapid desensitisation leading to fast synaptic transmission (Sugita *et al.*, 1992). An alternatively spliced variant (5-HT<sub>3As</sub>; Hope *et al.*, 1993) and species homologues have been reported in rat, guinea pig and human (Johnson & Heineman, 1992; Isenberg *et al.*, 1993; Belelli *et al.*, 1995; Miyake *et al.*, 1995; Lankiewicz *et al.*, 1998). mRNA for the short form of the splice variant predominates in mouse neuronal tissue, whereas the long form of the 5-HT<sub>3A</sub> subunit is not expressed in humans (Werner *et al.*, 1994). Recently an additional 5-HT<sub>3</sub> receptor subunit has been identified (the human 5-HT<sub>3B</sub> receptor subunit; Davies *et al.*, 1999) which requires co-expression of the 5-HT<sub>3A</sub> subunit for functionality resembling native 5-HT<sub>3</sub> receptors. Several antagonists such as GR65630, MDL72222, ICS 205 930 (tropisetron), odansetron, granisetron and zacopride show high affinity selectivity for the 5-HT<sub>3</sub> receptor. MDL 72222 does however display species differences in affinity being considerably less potent at the guinea pig variant

of the 5-HT<sub>3</sub> receptor (Kilpatrick & Tyers, 1992; Lankiewicz *et al.*, 1998). Despite the large number of selective antagonists there are no current selective 5-HT<sub>3</sub> agonists, although phenylbiguanide (PBG) and 2-methyl 5-HT have moderate affinity (Milburn & Peroutka, 1989; Belelli *et al.*, 1995; Miyake *et al.*, 1995). Despite chlorophenylbiguanide (CPBG; a higher affinity agonist) and PBG both having greater selectivity than 2-methyl 5-HT for the 5-HT<sub>3</sub> receptor, they both potently block dopamine re-uptake (Kilpatrick *et al.*, 1990a & b). Kilpatrick *et al.*, (1987) were the first to demonstrate the presence of 5-HT<sub>3</sub> binding sites using the radioligand [<sup>3</sup>H]GR 65630 and a rat brain homogenate preparation. Since then this radioligand and others have been used to assess the distribution of the 5-HT<sub>3</sub> receptor. In human and rat brain, 5-HT<sub>3</sub> receptors are relatively sparse in comparison to other 5-HT receptors, with highest levels of binding sites detected in the area postrema, nucleus of the solitary tract, dorsovagal complex, trigeminal nucleus caudalis, substantia gelatinosa of the spinal cord and lower densities in limbic areas such as the hippocampus, entorhinal cortex and hippocampus (Waeber *et al.*, 1989; Kilpatrick *et al.*, 1987, 1988 & 1989). These autoradiographic experiments have been confirmed using a 5-HT<sub>3</sub> receptor specific antibody (Morales *et al.*, 1996). One of the functions of the 5-HT<sub>3</sub> receptor is that it can also regulate 5-HT neurotransmission upon its activation. Unlike 5-HT<sub>1B/1D</sub> receptors, these receptors facilitate 5-HT release in guinea pig and rat cortex and hippocampus (Galzin *et al.*, 1990; Barnes *et al.*, 1992; Martin *et al.*, 1992; Blier & Bouchard, 1993). They are not however 5-HT nerve terminal autoreceptors and are thought to be located on an interneuron (Blier *et al.*, 1993).

### **1.5.5 The 5-HT<sub>4</sub> Receptor**

The 5-HT<sub>4</sub> receptor was initially identified in cultured mouse colliculi neurones and guinea-pig hippocampus membranes using a functional assay-stimulation of adenylate cyclase activity (Dumuis *et al.*, 1988a & b). This receptor was cloned from a rat brain cDNA library and shown to identify two full length cDNA sequences which existed as C-terminal splice variants displaying different distributions; 387 (short) and 407 (long) amino acids (Gerald *et al.*, 1995). RT-PCR revealed transcripts of the long form throughout the brain, whereas the short form

was restricted to the striatum (Gerald *et al.*, 1995). A single extra amino acid in the long (5-HT<sub>4L</sub> renamed 5-HT<sub>4(b)</sub>) and short (5-HT<sub>4S</sub> renamed 5-HT<sub>4(a)</sub>) forms was elucidated in the rat after the cloning of the human 5-HT<sub>4L</sub> subtype identified a frameshift produced by an open reading frame in the previously reported rat 5-HT<sub>4L</sub> receptor (Van den Wyngaert *et al.*, 1997). Two additional splice variants of the 5-HT<sub>4</sub> receptor have since been identified in tissues from mouse, rat and human, 5-HT<sub>4(c)</sub> and 5-HT<sub>4(d)</sub> which encode polypeptide sequences of 380 and 360 amino acids respectively, with all four isoforms diverging after Leu<sup>358</sup> (Blondel *et al.*, 1998; Bockaert *et al.*, 1998). The pharmacology of the human receptor isoforms has been reported to be similar (Blondel *et al.*, 1998). BIMU-1 and BIMU-8 have been described as selective agonists (Turconi *et al.*, 1989) compared to the moderate affinity partial agonists zacopride and cisapride (Bockaert *et al.*, 1992; Grossman *et al.*, 1993; Gerald *et al.*, 1995; Van den Wyngaert *et al.*, 1997). Tropisetron acts as a 5-HT<sub>4</sub> antagonist with moderate potency. There are now several highly potent and selective 5-HT<sub>4</sub> receptor antagonists available such as, GR113808, RS 23597-190, LY 297582, SB207266, SB207710, SB2047266, SB204070, SB203186 and SDZ 205557 (Grossman *et al.*, 1993; Medhurst & Kaumann, 1993; Gerald *et al.*, 1995; Wardle *et al.*, 1996; Van den Wyngaert *et al.*, 1997; Bockaert *et al.*, 1998). The autoradiographic localisation of binding sites using radioligands such as [<sup>3</sup>H]GR113808 or [<sup>3</sup>H]BIMU-1, and localisation of mRNA in the rat CNS is identical with high levels in the basal ganglia (especially in caudate-putamen, globus pallidus and substantia nigra) and lower levels in the limbic regions of the hippocampus, amygdala and frontal cortex (Gerald *et al.*, 1995; Claeysen *et al.*, 1996; Mengod *et al.*, 1996). All isoforms are expressed in the gut, but only 5-HT<sub>4(a)/(b)</sub> and 5-HT<sub>4(c)</sub> are found in the brain and heart (Blondel *et al.*, 1998). 5-HT<sub>4</sub> receptor-mediated cAMP accumulation activates protein kinase A (Gerald *et al.*, 1995; Van den Wyngaert *et al.*, 1997), with subsequent phosphorylation of potassium channels leading to their closure and reduced potassium conductance producing depolarisation in mouse collicular and hippocampal neurones (Andrade & Chaput, 1991; Fagni *et al.*, 1992). In cardiac muscle cells 5-HT<sub>4</sub> receptor stimulation activates voltage-sensitive calcium channels via protein kinase A (Kaumann *et al.*, 1990; Ouadid *et al.*, 1992).

### 1.5.6 The 5-HT<sub>5</sub> Receptor Family

The 5-HT<sub>5</sub> receptor family encompasses two-intron containing recombinant receptors (5-HT<sub>5A</sub> and 5-HT<sub>5B</sub>) which share 68% amino acid sequence identity (Erlander *et al.*, 1993). It is the least well understood of all the 5-HT receptor classes, with no reports of specific binding to native 5-HT<sub>5</sub> binding sites or of functional responses.

#### The 5-HT<sub>5A</sub> Receptor

The 5-HT<sub>5A</sub> receptor gene has been cloned from mouse, rat and man (Plassat *et al.*, 1992; Erlander *et al.*, 1993; Rees *et al.*, 1994). In transfected cells the recombinant 5-HT<sub>5A</sub> receptor displays high affinity [<sup>125</sup>I]LSD binding which is displaceable by ergotamine, 5-CT, 5-HT methysergide and methiothepin (Plassat *et al.*, 1992; Erlander *et al.*, 1993; Rees *et al.*, 1994). The presence of high and low affinity sites for [<sup>3</sup>H]5-CT binding suggests that this subtype is G protein-coupled (Plassat *et al.*, 1992), and a negative coupling to adenylate cyclase has been observed (Carson *et al.*, 1996). *In situ* hybridisation experiments have revealed a widespread distribution of 5-HT<sub>5A</sub> receptor mRNA throughout the mouse, rat and human brain, in areas such as the cerebral cortex, dentate gyrus, hippocampus and the cerebellum (Plassat *et al.*, 1992; Erlander *et al.*, 1993; Pasqualetti *et al.*, 1998). Site specific antibodies designed to the 5-HT<sub>5A</sub> receptor have revealed 5-HT<sub>5A</sub> immunoreactivity in the rat hypothalamus, hippocampus, corpus callosum and olfactory bulb, and appear to be associated primarily with astrocytes (Carson *et al.*, 1996). 5-HT<sub>5A</sub> protein has yet to be mapped in the brain due to the lack of selective ligands.

#### The 5-HT<sub>5B</sub> Receptor

The 5-HT<sub>5B</sub> receptor gene has been cloned from mouse, rat and man (Plassat *et al.*, 1992a & b; Erlander *et al.*, 1993; Rees *et al.*, 1994). The pharmacological profile of this subtype is comparable, but distinguishable, to that of the 5-HT<sub>5A</sub> receptor (Erlander *et al.*, 1993; Matthes *et al.*, 1993). Both these receptor subtypes do however show pharmacological similarities to the 5-HT<sub>1D</sub> receptor subtype, with 5-CT, LSD, ergotamine, methiothepin and sumatriptan all showing relatively high affinity. A discrete expression of 5-HT<sub>5B</sub> mRNA is apparent in the CA1 field of the hippocampus, medial and lateral habenula, dorsal raphe in the mouse and rat brain (Matthes *et al.*, 1993; Wisden *et al.*, 1993). However 5-HT<sub>5B</sub> receptor protein has yet



to be mapped in the brain due to the lack of selective ligands. As for the 5-ht<sub>5A</sub> receptor, [<sup>3</sup>H]5-CT labels two affinity states of the 5-ht<sub>5B</sub> receptor which are sensitive to guanine nucleotide regulation (Wisden *et al.*, 1993). Despite this evidence of G protein coupling, no data is currently available for second messenger linkage in native tissue.

### 1.5.7 The 5-ht<sub>6</sub> Receptor

The 5-ht<sub>6</sub> receptor was cloned from a rat cDNA library, initially revealing two different sequences (Monsma *et al.*, 1993; Ruat *et al.*, 1993a), which after the cloning of the human 5-ht<sub>6</sub> receptor (Kohen *et al.*, 1996), were considered to be identical. The 5-ht<sub>6</sub> receptor has received considerable attention due to the interaction of a high number of anti-psychotic (notably clozapine, olanzipine, rilapine, fluperlapine & seroquel) and antidepressant drugs (clomipramine, amitriptyline, doxepin & nortriptyline) with this receptor at clinically relevant concentrations (Hoyer *et al.*, 1994; Sebben *et al.*, 1994; Glatt *et al.*, 1995; Kohen *et al.*, 1996). Transfected receptors can be labelled with [<sup>3</sup>H]LSD and [<sup>3</sup>H]5-HT (Monsma *et al.*, 1993; Roth *et al.*, 1994; Kohen *et al.*, 1996). Only recently have two selective antagonists been identified (Ro 04-6790 & Ro 63-0563; Sleight *et al.*, 1998). Ro 63-0563 is however unsuitable for native labelling of the 5-ht<sub>6</sub> receptor due to the high (70-90%) non-specific binding and the low expression of this subtype (Boess *et al.*, 1998). Specific site directed antibodies have revealed a brain distribution with highest levels in the cerebral cortex, nucleus accumbens, striatum, CA1 region and dentate gyrus of the hippocampus and lower levels in the thalamus and substantia nigra (Gerald *et al.*, 1997). The distribution of 5-ht<sub>6</sub> mRNA appears largely to be confined to the brain though low levels are found in the stomach and adrenal glands (Ruat *et al.*, 1993a) and closely matches 5-ht<sub>6</sub> receptor protein expression as revealed by immunological studies (Gerard *et al.*, 1997). The 5-ht<sub>6</sub> receptor stimulates adenylate cyclase activity (Ruat *et al.*, 1993a; Sebben *et al.*, 1994) but no functional correlates have been positively identified. However studies using antisense oligonucleotides induced a behavioural syndrome of yawning and stretching that could be dose dependently blocked by atropine, suggesting an involvement of the 5-ht<sub>6</sub> receptor on the cholinergic system (Bourson *et al.*, 1995).

### 1.5.8 The 5-HT<sub>7</sub> Receptor

The 5-HT<sub>7</sub> receptor is the most recently identified 5-HT receptor. Using degenerate oligonucleotides corresponding to conserved sequences amongst other receptor families, 5-HT<sub>7</sub> receptor cDNA has been identified from a number of species including human, rat, mouse, guinea pig and toad (Bard *et al.*, 1993; Lovenberg *et al.*, 1993a & b; Meyerhof *et al.*, 1993; Plassat *et al.*, 1993; Ruat *et al.*, 1993b; Shen *et al.*, 1993; Tsou *et al.*, 1994; Nelson *et al.*, 1995). The 5-HT<sub>7</sub> receptor appears to be the mammalian species homologue of the 5-HT<sub>dro1</sub> receptor identified in *Drosophila melanogaster* (Witz *et al.*, 1990). Of the two introns in the 5-HT<sub>7</sub> receptor gene, one is responsible for the presence of a second extracellular loop (Heidman *et al.*, 1997) and the other corresponds to the C-terminal and is responsible for the generation of at least four currently recognised splice variants (5-HT<sub>7(a)</sub>, 5-HT<sub>7(b)</sub>, 5-HT<sub>7(c)</sub>, and 5-HT<sub>7(d)</sub>; Heidmann *et al.*, 1997). The pharmacological profile of 5-HT<sub>7</sub> receptors is unique (reviewed in Eglen *et al.*, 1997) with the non-selective agonists 5-CT and 5-MeOT, the non-selective antagonists methiothepin and metergoline, the 5-HT<sub>2</sub> antagonists mesulergine and ritanserin having high affinity and the 'selective' 5-HT<sub>1A</sub> receptor agonist, 8-OH DPAT displaying moderate affinity (Shen *et al.*, 1993; Ruat *et al.*, 1993b, Tsou *et al.*, 1994; To *et al.*, 1995). The pharmacology of the different receptor isoforms have not been directly compared, though preliminary experiments reveal a similar pharmacology between the 5-HT<sub>7(a)</sub> and 5-HT<sub>7(b)</sub> isoforms (Jasper *et al.*, 1997). To date only one selective antagonist has been identified SB-258719 (Forbes *et al.*, 1998; Thomas *et al.*, 1998). Previous autoradiographic studies have relied on using non-selective ligands such as [<sup>3</sup>H]5-CT in the presence of masking drugs to attempt to block non-5-HT<sub>7</sub> binding. In rat and guinea pig brain, both the mRNA and receptor binding sites display a similar distribution, and expression is relatively high within regions of the thalamus, hypothalamus and hippocampus with generally lower levels in areas such as the cerebral cortex and amygdala (To *et al.*, 1995; Gustafson *et al.*, 1996; Stowe & Barnes, 1998b). The 5-HT<sub>7</sub> receptor transcript has also been identified in several other organs including the spleen, kidney, pancreas, stomach and ileum of both human and rat, (Ruat *et al.*, 1993b; Shen *et al.*, 1993; Bard *et al.*, 1993; Stam *et al.*, 1997) and more recently in the rat adrenal gland (Contesse *et al.*, 1999). Additionally

5-HT<sub>7</sub> mRNA has been detected in a variety of vascular tissues, including microvessels in human cerebral cortex, saphenous vein, aortic and pulmonary artery smooth muscle, and in the vena cava, femoral vein, aorta and renal artery of the rat (Ullmer *et al.*, 1995; Cohen & Hamel, 1996; Hamblin & Heidmann, 1996). The 5-HT<sub>7</sub> receptor isoforms show differential species and regional distribution and expression levels as reviewed by Heidmann *et al.*, (1997 & 1998). Both the recombinant and the native 5-HT<sub>7</sub> receptor stimulate adenylate cyclase (Shen *et al.*, 1993; Heidmann *et al.*, 1997 & 1998). At present no direct functional evidence is apparent for the behavioural, biochemical or neuroendocrine effects in Table 1.5 as seen with activation of the 5-HT<sub>1-4</sub> receptor subtypes. However activation of the 5-HT<sub>7</sub> receptor has been shown to produce effects on vasculature, gastric motility, and circadian rhythms. The 5-HT<sub>7</sub> receptor has recently been shown to be responsible for mediating smooth muscle relaxation in a number of vascular tissues (Martin & Wilson, 1996; Cushing *et al.*, 1996; Leung *et al.*, 1996; Terrón, 1996 & 1997a; Terrón & Falcón-Neri, 1999). It is also now known that the 5-HT<sub>7</sub> receptor mediates 5-HT induced hypotension (De Vries *et al.*, 1997; Terrón, 1997b) without affecting cardiac output or blood flow to vital organs (De Vries *et al.*, 1998). This 5-HT<sub>7</sub> receptor subtype may therefore be a target for anti-hypertensive therapy. 5-HT<sub>2B</sub> receptor activation can cause stomach fundus contraction (Kursar *et al.*, 1992), and 5-HT<sub>7</sub> receptor activation can cause smooth muscle contraction in the rat jejunum (McClellan & Coupar, 1996), smooth muscle relaxation in the guinea-pig ileum (Carter *et al.*, 1995) and porcine myometrium (Kitizawa *et al.*, 1998). The finding that 8-OH DPAT induces a phase shift in neuronal activity in the suprachiasmatic nucleus has now been shown to be associated with 5-HT<sub>7</sub> receptor activation (Lovenberg *et al.*, 1993a; Ying & Rusack, 1997) rather than 5-HT<sub>1A</sub> receptor stimulation as first thought (Prosser *et al.*, 1993). The development and use of more selective antagonist and agonist ligands will help to further evaluate the functional roles of this 5-HT receptor subtype.

## 1.6 The 5-HT Transporter

After transmitter release from activated nerve terminals, the principle mechanism involved in the rapid clearance from the synapse is transport of the transmitter back into presynaptic nerve terminals by one of a large number of structurally and pharmacologically distinguishable transport proteins (Amara & Kuhar, 1993).

### 1.6.1 Cloning, Structure, Localisation and Classification

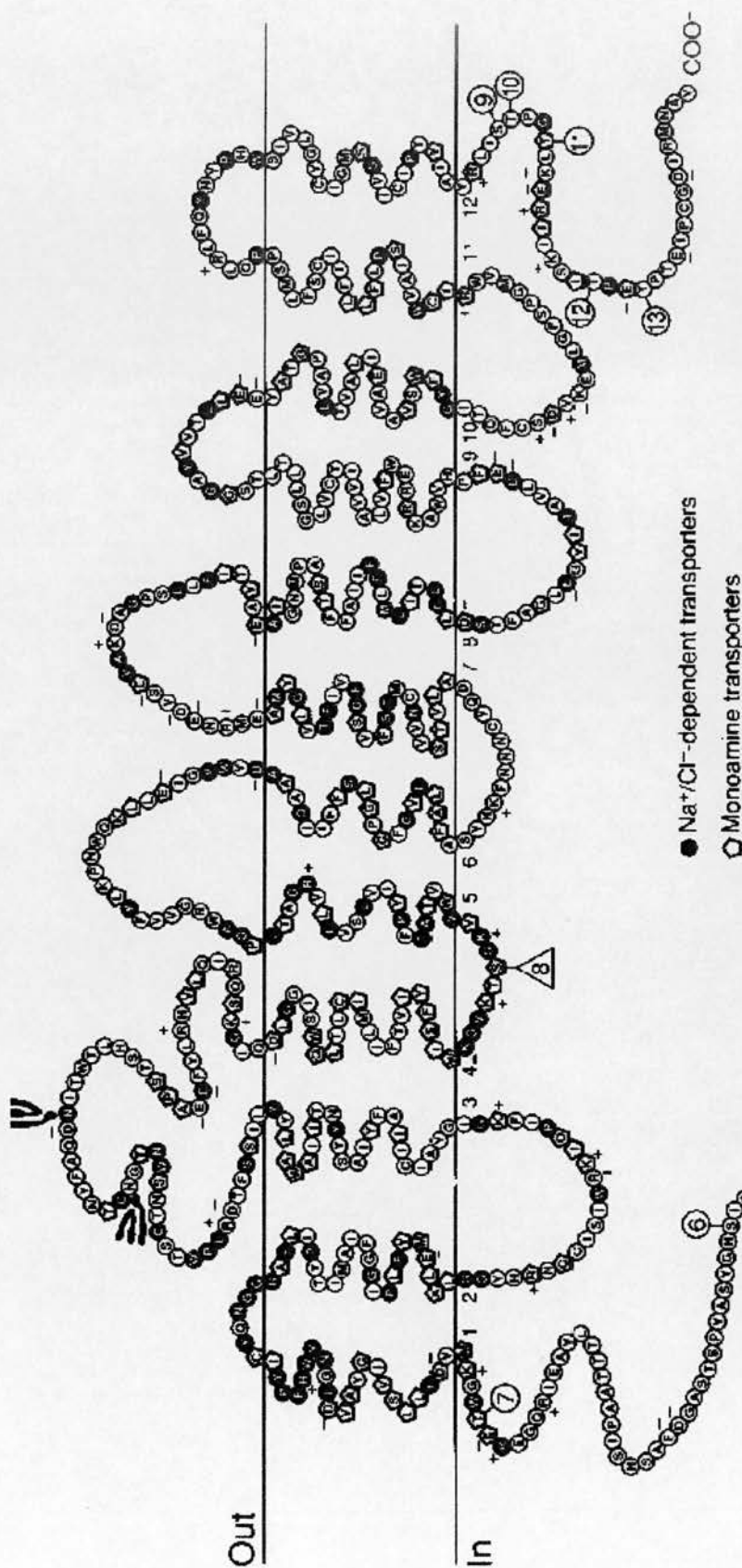
#### Cloning

The molecular characterisation of neurotransmitter transporters began with the purification, amino acid sequencing, and cloning of the rat and human  $\gamma$ -aminobutyric acid (GABA) transporter (GAT; Radian *et al.*, 1986; Guastella *et al.*, 1990; Nelson *et al.*, 1990). Within 6 months, a cDNA clone for the cocaine and antidepressant sensitive human noradrenaline transporter (NET) was cloned (Pacholczyk *et al.*, 1991). Alignment of GAT and NET sequences demonstrated a 46% amino acid identity and revealed the sequence motifs of a novel neurotransmitter transporter gene family with a similar inferred membrane topography of 12 regions of high hydrophobicity in spans long enough to form transmembrane domains (TMDs). Degenerate oligonucleotides designed to the conserved sequences of GAT and NET served as probes for homology cloning, resulting in the identification of eight distinct transporter homologues expressed in rodent and human brain using PCR techniques (Peek *et al.*, 1991). One of these PCR products recognised mRNA overlying cells of the midbrain and brain stem raphe nuclei (Blakely *et al.*, 1991), which are the principle sites of serotonergic neurons in the rat brain (Steinbusch, 1984). This clone was shown to be a functional 5-HT selective transporter (SERT) due to its sensitivity to selective uptake blockers, including paroxetine, citalopram and fluoxetine and also by the ability of low concentrations of addictive amphetamines and cocaine to block 5-HT uptake in transfected cells (Blakely *et al.*, 1991). A cDNA encoding a functional SERT was also identified in the mast cell line RBL (rat basophilic leukaemia; Hoffman *et al.*, 1991) and shown to be identical to rat brain SERT (Blakely *et al.*, 1991). This suggested that neuronal and non-neuronal SERTs are encoded by a common gene

(Blakely *et al.*, 1993). Cloning of cDNAs encoding functional SERTs from human placental (Ramamoorthy *et al.*, 1993a), brain (Blakely *et al.*, 1993; Lesch *et al.*, 1993b) and platelets (Lesch *et al.*, 1993c) and from other rodent species (mouse: Chang *et al.*, 1996; guinea pig: Chen *et al.*, 1997), have revealed that rodent and human SERTs show >90% cross-species sequence identity. Furthermore, peripheral and CNS SERT are identical, suggesting that they encoded by a common gene, a hypothesis supported by the identification of a single SERT loci in human (Ramamoorthy *et al.*, 1993a) and mouse (Gregor *et al.*, 1993). SERT genes are fragmented by multiple introns (Lesch *et al.*, 1994) and thus could give rise to multiple transcripts by alternative RNA processing. Peripheral and CNS SERT do show differences in size, which may involve differential post-translational modifications. For example, in SDS western blots, rat platelet SERT migrates to 94 kDa, whereas brain SERT migrates to 76 kDa (Qian *et al.*, 1995). This difference in electrophoretic mobility is due to differential deglycosylation, with platelet SERT being more heavily glycosylated. Sequential stages of transporter glycosylation are also apparent for SERTs transfected in HeLa cells (Melikian *et al.*, 1994). Glycosylation plays a role in receptor and transporter assembly and trafficking (Rands *et al.*, 1990; Asano *et al.*, 1993; Collier *et al.*, 1993), and in some cases contribute to functional properties (Boege *et al.*, 1988; Leconte *et al.*, 1992). However in the case of SERT, glycosylation is required for optimal stability of the SERT in the membrane but not for serotonin transport or ligand binding *per se* (Tate & Blakely, 1994).

### **Structure**

The structure of SERT consists of 630 amino acids arranged in 12  $\alpha$ -helical transmembrane domains (TMDs), with intracellular and extracellular loops, similar to the structure of GAT and NET (Figure 1.6). The carboxy and amino terminals are predicted to be intracellular (Figure 1.6). Multiple sites for N-linked glycosylation are located on the large extracellular loop between TMDs 3 and 4, whereas multiple consensus sequences for possible phosphorylation are found in the intracellular domains (Figure 1.6). The greatest sequence identity to NET is in TMDs 1-2 and 5-8,



**Figure 1.6: Topological Representation of 5-HT Transporter.**

Residues conserved among all members of the  $\text{Na}^+/\text{Cl}^-$ -dependent transporter family are encoded as black circles with white letters, while residues conserved among mammalian (mouse, rat, human) 5-HT, DA, and NE transporters are encoded as pentagons with black letters. Transmembrane domains are numbered 1 through 12 at the putative intracellular/membrane interface. Potential phosphorylation sites are symbolized by numbers in circle and stem structures or a number in a triangle and stem structure. The number "8" in the stem and triangle structure is conserved among all of the monoamine plasma membrane transporters. The enclosed numbers correspond to canonical phosphorylation sites as follows: 1, proline kinase; 2, protein kinase C; 3, protein kinase A and cGMP-dependent kinase; 4, cGMP-dependent kinase; 5, casein kinase I; 6, glycogen synthase kinase 3; 7, cGMP-dependent kinase; 8, protein kinase C and cGMP-dependent kinase; 9, proline kinase A and cGMP-dependent kinase; 10, proline kinase; 11, proline kinase C; 12, protein kinase A and cGMP-dependent kinase; 13, proline kinase. Potential glycosylation sites are shown as "tree" structures on the extracellular loop between transmembrane domains 3 and 4. Figure taken from Hoffman *et al.*, 1998.

whereas the greatest sequence divergence is in regions between putative TMDs as well as in the predicted cytoplasmic NH<sub>2</sub> and COOH-terminals (Figure 1.6).

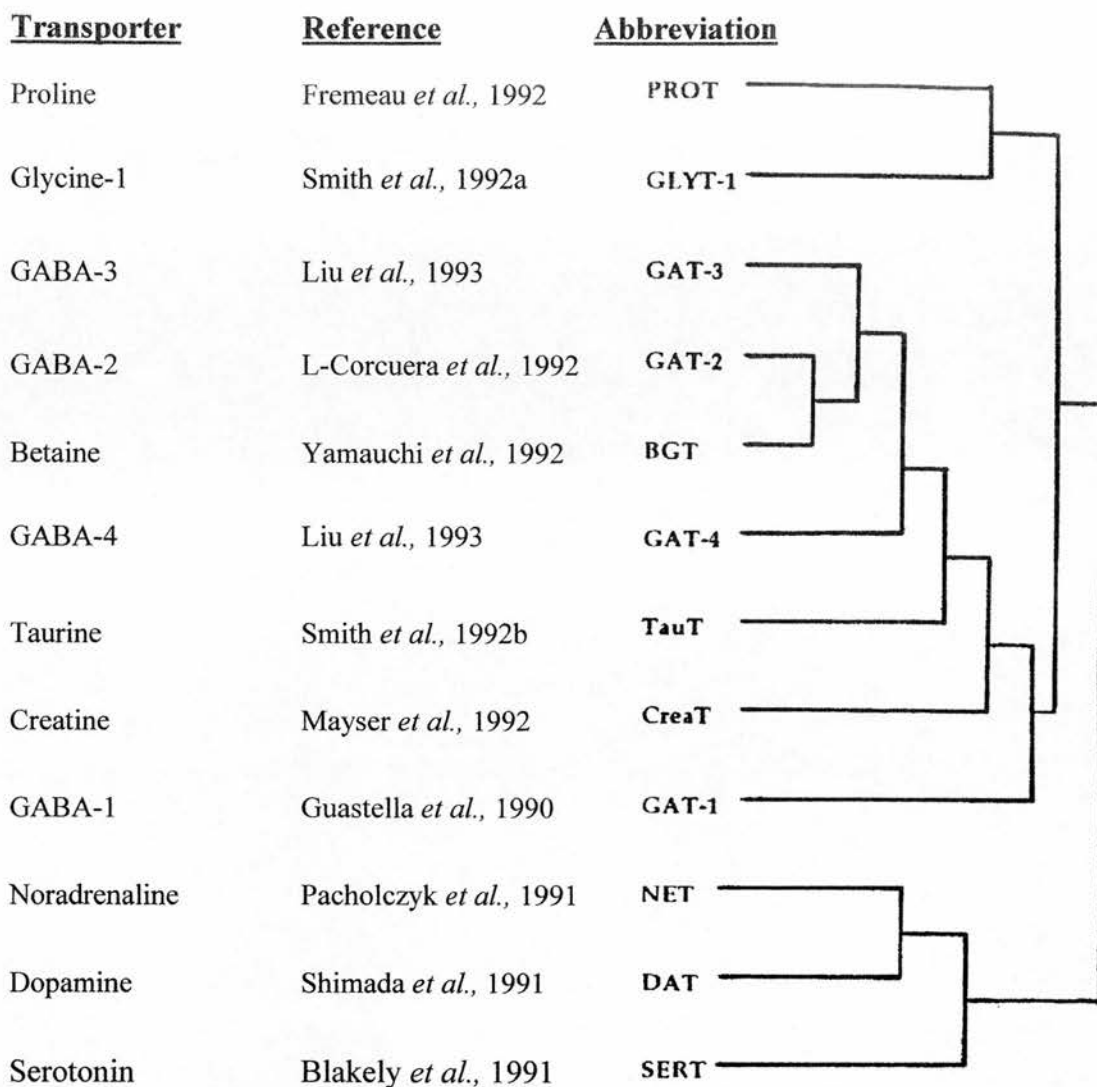
### **Localisation**

In the CNS, cells in the raphé nuclei express high levels of SERT mRNA with lower but detectable levels in the projectional terminal fields of areas such as the cortex, hippocampus and striatum (Blakely *et al.*, 1991; Fujita *et al.*, 1993; Austin *et al.*, 1994). Though SERT activity has been reported in astrocytes (Bel *et al.*, 1997), there is no evidence of glial SERT protein (Hoffman *et al.*, 1998; Masson *et al.*, 1999). In addition, SERT activity has been described in certain peripheral tissues, including platelets (Sneddon, 1973; Rudnick, 1977), lung (Paczkowski *et al.*, 1996) and in human resting lymphocytes (Mazarreti *et al.*, 1998). SERT mRNA has also been detected in the rat adrenal gland (Blakely *et al.*, 1991; Hoffman *et al.*, 1991).

### **Classification**

The cloning of other neurotransmitter transporters has revealed a family of transporters all with a 12 TMD structure. These neurotransmitter transporters form three distinct families based on their amino acid sequence homology, membrane location and ionic dependence: (1) Na<sup>+</sup> and Cl<sup>-</sup> dependent transporters that operate on the plasma membrane; (2) Na<sup>+</sup> and K<sup>+</sup> dependent transporters that function on the plasma membranes, especially in glutamate transport (3) vesicular transporters that function in uptake into synaptic vesicles and granules.

The Na<sup>+</sup> and Cl<sup>-</sup> dependent neurotransmitter transporter family is further subdivided based upon amino acid sequence conservation (Figure 1.7) and gene structure into 3 subfamilies including: (1) the GABA, betaine, taurine and creatine transporters; (2) the amino acid (L-proline and glycine) transporters; and (3) the biogenic amine transporters which includes transporters for dopamine, noradrenaline and 5-HT (DAT, NET and SERT respectively). For example, DAT, NET and SERT genes encode a large extracellular loop on an exon that also encodes the third transmembrane domain, whereas these domains are split by an intron in the murine GAT gene (Lesch *et al.*, 1994; Liu *et al.*, 1993). Recently four new members of this family have been identified, though these are still to be established as actual transporters since their respective substrates have not been identified. These



**Figure 1.7: Dendrogram of Amino Acid Relationships Between Members of the Na<sup>+</sup> and Cl<sup>-</sup> Dependent Plasma Membrane Transporters.**

Figure taken and adapted from Shafqat *et al.*, 1993. Dendrogram shows that three distinct subfamilies can be resolved based upon amino acid sequence similarities: the subfamily of amino acid (L-proline and glycine) transporters; the subfamily of GABA, betaine, taurine and creatine transporters; and the subfamily of biogenic amine transporters. GLYT-2 is identical to GLYT-1 except for the first 15 amino acids (Borowsky *et al.*, 1993) and is not shown.



“orphan” transporters exhibit significant homology with the “classical” members such as DAT, SERT, GAT and glycine transporters despite distinct membrane topographies with large second and fourth extracellular loops with the presence of an additional site for N-linked glycosylation in the later (see Masson *et al.*, 1999).

The details of the biogenic amine transporters are discussed briefly below due to SERT being present in this sub family of the Na<sup>+</sup> and Cl<sup>-</sup> dependent family of neurotransmitter transporters. The vesicular monoamine transporters are also briefly reviewed below due to their involvement in 5-HT neurotransmission. More comprehensive reviews are available (Henry *et al.*, 1994; Liu & Edwards, 1997; Peter *et al.*, 1998; Masson *et al.*, 1999). SERT is not a member of the Na<sup>+</sup> and K<sup>+</sup> dependent transporters which are predominantly involved in glutamate uptake and may be reviewed with the other neurotransmitter transporters elsewhere (Shafqat *et al.*, 1993; Amara & Kuhar, 1993; Kanner, 1994; Rudnick, 1997; Masson *et al.*, 1999).

### **The Biogenic Amine and Vesicular Monoamine Transporters**

Dopamine (DA), noradrenaline (NA) and 5-HT are the main substrates for DAT, NET and SERT respectively. However these transporters may also transport each others main substrates, albeit with a lower affinity, and a variety of neurotoxins including addictive amphetamines (Table 1.6). The uptake ability of these biogenic transporters may also be blocked by a variety of specific uptake inhibitors and cocaine (Table 1.6). For example citalopram and fluoxetine are considered serotonin specific reuptake inhibitors (SSRIs) due to their higher affinity for SERT, whereas nomifensine has reasonably high affinity for both NET and DAT and is not considered a specific uptake inhibitor. Bupropion though weak in potency is more specific for DAT, whereas DMI and nisoxetine are more selective for NET (Table 1.6). The vesicular monoamine transporters (VMATs), in contrast to those at the plasma membrane, translocate transmitters from the cytoplasm into vesicles in preparation for regulated release by exocytosis. In addition, a single vesicular transport activity recognises all of the monoamine transmitters with similar sub-micromolar affinity, occurring in DA, NA and 5-HT neurons with 5-HT having slightly higher affinity (~0.2µM; Liu & Edwards, 1997). Indeed one vesicular transporter, VMAT<sub>2</sub> is responsible for these re-uptake

Substrates "Classical"	Biogenic system (Affinity of uptake $K_m$ in $\mu M$ )		
	Serotonergic	Noradrenergic	Dopaminergic
5-HT	0.1	8	>100
Noradrenaline	>50	0.1	0.1
Dopamine	50	0.1	0.1

**Neurotoxins are substrates and examples are listed below:  $K_{ms} \sim 1\mu M$**

	Amphetamine	Amphetamine	Amphetamine
	p-chloroamphetamine	p-chloroamphetamine	p-chloroamphetamine
	MDMA	MDMA	MDMA
	fenfluramine	fenfluramine	fenfluramine
	MPTP (MPP <sup>+</sup> )	MPTP (MPP <sup>+</sup> )	MPTP (MPP <sup>+</sup> )
	5,7 DHT*	6-OH DA	6-OH DA**

Uptake Inhibitors	Biogenic system (Affinity of inhibition of uptake $K_i$ in nM)		
	Serotonergic	Noradrenergic	Dopaminergic
Citalopram	1.3	4000	280000
Fluoxetine	12	280	1600
DMI	340	0.9	5200
Nisoxetine	310	1.3	510
Nomifensine	1280	5	51
Bupropion	15600	2300	630
Cocaine	180	155	270

**Table 1.6: The Main Substrates and Examples of Inhibitors of Biogenic Amine Transporters**

Substrate data from Amara & Kuhar (1993). Inhibitor data from Richelson & Pfenning (1984). Amphetamine and its derivatives (p-chloroamphetamine, MDMA = 3,4-methylenedioxymetamphetamine (Ecstasy) and fenfluramine) are also substrates for SERT along with the classical substrates (5-HT, noradrenaline and dopamine). MPTP = N-methyl-1,2,3,6-tetrahydropyridine. MPP<sup>+</sup> = N-methyl-4-phenylpyridinium and is the active metabolite of MPTP. 5,7-DHT = 5,7 dihydroxytryptamine. 6-OH DA = 6-hydroxy dopamine. \* = in the presence of DMI to block NET. \*\* = in the presence of DMI to block NET and pargyline to prevent oxidation.

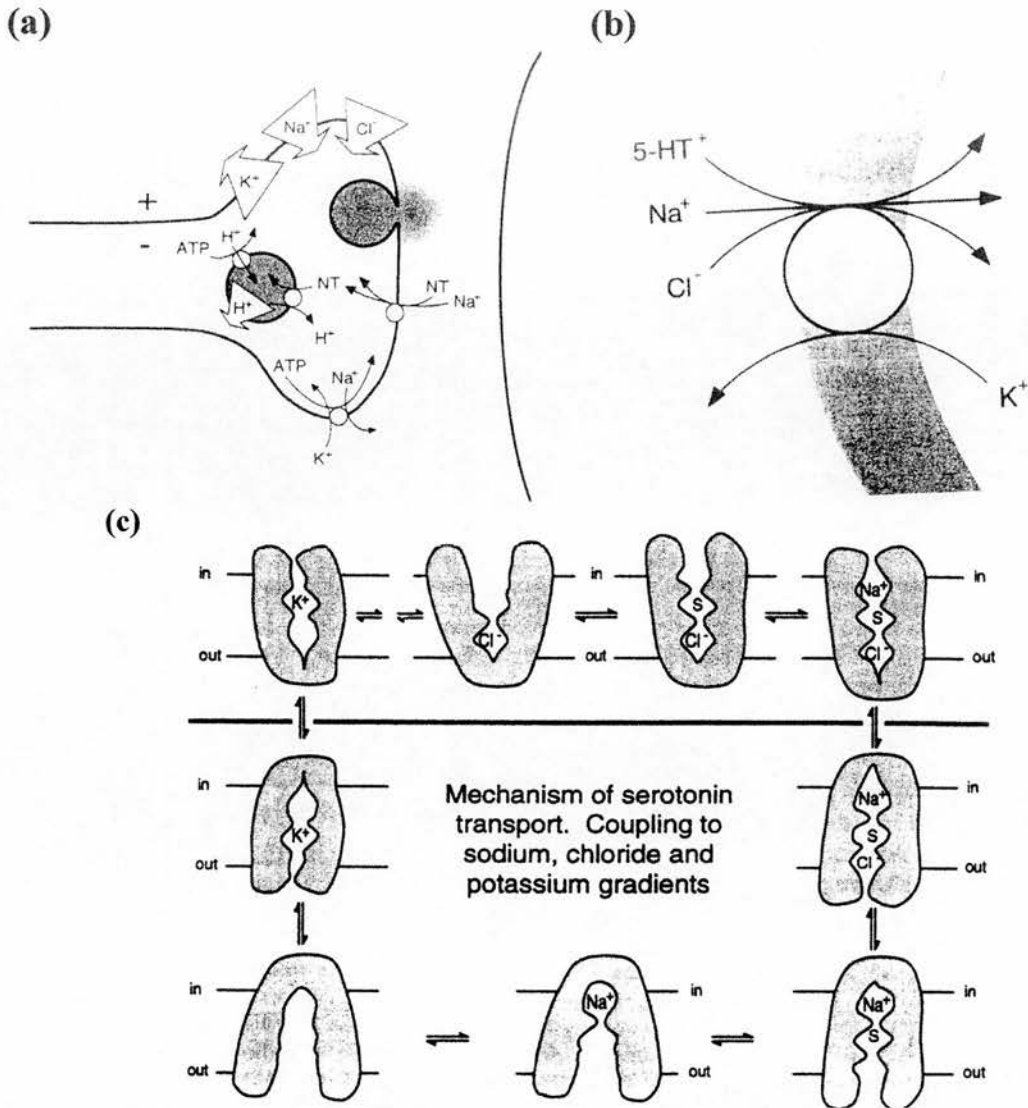
properties in the brain, which is sensitive to inhibition by both reserpine and tetrabenazine (see Liu & Edwards, 1997; Masson *et al.*, 1999). Other vesicular transporters, with different specificities are found in neurons that release amino acid neurotransmitters and acetylcholine (see Usdin *et al.*, 1995; Liu & Edwards, 1997; McIntire *et al.*, 1997)

## 1.6.2 Mechanisms of 5-HT Uptake

Outlined in Figure 1.8a is the recapture of released neurotransmitter, which via the plasma membrane is only the first of two steps required for efficient neurotransmitter recycling. A second transport process sequesters cytoplasmic transmitters within synaptic vesicles via VMAT, in preparation for their release by exocytosis.

### At the Plasma Membrane

As outlined in Figure 1.8b inwardly-directed  $\text{Na}^+$  and  $\text{Cl}^-$  gradients, and outwardly-directed  $\text{K}^+$  or  $\text{H}^+$  gradients serve as driving forces for 5-HT transport across the plasma membrane (Rudnick & Clark, 1993). These electrochemical gradients are created and maintained by the plasma membrane  $\text{Na}^+/\text{K}^+$  ATPase (Kanner & Schuldiner, 1987; Figure 1.8a). When these appropriate transmembrane ion gradients are imposed, the nerve terminal accumulates 5-HT concentrations several hundred fold higher than in the external medium. Using stably transfected cells, the ionic dependence of monoamine reuptake has been established (Gu *et al.*, 1994, 1996 & 1998). For DA, two  $\text{Na}^+$  ions are cotransported with the substrate while NA and 5-HT are cotransported with only a single  $\text{Na}^+$  ion (Gu *et al.*, 1994, 1996 & 1998). All three transporters also cotransport a single  $\text{Cl}^-$  ion with the substrate (Gu *et al.*, 1998). A theoretical model for substrate translocation has been suggested whereby SERT may assume open and closed channel-like states which differ only in the accessibility of the central binding site (see Rudnick & Clark, 1993; Figure 1.8c). Thus the transporter may behave like a channel with a gate at each face of the membrane, but only one gate may be open at any point in time (the gate-lumen gate theory). Electrophysiological methods have uncovered a  $\text{Na}^+$  flux not accounted for by the ionic dependence of substrate translocation. Using both *Xenopus* oocytes (Lester *et al.*, 1994; Mager *et al.*, 1994; Deflice & Blakely, 1996; Lin *et al.*, 1996;



**Figure 1.8: Mechanism of 5-HT Re-uptake at the Plasma Membrane**  
 In (a) Neurotransmitter recycling is represented. Gradients of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> generated by the plasma membrane Na<sup>+</sup>/K<sup>+</sup>-ATPase drive influx of released neurotransmitter (NT) across the plasma membrane. Once inside the nerve terminal, the transmitter is accumulated within synaptic vesicles by exchange with intravesicular H<sup>+</sup> ions supplied by the vacuolar H<sup>+</sup>-pumping ATPase. In (b) Driving forces of 5-HT transport are shown. Inwardly-directed gradients of Na<sup>+</sup> and Cl<sup>-</sup> are coupled by SERT to serotonin influx by a process of symport (or cotransport). The K<sup>+</sup> gradient also serves as a driving force for the transporter, which catalyses exchange (countertransport or antiport) of internal K<sup>+</sup> for external 5-HT. In (c) a model for Na<sup>+</sup>, Cl<sup>-</sup> and K<sup>+</sup> coupling to 5-HT transport is shown. Counter clockwise from the lower left, SERT is shown binding external Na<sup>+</sup>, 5-HT (s) and Cl<sup>-</sup> and then interconverting to a form (above the line) in which the binding sites for these ligands is accessible from the cytoplasm. This interconversion represents the transport process. Following dissociation of Na<sup>+</sup>, 5-HT and Cl<sup>-</sup>, the transporter binds internal K<sup>+</sup> (upper left) and converts to a form which can release K<sup>+</sup> to the external medium (left side) to return to the starting point. Figure from Rudnick & Clarke (1993).

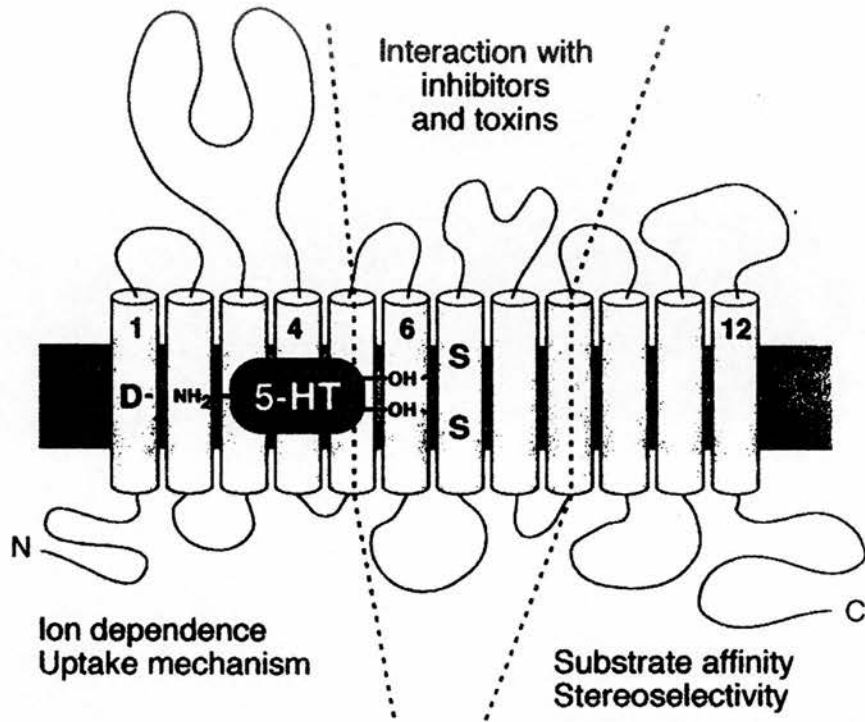
Zhu *et al.*, 1997; Zahiser *et al.*, 1998) and mammalian cells (Galli *et al.*, 1995, 1996 & 1997) expressing these transporters, two different antidepressant-sensitive  $\text{Na}^+$  fluxes were detected, one associated with the transport cycle and the second a leak current in the absence of substrate. These data suggest these transporters have a channel-like activity and are reviewed by Lester *et al.*, (1996) and Sonders & Amara, (1996). It has also now been suggested that the transporter-associated current is because of both gates remaining open (Cao *et al.*, 1998).

### **At the Vesicular Membrane**

The transport system consists of two components: (i) a vacuolar ATP-driven  $\text{H}^+$  pump that acidifies the vesicle interior creating a transmembrane pH difference in the absence of ATP and the development of a transmembrane electrical potential, and (ii) the reserpine-sensitive vesicular amine which couples efflux of two  $\text{H}^+$  ions to the uptake of each molecule of the 5-HT (see Rudnick & Clark, 1993). Because the amine substrate is exchanged for the equivalent of two  $\text{H}^+$  ions, a 10-fold  $\text{H}^+$  concentration gradient (one pH unit) will lead to a 100-fold gradient of substrate. Membrane potential is not as strong a driving force, since only one charge crosses the membrane with each catalytic cycle (see Rudnick & Clark, 1993).

### **1.6.3 Functional Domains of SERT**

A hypothetical model for functional domains of SERT has been proposed based on the known structure of this transporter (Figure 1.9). The combination of molecular biology and pharmacological techniques has allowed this model to be assessed. A useful strategy for identifying ligand binding domains of G-protein-coupled receptors and ion channels has been the exploitation of species differences in antagonist potencies, in which cross-species chimeras and site-directed mutagenesis localise domains and residues critical for ligand interaction (Jackson *et al.*, 1991; Oksenberg *et al.*, 1992; Olah & Stiles, 1997). Using chimeras of rat and human SERTs, the carboxy region of SERT distal to TMD11 was identified as the domain involved in the species selectivity of imipramine and d-amphetamine (Barker *et al.*, 1994) in agreement with the hypothetical model (Figure 1.9). Follow-up studies using site-directed mutagenesis identified a single amino acid in TMD12 (phenylalanine 586) for the species selectivity (favouring human SERT) of the



**Figure 1.9: Hypothetical Model of SERT Functional Domains**

Taken from Lesch (1998).

tricyclic antidepressants and cocaine (Barker & Blakely, 1996). Previous mutagenesis studies of the dopamine transporter have shown a conserved aspartate 79 located in TMD1 as being critical for dopamine uptake and cocaine analogue recognition (Kitayama *et al.*, 1992). Corresponding studies in the equivalent rat SERT TMD 1, aspartate 98, suggested that this residue may indeed serve as a direct contact site for substrates and some uptake inhibitors and hence may be directly involved in the uptake mechanism (Barker *et al.*, 1998). Data from numerous studies on chimeric transporters have suggested that maintaining proper interactions between TMD7 and other nearby spans is critical for transporter function (Giros *et al.*, 1994; Buck & Amara, 1994 & 1995). Mutagenesis studies of the rat brain SERT have revealed critical amino acid residues in TMD7 that play a role in Na<sup>+</sup> binding or coupling (Penado *et al.*, 1998) and may form part of an aromatic binding pocket similar to that found in the crystal structure of acetylcholine esterase (Sussman *et al.*, 1993). The absence of crystal structure data for SERT however has further reinforced the importance of such techniques for future studies aimed at understanding the three-dimensional structure and mechanism of transporter function and inhibition. Chimera studies have also revealed that 2<sup>nd</sup> extracellular loop is involved in conformational changes (Stephanet *et al.*, 1997). None of the remaining extracellular loops seem to be directly involved in substrate or inhibitor binding, though the 1<sup>st</sup>, 5<sup>th</sup>, and 6<sup>th</sup> extracellular loops appear to be involved in the transport reaction as revealed by mutagenesis studies of cysteine and leucine residues in these regions (Chen *et al.*, 1997; Chen *et al.*, 1998; Smicun *et al.*, 1999).

The distantly related *Drosophila melanogaster* SERT (Demchyshyn *et al.*, 1994; Corey *et al.*, 1994) has further helped investigators unravel studies on the functional domain of SERT. Despite human and *Drosophila* SERTs only showing 49% sequence identity (extending to 58% when considering only the TMDs), both transporters exhibit essentially equivalent transport kinetics for 5-HT; however, vastly different pharmacological profiles are observed (Barker & Blakely, 1998). *Drosophila*/human SERT chimeras have helped to identify the TMDs 1-2 region as being potentially involved in the recognition of the uptake inhibitors mazindol and citalopram as well as interactions with the cotransported Na<sup>+</sup> ions (Barker *et al.*, 1998). Another important difference in these different SERT species is in their

transporter mediated currents. Transport of 5-HT by *Drosophila* SERT appears to be voltage dependent, whereas human SERT exhibits voltage-independent transport activity with a magnitude of current 5-10 times lower than in *Drosophila* (Corey *et al.*, 1994). This is possibly due to the stoichiometry of  $2\text{Na}^+$ : 1 5-HT coupling of *Drosophila* SERT (Barker & Blakely, 1998). Therefore chimera studies may reveal domains involved in these properties. Indeed, replacement of serine at position 545 in TMD11 in the recombinant rat SERT by alanine has been found to alter the cation dependence of serotonin uptake (Sur *et al.*, 1997) and has been suggested to be involved in the ligand-dependent gating processes that are indicative of ion channels (Magers *et al.*, 1994 & Sonders *et al.*, 1996).

#### **1.6.4 Regulation of 5-HT Transporter Function**

Cool *et al.*, (1990) have demonstrated that SERT activity in human placental JAR cells can be modulated by chronic exposure to cAMP elevating agents including cholera toxin (CTX). This effect despite taking many hours to manifest is inhibited by translation and transcription inhibitors. SERT abundance is increased in parallel with SERT mRNA after CTX treatment (Ramamoorthy *et al.*, 1993b), suggesting a transcriptional mediated increase in SERT abundance. Human SERT gene expression is significantly regulated by activation of both PKA and PKC pathways (Blakely *et al.*, 1997 & 1998), and potential target sites for second messenger-mediated regulation of gene expression have been identified at or near transcription initiation and mRNA splicing sites (Lesch *et al.*, 1994; Heils *et al.*, 1996; Bradley & Blakely, 1997). Platelet, endothelial and brain SERT are down-regulated within minutes by PKC activation (Myers *et al.*, 1989; Anderson & Horne, 1992). PKC-mediated down-regulation of 5-HT uptake in stably transfected HEK-293 cells occurs via a specific reduction in cell-surface transporter protein (Qian *et al.*, 1997). The presence of multiple, canonical serine and threonine phosphorylation sites on SERT cytoplasmic domains and the ability of  $\text{NH}_2$  and  $\text{COOH}$  termini, where most of these sites lie, to serve as substrates for purified protein kinases has been shown (Figure 1.6). Rapid kinase-mediated regulation of 5-HT uptake, as a consequence of SERT phosphorylation is paralleled with reductions in SERT surface abundance (Ramamoorthy *et al.*, 1998) though the possibility of PKC induced internalisation of



cell-surface SERT protein may underlie this mechanism by which transmitter clearance is mediated (Qian *et al.*, 1997).

Another regulating mechanism of transporter function is the direct interaction of various uptake inhibitors (see section 1.8.3). It is believed that uptake inhibitors bind to the same or closely overlapping site in SERT as 5-HT itself (Bäckström *et al.*, 1989; Graham *et al.*, 1989), thereby inhibiting the transport activity of SERT and increasing synaptic availability of the neurotransmitter, enhancing receptor activation. This effect has been thought to be due to a decrease in SERT density and a desensitisation of its function following chronic treatment with uptake inhibitors such as paroxetine (Piñeyro *et al.*, 1994). Furthermore chronic treatment with uptake inhibitors reduce SERT mRNA levels in rat brain (Lesch *et al.*, 1993a), suggesting a sensitivity of SERT at the level of gene expression.

A class of amphetamine derivatives, including p-chloroamphetamine, fenfluramine, and 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”; see Section 1.9) can also bind to SERT, on the same site as antidepressants, and catalyse the exchange of one molecule of 5-HT to the outside of the membrane for one molecule of drug to the inside (Rudnick & Wall, 1992). Therefore these drugs can lead to large concentrations of synaptic 5-HT, that cannot be retaken up into the presynaptic neuron.

## **1.7 Physiological and Pathophysiological Roles of 5-HT**

Neurons in the entire mammalian CNS number in the billions, whereas serotonergic cells number in the thousands. Therefore serotonergic neurons constitute approximately 1000000<sup>th</sup> of all CNS neurons. However their diverse projections and branching account for approximately 500<sup>th</sup> of all axon terminals in the rat cortex (Audet *et al.*, 1989). This extensive network of its projections and the diverse function of different 5-HT receptor types has implicated the 5-HT system in a wide range of physiological functions including neuronal development, thermoregulation, pain, motor regulation, sleep, appetite, sexual behaviour, aggression, anxiety and mood (see Kean & Soubrie, 1998). Some of these have been assigned to specific receptor subtypes on the basis of the action of often non-selective drugs in a variety of behavioural and physiological experiments (as outlined

in Table 1.5). In addition to the effects named above, there is also evidence for a role of the serotonergic system in neuroendocrine function, the immune system and in neuroimmune interactions (see reviews Van de Kar, 1991; Mössner & Lesch, 1998; Raap & Van de Kar, 1999). Due to the large array of physiological responses attributed to the 5-HT system there are a variety of current applications for 5-HT compounds in the clinic to treat symptoms associated with alterations in the 5-HT system (Table 1.7). However the lack of selective compounds for the ever increasing number of 5-HT receptor subtypes discovered and the multifunctional modulatory effects of 5-HT (Elliot *et al.*, 1994), may explain why an even greater number of drugs are not currently available to physicians.

Two common physiological effects that can be treated using selective serotonergic drugs are migraine and nausea. Migraine represents a disorder of cerebral vascular regulation and may be the result of a marked, prolonged phase of cranial vasodilation. During an attack, extravasation of plasma proteins and development of localised inflammation in intracranial vessels also occur. Some 5-HT receptor subtypes located on blood vessels have a powerful influence over smooth muscle contractibility of blood vessels (see Martin, 1994). The 5-HT<sub>1D</sub> agonist, sumatriptan (Table 1.7) was developed by screening compounds for vasoconstrictor activity in dog saphenous vein (Humphrey *et al.*, 1988). Injection of sumatriptan reverses the dilation of the middle cerebral artery on the headache side (Friebert *et al.*, 1991). It is also believed that it may activate a prejunctional receptor, which resembles the 5-HT<sub>1D</sub> receptor on perivascular fibers, resulting in an inhibition of release of inflammatory neuropeptides that mediate pain, such as substance P and CGRP (Moskowitz, 1992).

Unlike migraine, nausea may be treated with specific 5-HT receptor antagonists. Nausea and vomiting have consistently appeared among the severe side effects associated with radiation and chemotherapy in cancer patients. During the course of these therapies, mucosal enterochromaffin cells release 5-HT which stimulates 5-HT<sub>3</sub> receptors on enteric neurons such as the vagus and sympathetic nerves (Cubeddu *et al.*, 1990). The resultant vagal efferent discharge induces emesis by activation of the parvicellular reticular formation, the so-called emetic centre of the brain. 5-HT<sub>3</sub> receptors are also found centrally in high concentrations in cortical

Clinical Indication	Drug	Class of Compound
generalised anxiety disorder	buspirone	5-HT <sub>1A</sub> partial agonist
panic disorder	paroxetine citalopram	SSRI SSRI
obsessive-compulsive disorder	fluoxetine fluvoxamine paroxetine	SSRI SSRI SSRI
bulimia	fluoxetine	SSRI
schizophrenia	clozapine risperidone	5-HT <sub>2A/2C</sub> antagonist 5-HT <sub>2A/2C</sub> antagonist
chemotherapy-induced nausea	ondansetron granisetron tropisetron	5-HT <sub>3</sub> antagonist 5-HT <sub>3</sub> antagonist 5-HT <sub>3</sub> antagonist
post-operative nausea and vomiting	ondansetron granisetron	5-HT <sub>3</sub> antagonist 5-HT <sub>3</sub> antagonist
gatroparesis	cyproheptadine cisapride	5-HT <sub>2</sub> antagonist 5-HT <sub>4</sub> agonist
acute migraine	sumatriptan naratriptan zolmitriptan rizatriptan	5-HT <sub>1D</sub> agonist 5-HT <sub>1D</sub> agonist 5-HT <sub>1D</sub> agonist 5-HT <sub>1D</sub> agonist
migraine prophylaxis	methysergide pizotifen cyroheptidine amitriptyline	5-HT <sub>2A/2C</sub> antagonist 5-HT <sub>2</sub> antagonist 5-HT <sub>2</sub> antagonist atypical antidepressant
depression	phenelzine, isocarboxaid, trancylcypromine, moclobemide tryptophan amitriptyline, amoxapine, clomipramine, dothepin, doxepin, imipramine, lotepramine, nortriptyline, protriptyline, trimipramine maprotiline, mianserin citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline nefazodone, trazodone venlafaxine, viloxazine mirtazapine	monoamine oxidase Inhibitors  amino acid tricyclic antidepressants  atypical antidepressants SSRIs  5-HT uptake and 5-HT <sub>2</sub> antagonists SNRIs NaSSA

**Table 1.7: Therapeutic Indications of 5-HT Compounds.**

Taken from the British National Formulary, September 1999 issue. SSRI = serotonin specific reuptake inhibitor; SNRI = serotonin/noradrenaline reuptake inhibitor; NaSSA = noradrenergic and specific serotonergic antidepressant.

and limbic regions and in or near the chemoreceptor trigger zone, all of which are implicated in the vomiting reflex induced by serotonin (Kilpatrick *et al.*, 1989; Andrews, 1990). Both ondansetron and granisetron are potent and selective inhibitors of 5-HT<sub>3</sub> receptors both centrally and peripherally, and hence are used for the treatment of post-operative nausea and vomiting and chemotherapy-induced nausea (Table 1.7; see Perez, 1995).

5-HT has also been implicated in many neurophysiatric disorders such as anxiety, schizophrenia, obsessive compulsive disorders and depression (see Blier & De Montigny, 1998). The treatment of depression is one of these disorders which currently assigns the most clinically used drugs that act on the 5-HT system as stated by The British National Formulary (September 1999; BNF No. 38 issue; Table 1.7). The management of the depressive state is reviewed in the following section (section 1.8).

## **1.8 Models of Depression**

Depressive disorders are serious illnesses that are characterised by similar signs and symptoms irrespective of race and socio-economic status throughout the world, and are considered to be a major health problem (Ünstün & Sartorius, 1993). Today it is well established that major depression is the most common of psychiatric disorders, which has lifetime prevalence rates between 4.4 and 19.6% (Angst, 1992). It can be treated effectively in many cases with a variety of methods, such as psychotherapeutic techniques, pharmacotherapy or electroconvulsive therapy (ECT).

### **1.8.1 Monoamine Hypothesis of Depression**

One of the first indicators of a monoamine in depression was the finding that reserpine, which blocks vesicular amine transport, although effective in the treatment of high blood pressure, produces a behavioural syndrome that resembles depression (Frize, 1954). The subsequent monoaminergic model of depression was suggested by Schildkraut (1965), which stated that depression is caused by a functional deficit of monoamine transmitters at certain sites in the brain, whereas mania results from functional excess. Imipramine was established as possessing antidepressive properties (Kuhn, 1958), despite it and its congeners (tricyclic antidepressants; TCAs) being originally synthesised as antihistamines. Antagonism of the behavioural

effects of reserpine and tetrabenazine by the TCA drugs was suggested to be associated with the inhibition of NE reuptake in the brain (Ross & Renyi, 1967) and these behavioural tests were considered predictive of antidepressant activity (Corrodi & Fuxe, 1968). However, imipramine was also found to block reserpine-sensitive accumulation of 5-HT in brain (Blackburn *et al.*, 1967; Carlsson *et al.*, 1968). Researchers discovered that most TCAs were inhibitors of both serotonin and noradrenaline reuptake into the presynaptic nerve terminal (Ross & Renyi, 1969). This combined role was based on findings that N-demethylation of these tertiary amines (parent drugs) to the secondary amine metabolites (e.g. imipramine to desipramine (DMI) and amitriptyline to nortriptyline) increased noradrenaline reuptake affinity *in vitro* (Table 1.12) and inhibition of noradrenaline uptake in rat brain was mainly observed when the tertiary amine drugs were administered *in vivo* (Carlsson *et al.*, 1969; Ross & Renyi, 1969). The affinity of the imipramine series of drugs for 5-HT reuptake inhibition was shown as DMI < imipramine < clomipramine suggesting that inhibitors of 5-HT uptake may be responsible for the mood elevating effect of the TCAs (Carlsson *et al.*, 1969). This subsequently led to the formulation of the indoleamine hypothesis of depression, suggesting that 5-HT neurotransmission is also decreased in depressed patients (Lapin and Oxenkrug, 1969; Carlsson *et al.*, 1969). Later, it was thought that there might be two types of depression linked with decreases in either NA or 5-HT neurotransmission, (Maas, 1975). However this hypothesis was not supported by clinical findings, and antidepressant agents inhibiting either 5-HT or NA reuptake, or both, are equally effective and are not selective for subtypes of depression (De Johnge *et al.*, 1991; Yazici *et al.*, 1993).

### **1.8.2 Involvement of The 5-HT System in Depression**

The inaccessibility of the human brain to investigation has been a major obstacle in understanding mental illness. Most investigations have been restricted to studies on post-mortem brain tissue, or in looking for biochemical abnormalities in the cerebrospinal fluid (CSF), blood or urine from depressed patients. For example concentrations of 5-HT and its metabolite, 5-HIAA, were found to be lower in the hindbrains of suicide victims suffering from depression compared to sudden death victims (Shaw *et al.*, 1967; Lloyd *et al.*, 1974) or those that suffered coronary

occlusion (Bourne *et al.*, 1968). Additionally, the concentration of 5-HIAA in CSF was lower in depressed patients (Ascroft *et al.*, 1966; Dencker *et al.*, 1966), particularly among those who had an increased incidence of suicide attempts and subsequently committed suicide (Asberg *et al.*, 1986). Treatment with amino acid precursors of 5-HT, tryptophan (Coppen *et al.*, 1967; Hertz & Sulman, 1968) and 5-hydroxytryptophan (Sano, 1977), showed antidepressive effects. In a more recent study remitted depressed patients receiving serotonergic antidepressants were given a low tryptophan diet and found to promptly relapse, which was reversed on tryptophan supplementation (Delgado *et al.*, 1990).

Precursors, metabolites and 5-HT itself are not alone in the evidence for a role of the 5-HT system in depression. SERT and the 5-HT receptors have also been implicated in the pathophysiology of depression. Studies using post-mortem material are often difficult to obtain in large numbers, and so many studies have been conducted in the non-invasive model of SERT, namely blood platelets. Like SERT, 5-HT<sub>2</sub> receptors are also found on both 5-HT containing neurons and blood platelets. Studies in depressed and suicidal patients for both these types of tissue have revealed a decrease in SERT binding density and an increase in 5-HT<sub>2</sub> receptor density in many studies (see Owens & Nemeroff, 1994). These respective changes in density have been shown to return to control levels upon clinical improvement, but low densities for SERT persist until clinical improvement is achieved (Berrettini *et al.*, 1982; Suranyi-Cadotte *et al.*, 1984; Langer *et al.*, 1987).

Hormone responses to indirect serotonin agonists like L-tryptophan also lend evidence to the indoleamine hypothesis. Upon stimulation of the serotonin system, the hormones, prolactin and growth hormone are released. Neuroendocrine responses to intravenous L-tryptophan were examined to compare the serotonergic function in depressed patients and healthy comparison subjects. Prolactin and growth hormone responses to the intravenous injection of L-tryptophan were decreased in depressed patients (Price *et al.*, 1991), supporting the evidence that the serotonin function is abnormal in depression. Stimulation of the serotonergic system also activates the hypothalamic-pituitary and adrenal (HPA) axis (Calogero *et al.*, 1990; López *et al.*, 1997), which has been implicated in depression (Barden *et al.*, 1995). Corticotropin-releasing hormone (CRH) in the hypothalamus is the main driving force behind the

activation of the HPA axis. Subsequent release of pituitary pro-opiomelanocortin-derived peptides (especially adrenocorticotrophic hormone, ACTH) stimulate the production of adrenal steroids which can influence steroidal receptors in the brain or cause a series of feedback inhibitions that regulate these neuroendocrine responses (Barden *et al.*, 1995). In depressed patients there is a hyperactivity of the HPA axis, which can be reversed by antidepressant treatment following a similar time course to clinical improvement (Barden *et al.*, 1995). Densitisation of 5-HT<sub>1A</sub> receptor-mediated and a potentiation of the 5-HT<sub>2A</sub> receptor-mediated hormone responses after chronic SSRI treatment in rats are believed to account for the normalisation of the HPA axis (Raap & Van der Kaar, 1999).

The molecular cloning and sequencing of the human SERT gene located on chromosome 17 (Table 1.3) has added evidence for the involvement of the serotonergic system in depression. Although there is no evidence of an abnormality in any of the 14 exons of the human SERT gene (Lesch *et al.*, 1995), there is evidence of a number of polymorphisms in the promotor region (Heils *et al.*, 1996) and second intron (Lesch *et al.*, 1994; Battersby *et al.*, 1996) containing differential numbers of variable number tandem repeats (VNTR) in depression. The first association study of this kind revealed a difference in the frequency in the VNTR of the 2<sup>nd</sup> exon in depressed patients, suggesting that this polymorphism was associated with the susceptibility to major depression (Ogilvie *et al.*, 1996). This was supported by evidence from other groups (Battersby *et al.*, 1996; Stober *et al.*, 1996; Collier *et al.*, 1996a; Kunugi *et al.*, 1997; Rees *et al.*, 1997), but was disputed by others (Kunugi *et al.*, 1996; Esterling *et al.*, 1998; Ewald *et al.*, 1998; Gutiérrez *et al.*, 1998; Hoehe *et al.*, 1998). A similar controversial story is apparent for the VNTR close to the promotor region of the SERT gene whereby some groups have reported weak associations between these alleles and affective disorder (Collier *et al.*, 1996b; Furlong *et al.*, 1998) whereas others have not (Mendes de Oliveira *et al.*, 1998). It is likely that depression may therefore be associated with a locus at or near the SERT gene, just as in migraine (Ogilvie *et al.*, 1998). To answer this question, research is currently geared towards finding more polymorphic regions, such as that recently identified in the 3' untranslated region of human SERT (Battersby *et al.*, 1999) and

to investigating other markers surrounding the SERT gene as suggested by Collier (1998).

Direct evidence for the role of 5-HT in depression has been more recently shown using *in vivo* human studies. Mann *et al.*, (1996) developed a method which enabled the visualisation of *in vivo* brain responses to serotonin release by comparing regional brain glucose metabolism, measured using [<sup>18</sup>F]deoxyglucose, after administration of fenfluramine, a serotonin releasing drug. Significant increases in glucose metabolism, namely in the left prefrontal and temporoparietal cortex, and decreases in metabolism in the right prefrontal cortex were observed in healthy patients. Depressed patients, however, had no areas of decrease or increase in glucose metabolism. The development of ligands for positron emission tomography (PET; e.g. [<sup>11</sup>C](+)MCN5652 & [<sup>125</sup>I]5-iodo-6-nitroquipazine) or single photon emission tomography (SPECT; e.g. [<sup>123</sup>I]CIT, (2 $\beta$ -carbomethoxy-3 $\beta$ -(4-iodophenyl)-tropane)) have enabled the direct visualisation of SERT and 5-HT receptors in the living brain (Fletcher *et al.*, 1995). To date there have been reports of decreased SERT density in living brains of depressed patients (Malison *et al.*, 1998; Staley *et al.*, 1998). 5-HT<sub>2</sub> receptors however appear to be unaffected, in contrast to the post-mortem studies described above (Staley *et al.*, 1998; Meyer *et al.*, 1999). Although this field is at a relatively early stage, the TCA desipramine has been shown to decrease the density of 5-HT<sub>2</sub> binding sites in the living human brain of depressed patients following a 3-4 week treatment using [<sup>18</sup>F]setoperone (Yatham *et al.*, 1999). No comparable studies have yet been carried out to investigate SERT or other 5-HT receptors during or after antidepressant treatment. Other technical advances have enabled serotonin transporter function *in vivo* to be assessed by chronoamperometry, which measures the disappearance of extracellular monoamine signals, but so far these studies are limited to rodents and nonhuman primate brains (see Frazer & Daws, 1998).

The other main monoamines have also been implicated in depression, such as noradrenaline (see review Leonard, 1997) and dopamine (see review Fibiger, 1995). However according to the BNF, only reboxetine, a specific noradrenergic reuptake inhibitor (Healy & Healy, 1998) is currently used as an alternative to the serotonergic drugs for the treatment of depression.



### 1.8.3 Antidepressant Drugs Linked With The 5-HT System

Despite biochemical, molecular, genetic and *in vivo* studies, probably the most striking evidence for the involvement of the 5-HT system in depression is the fact that so many of the clinically effective antidepressants interact with the 5-HT system (Blier *et al.*, 1990; Cowen, 1990; Goodwin, 1996; Goodnick & Goldstein, 1998). There are three main classes of antidepressants (MAOIs, TCAs and SSRIs) all of which are effective in relieving depressive symptoms and interact with the 5-HT system. However they differ not only in their mode of action via different receptor/transporter or enzymatic systems, but also in their duration of action, immediate effect on mood, unwanted side effects, risk with acute overdose, risk of drug interactions and delay in therapeutic onset of action as shown in Table 1.8. Electrophysiological experiments have provided evidence for an enhancement of 5-HT neurotransmission following various long-term drug treatments (Table 1.8).

#### MAOIs

MAO-A inhibitors such as clorgyline produce an initial decrease in the firing activity of 5-HT containing neurons in the raphé nuclei, which is followed by a progressive recovery during a three week treatment due to desensitisation of somatodendritic 5-HT<sub>1A</sub> receptors (Blier *et al.*, 1990) but not desensitisation of terminal 5-HT autoreceptors (Blier *et al.*, 1988b). MAO-A inhibitors however desensitise  $\alpha_2$ -adrenoceptors on the nerve terminals of 5-HT containing neurons (Mongeau *et al.*, 1994), as this isoform has a substrate preference for both 5-HT and noradrenaline.

#### TCAs

TCAs are a group of antidepressants that lack reasonable affinity for DAT but have moderate to high affinity for SERT (e.g. imipramine) but in the case of the secondary amine metabolites have higher affinity for NET (e.g. desipramine; Table 1.9). The sensitivity of postsynaptic neurons to 5-HT is enhanced by TCA and also ECT, following 2-4 week treatment (De Montigny & Aghajanian, 1978; De Montigny 1984; Chaput *et al.*, 1991). However it was soon realised that the interaction of such uptake inhibitors with receptors of neurotransmitters may produce unwanted side effects that complicate antidepressive therapy (Synder & Yamamura, 1977). This is particularly the case for TCAs, such as imipramine, which have high

**Characteristics of the main classes of antidepressants**

	<b>TCA</b>	<b>MAOI</b>	<b>SSRIs</b>
<b>Duration of action</b>	1-3 days	2-4 weeks	1-3 days
<b>Delay in therapeutic effect</b>	2-4 weeks	2-4 weeks	2-4 weeks
<b>Immediate effect on mood</b>	Sedation, dysphoria	Euphoria	None
<b>Main unwanted effects</b>	Sedation, anticholinergic effects, postural hypotension, seizures, mania, impotence	Sedation, postural hypotension, insomnia, weight gain, liver damage (rare)	Nausea, diarrhoea, anxiety & restlessness, insomnia
<b>Risk with acute overdose</b>	High (cardiac dysrhythmias, seizures, mania)	Moderate (seizures and mania)	Low
<b>Risk of drug interactions</b>	Many (eg. alcohol)	Many (eg. ephedrine, pethidine)	Must not be used with MAOIs

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**Effects of Long-term administration of antidepressant treatments**

<b>Responsiveness of somatodendritic 5-HT<sub>1A</sub> autoreceptors</b>	No change	Decrease	Decrease
<b>Function of terminal autoreceptors</b>	No change	-	Decrease
<b>Function of terminal <math>\alpha_2</math>-adrenoceptors</b>	-	Decrease	No change
<b>Responsiveness of postsynaptic 5-HT receptors</b>	Increase	No change or decrease	No change
<b>Net 5-HT neurotransmission</b>	Increase	Increase	Increase

**Table 1.8: Characteristics and Effects of Long Term Administration of The Main Classes of Antidepressants**

TCA = tricyclic antidepressants. MAOI = monoamine oxidase inhibitors. SSRI = serotonin specific reuptake inhibitors. Adapted from Rang *et al.*, (1995) and Blier & De Montigny (1994).

affinity for various neuronal receptors including  $\alpha_1$ -adrenergic, muscarinic and histaminergic receptors (Hall and Ogren, 1981; Wong *et al.*, 1983). Interaction of TCAs with these receptors may relate to the side effect profiles of constipation, urinary retention, blurred vision, postural hypotension and sedation (Snyder & Yamamura, 1977; U'Prichard *et al.*, 1978; Richelson & Nelson, 1984; Table 1.8). This prompted the search for much more selective drugs which would have a more tolerable side effect profile compared to the TCAs and MAOIs.

### **SSRIs**

The development of serotonin specific reuptake inhibitors (SSRIs) which had a marked selectivity for inhibiting serotonin uptake, such as paroxetine, citalopram and fluoxetine (Table 1.9), compared to other reuptake systems, did not improve clinical efficacy, but did improve the side effect profile by reducing the cardiotoxicity and anticholinergic side effects associated with TCAs (Anderson & Tomenson, 1993; Goldstein & Goodnick, 1998). Comparing the uptake blocking profile of the SSRIs in Table 1.9, sertraline despite having high affinity for SERT also has high affinity for DAT. It is structurally related to the atypical antidepressant nomifensine, which similarly has high affinity for DAT (Table 1.9). Though the clinical relevance of this blockade is currently unknown, it may help to explain why sertraline was more effective in improving cognitive function over fluoxetine in a group of depressed patients (Oxman, 1996). When acutely administered, SSRIs reduce the firing activity of 5-HT neurons (Chaput *et al.*, 1986). With long-term treatment firing activity is recovered leading to increases in extracellular 5-HT levels, controlled by the desensitisation of somatodendritic 5-HT<sub>1A</sub> autoreceptors (Chaput *et al.*, 1986). Terminal 5-HT autoreceptors which exert a negative influence on 5-HT release are also desensitised after chronic antidepressant treatment (see Piñeyro & Blier, 1999). In the case of paroxetine, a desensitisation of the neuronal 5-HT transporter is also believed to be involved, as shown by a reduction in the density of SERT and [<sup>3</sup>H]5-HT uptake (Piñeyro *et al.*, 1994). This is also apparent for the noradrenaline transporter after long term desipramine treatment (Bauer & Tejani-Butt, 1992) suggesting that monoamine transporters share a common adaptive mechanism caused by their long term blockade. Therapeutic relief in patients taking SSRIs is usually not attained until 2-3 weeks after commencement of treatment

**Affinity at Human Transporters pK<sub>D</sub> (-Log M)**

	Serotonin	Noradrenaline	Dopamine
<b>Tricyclics</b>			
Amitriptyline	8.37	7.46	5.49
Nortriptyline	7.74	8.36	5.94
Imipramine	8.85	7.43	5.07
Desipramine	7.75	9.08	5.50
Chloroimipramine	9.55	7.42	5.66
<b>Atypical</b>			
Oxaprotiline	5.41	8.31	5.36
Maprotiline	5.24	7.95	6
Mianserin	5.40	7.15	5.03
Nomifensine	6.00	7.81	7.25
<b>SSRIs</b>			
Paroxetine	9.89	7.40	6.31
Fluoxetine	9.09	6.62	5.44
Fluoxamine	8.66	5.89	5.04
Citalopram	8.94	5.39	4.55
Sertraline	9.53	6.38	7.60
<b>5-HT reuptake &amp; 5-HT<sub>2</sub> antagonists</b>			
Nefazodone	6.70	6.44	6.44
Trazodone	6.80	5.07	5.13
<b>SNRIs</b>			
Venlafaxine	8.05	5.97	5.03
Viloxazine	4.76	6.81	<4
<b>NaSSA</b>			
Mirtazapine	<4	5.34	<4

**Table 1.9: Affinity of Antidepressants at Human Transporters.**

Data taken from Tatsumi *et al.*, 1997. SSRI = serotonin specific reuptake inhibitor. SNRI = Serotonin/noradrenaline reuptake inhibitor. NaSSA = Noradrenergic and specific serotonergic antidepressant.

(Artigas *et al.*, 1996; Quitkin *et al.*, 1996). Indeed several lines of evidence from the kinetics of [<sup>3</sup>H]5-HT transport and binding studies on SERT have suggested that chronic but not short-term treatment with these drugs have a time-dependent regulatory effect on monoamine transporter systems. SERT mRNA transcription and/or stability is regulated by chronic treatment with selective and non-selective uptake inhibitors (Lesch *et al.*, 1993a). A more recent study, however, has shown fluoxetine to reduce dorsal raphé 5-HT<sub>1B</sub> mRNA levels in a time dependent manner whereas SERT mRNA was only transiently decreased within 21 days of treatment (Neumaier *et al.*, 1996). This reduction in 5-HT<sub>1B</sub> mRNA was specific to dorsal raphé nucleus and was not found in several postsynaptic (non-serotonergic) regions. Hence adaptive changes of SERT and/or 5-HT autoreceptors may underlie the therapeutic effectiveness of SSRIs. This characteristic delay in the onset of clinical improvement is a major concern since this period is associated with a high risk of suicide (Stockmeier, 1997). The mechanism of action of SSRIs is further reviewed elsewhere (Goodnick & Goldstein, 1998; Goldstein & Goodnick, 1998; Stahl, 1998a & b; Feighner, 1999; Piñeyro & Blier, 1999).

#### **Other Classes of Antidepressant Drugs**

Drugs which may have an improved clinical efficacy compared with the SSRIs and which have an improved side effect profile compared to both the TCAs and the SSRIs are currently sought. This search has led to the development of a variety of compounds which have been grouped into different classes based on their *in vitro* and *in vivo* mechanisms. These may be reviewed in Stahl, (1998b), Feighner, (1999) and Piñeyro & Blier, (1999). These include atypical antidepressants (e.g. maprotiline), 5-HT<sub>1A</sub> receptor partial agonists (e.g. buspirone; Sharp *et al.*, 1989), serotonin uptake and 5-HT<sub>2</sub> receptor inhibitors (e.g. nefazodone; Davis *et al.*, 1997), dual serotonin and noradrenaline uptake inhibitors (SNRIs; e.g. venlafaxine; Andrews *et al.*, 1996) and noradrenergic and specific serotonergic antidepressants (NaSSAs; e.g. mirtazapine; De Boer & Ruigt, 1995).

Atypical antidepressants represent a heterogeneous group of compounds, which, compared to TCAs, have fewer side effects, have a lower acute toxicity in overdose, a somewhat debatable improved onset of action and an improved efficacy in patients non-responsive to TCAs or MAOIs but show little improvement over the

SSRIs. However, unlike other classes of antidepressants, these clinically effective compounds do not share a common mechanism of action. They can however be split into broad classes: non-tricyclic structures which preferentially block noradrenaline-uptake (e.g. Oxaprotiline and maprotiline; Table 1.9), drugs that do not affect monoamine uptake such as Iprindole, those that weakly affect monoamine uptake but also blocks other receptor systems such as mianserin and those that actually enhance monoamine uptake such as tianeptine (Mennini *et al.*, 1987; Fattaccini *et al.*, 1990).

SNRIs show a slightly greater antidepressant efficacy than the SSRIs without the side effects of the TCAs (Morton *et al.*, 1995). Venlafaxine also has a favourable drug interaction profile due to the fact that it does not interact with other neurotransmitter receptors, unlike some SSRIs and TCAs, or inhibit cytochrome p450 enzymes which play a role in the metabolism of such drugs (Ereshefsky, 1996). However the first NaSSA identified, mirtazapine, which appears to have little affinity at monoamine transporters (Table 1.9) has the greatest benefit to date over SSRIs with an onset of action of only one week (Claghorn, 1995). In comparison to SSRIs, which reduce 5-HT cell firing initially, mirtazapine immediately and persistently enhances serotonergic cell firing (Blier & De Montigny, 1994; De Montigny *et al.*, 1995). The mechanism of action of mirtazapine is unique in that it enhances both NA and 5-HT neurotransmission. This NaSSA enhances noradrenergic transmission by blocking  $\alpha_2$  adrenergic autoreceptors, which when normally stimulated by noradrenaline, inhibit the release of noradrenaline. Noradrenergic neurons control the firing rate of serotonergic 5-HT neurons via  $\alpha_1$  adrenoceptors, located on 5-HT cell bodies. Stimulation of these receptors by noradrenaline leads to an increase in the firing rate of the 5-HT neurons. Due to very low affinity of mirtazapine for  $\alpha_1$  adrenoceptors, the increased levels of noradrenaline (caused by mirtazapine blockade of  $\alpha_2$  autoreceptors) leads to an enhancement of serotonergic cell firing. This increased firing rate raises 5-HT release at the nerve terminal. This release is not inhibited by the inhibitory effect of noradrenaline on serotonin release due to mirtazapine blockade of  $\alpha_2$  heteroreceptors and hence results in a net enhancement of serotonin neurotransmission, mediated by 5-HT<sub>1</sub> type receptors. Mirtazapine also blocks 5-HT<sub>2</sub> and 5-HT<sub>3</sub> like receptors, which probably accounts for the lower side

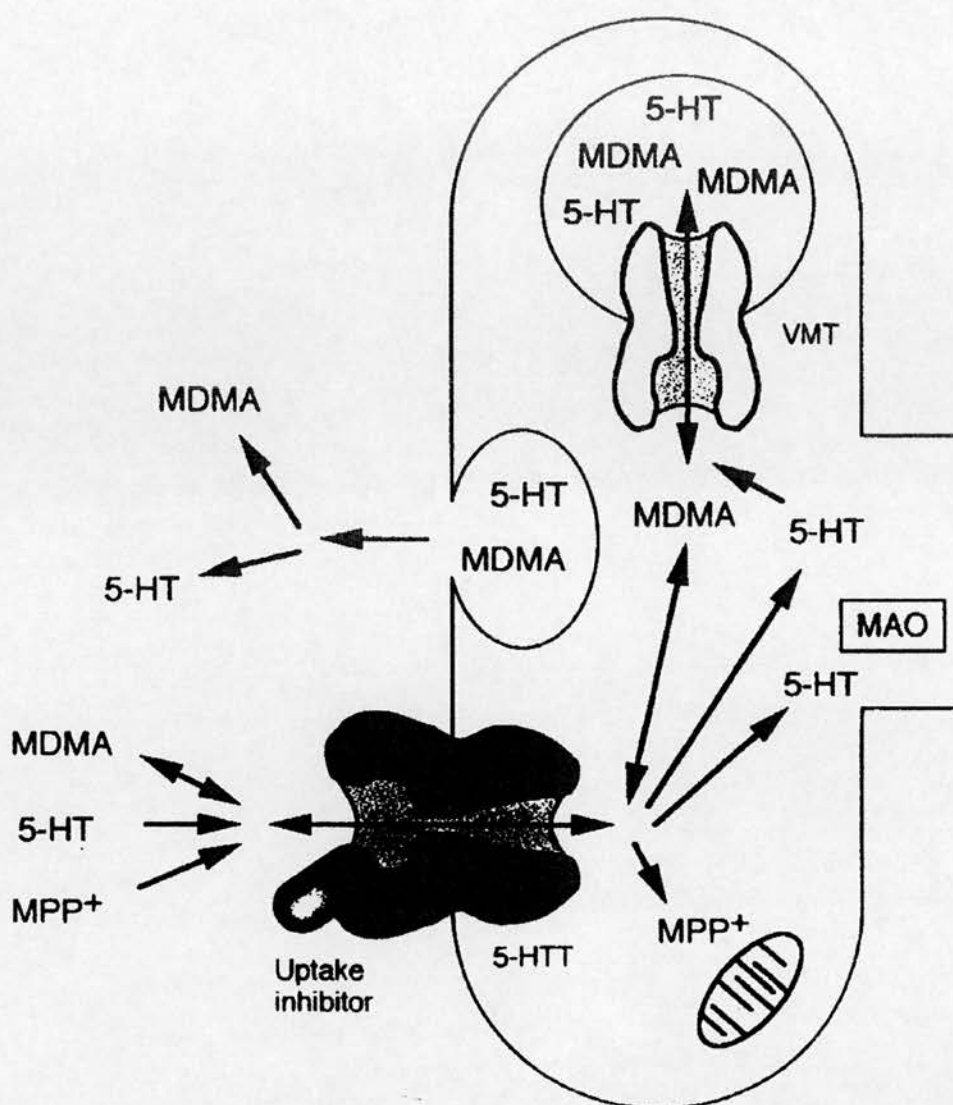
effect profile of this antidepressant compared to the SSRIs (see review De Boer, 1996).

#### **1.8.4 Animal Models of Depression**

In the laboratory the potential effectiveness of antidepressants is examined using a variety of biochemical, *in vivo* and *ex vivo* experiments, but also in behavioural models, which though not used in this thesis are included for completeness. Unlike some other psychological disorders such as psychosis and anxiety, affective disorders such as depression are only known to be experienced by man. However, animal models that introduce similar environmental factors that are thought to be associated with depression in humans have been validated, to assess the therapeutic efficacy of many potential antidepressant agents. These models may be reviewed in Willner (1995) and Kean & Soubrie (1998). The classical example is the model of learned helplessness in which an animal is exposed to a prolonged inescapable stress, but does not attempt to escape when later given the opportunity. Many types of antidepressants, including serotonin specific reuptake inhibitors, increase escape behaviour in animals displaying learned helplessness (Martin *et al.*, 1990).

#### **1.9 Consequences of MDMA Exposure**

The popular recreational drug of abuse 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') has two well established phases of action in experimental animals. The acute phase consists of a rapid release of 5-HT from neuronal stores, thereby producing many of the acute behavioural changes, including behavioural excitation and hyperthermia, that are seen in both experimental animals and humans (see reviews, Steele *et al.*, 1994; Green *et al.*, 1995; White *et al.*, 1996). The mechanism by which this occurs is summarised in Figure 1.10. Amphetamines and their highly lipophilic, neurotoxic analogues, such as MDMA, are substrates of SERT, displace the biogenic amine from its storage vesicle, and ultimately induce 5-HT efflux by a carrier-dependent process (Levi & Raiteri, 1993; Rudnick & Wall, 1993). MDMA induces efflux in a stereospecific, Na<sup>+</sup>-dependent and imipramine-sensitive manner, a characteristic of SERT-mediated exchange. MDMA-evoked vesicular 5-HT efflux on the otherhand is due to dissipation of the transmembrane



**Figure 1.10: The Role of The 5-HT Transporter in the Mechanism of MDMA and MPP<sup>+</sup> Neurotoxicity.**

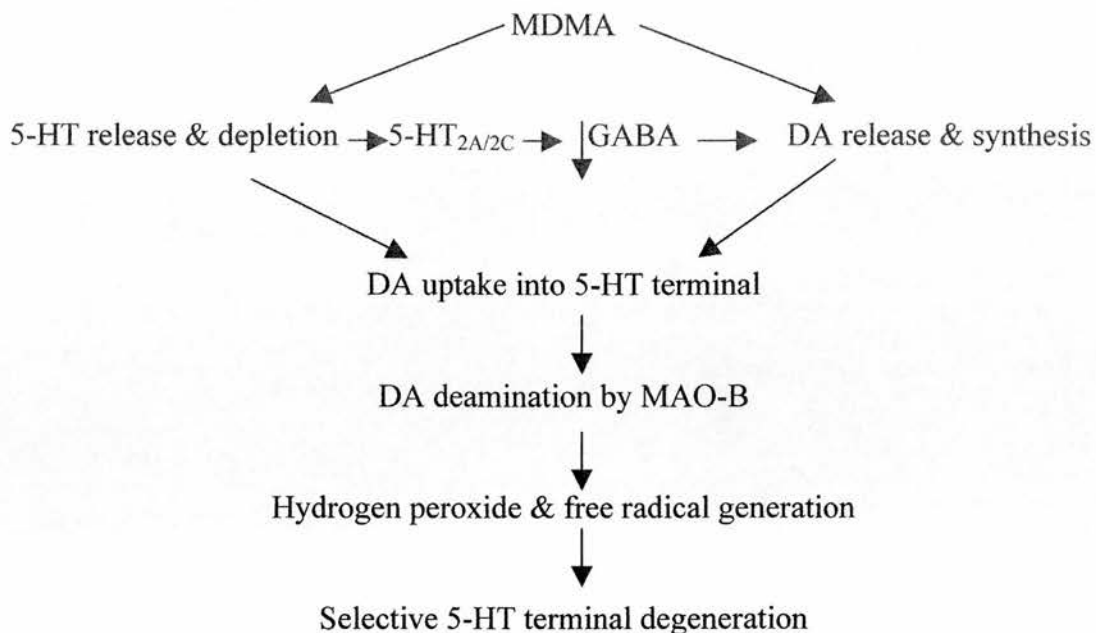
Amphetamines and their highly lipophilic, neurotoxic analogs, such as MDMA, are substrates of SERT (5-HTT), displace 5-HT from its storage vesicle, and ultimately induce 5-HT efflux by a carrier-dependent process. MDMA is therefore a potent releaser of 5-HT. Long-term administration results in toxic degeneration of serotonergic terminals. MDMA-induced 5-HT release and neurotoxicity can be prevented by reuptake inhibitors. MPP<sup>+</sup>, the neurotoxic metabolite of MPTP, also enters serotonergic neurons via SERT. In pathological conditions a dysfunctional transport process may contribute to an increased susceptibility to exogenous MPP<sup>+</sup>-like neurotoxins. The vulnerability of MPP<sup>+</sup> may be further aggravated by an impaired capacity of the vesicular monoamine transporter (VMT), which plays a central role in the sequestration of cytoplasmic toxins and thus in the limitation of mitochondrial damage. Figure from Lesch *et al.*, (1998).



pH difference generated by ATP hydrolysis (alkalinisation) and to direct interaction with the vesicular monoamine transporter (Rudnick & Wall, 1992). Recently the mechanism of MDMA induced release of 5-HT has been extended to include a  $\text{Ca}^{2+}$ -dependent process, which is possibly exocytotic-like (Crespi *et al.*, 1997).

The second action of MDMA in the brain of experimental animals is to produce a long lasting neurotoxic loss of 5-HT nerve terminals in several areas of the brain, an effect demonstrated both histologically (O'Hearn *et al.*, 1988; Molliver *et al.*, 1990) and biochemically (Battaglia *et al.*, 1987; Sharkey *et al.*, 1991; Hewitt & Green, 1994). The main biochemical evidence for MDMA induced neurotoxicity is derived from reductions in [ $^3\text{H}$ ]citalopram or [ $^3\text{H}$ ]paroxetine (SERT) binding density (as markers of serotonergic nerve terminals; Battaglia *et al.*, 1987, 1988; Sharkey *et al.*, 1991). Other parameters are often used such as a reduction of 5-HT and its major metabolite 5-HIAA (Stone *et al.*, 1986; Schmidt, 1987), or a decline in the activity of tryptophan hydroxylase (Stone *et al.*, 1986) to confirm MDMA neurotoxicity which is specific to central 5-HT nerve terminals, and not DA or NA neurons. This has been derived from several species of experimental animal, including rat (Battaglia *et al.*, 1987), guinea pig (Battaglia *et al.*, 1988) and different species of primate, which appear to be four times more susceptible to MDMA than are rodents (Ricaurte *et al.*, 1988). Although initially it was thought that toxicity required multiple exposure to relatively high doses of MDMA, several subsequent studies have found that a single exposure to a high dose, or several exposures to lower doses can induce the same profile of toxicity (Colado *et al.*, 1993). More recently it has been found that even single treatments with doses close to those encountered by human users can also result in some of the manifestations of neuronal damage (Colado *et al.*, 1997a; O'Shea *et al.*, 1998).

Although the mechanism by which MDMA damages 5-HT terminals remains elusive, different drugs interfering with central serotonergic or dopaminergic systems, such as blockers of 5-HT or DA uptake, 5-HT<sub>2</sub> and DA receptor antagonists, DA synthesis inhibitors or previous dopaminergic lesioning, all prevent the depletion of brain 5-HT terminals following MDMA administration to rats (Schmidt, 1987; Stone *et al.*, 1988; Schmidt *et al.*, 1990a & b; Hewitt & Green, 1994). Recent data have also given substantial evidence to support the contention



**Figure 1.11: An Intergrated Hypothesis for the Development of Selective 5-HT Terminal Degeneration Following MDMA.**

MDMA induces an acute release of 5-HT (Nichols *et al.*, 1982, Schmidt *et al.*, 1987; Steele *et al.*, 1987) thought to be due to 5-HT-MDMA exchange (Rudnick & Wall, 1992; Zacek *et al.*, 1990), causing depletion of intraneuronal stores (Commins *et al.*, 1987; Schmidt *et al.*, 1987; Stone *et al.*, 1986). The 5-HT released activates post-synaptic 5-HT<sub>2A/2C</sub> receptors located on GABA interneurons (Cowan *et al.*, 1990; Kita *et al.*, 1990), causing a decrease in inhibitory GABAergic transmission (Yamamoto *et al.*, 1995) leading to an increase in dopamine (DA) release and synthesis (Nash, 1990). The excessive DA released is then transported into the depleted 5-HT terminal (Sprague & Nichols, 1995). Once concentrated within the 5-HT terminal, the DA is deaminated by MAO-B located within the 5-HT terminal (Levitt *et al.*, 1982). Hydrogen peroxide formed from this process may lead to lipid peroxidation and the selective destruction of the 5-HT terminal. Deamination of large quantities of DA would generate concentrations of reactive oxidising species sufficient to overwhelm the protective reductive capabilities of the terminal. Figure adapted from Sprague *et al.*, (1998).

that increased free radical production is also involved (Colado *et al.*, 1997b). This has led to the formulation of an integrated hypothesis of MDMA neurotoxicity involving SERT, DA and free radicals as outlined in Figure 1.11 (Sprague *et al.*, 1998).

Many users perceive this recreational drug of abuse as “safe” (Peroutka, 1987; Randall, 1992). However there is a growing evidence that both cognitive deficits and mood disturbances are amongst the negative, long-term consequences of MDMA exposure in humans (Curran & Travill, 1997; Davison & Parrott, 1997; Cohen & Cocores, 1997; Parrot & Lasky, 1998; McGann *et al.*, 1999). Furthermore there is direct evidence that MDMA causes a loss of serotonergic neurons in human users due to the reduction of 5-HT transporter sites recently shown by PET studies (McGann *et al.*, 1998). There is a general concern that the use and especially misuse of this drug may lead to negative mental consequences later on in human life.

### **1.10 Aims of Thesis**

The 5-HT transporter (SERT), which functions in the synaptic clearance of 5-HT within the nervous system and accumulation of 5-HT by platelets, is a molecular target not only for clinically effective antidepressants, but also for the popular recreational drug of abuse, 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy). Serotonin specific reuptake inhibitors (SSRIs), bind to and inhibit SERT causing an acute increase in synaptic 5-HT as an initial response to relieving depression. However the nature of the link between the acute actions of these drugs *in vitro* and the weeks of treatment required for clinical improvements remains unresolved. Neuroadaptive changes not only of SERT, but also of the 5-HT receptors regulating 5-HT neurotransmission, may account for this delayed onset of action. In contrast, MDMA significantly alters neurotransmission following uptake into serotonergic nerve terminals and has been shown to be selectively neurotoxic for serotonergic systems in both animals and humans, by an as yet unidentified or confirmed mechanism.

In this thesis the effects of chronic antidepressant treatments and repeated MDMA administration on the density and affinity of a variety of regional rat brain SERT and 5-HT receptors is investigated using a range of radioligand binding and

immunological techniques. The influence of potentially neuroprotective drugs on the effects of MDMA is also examined. Since SERT mRNA has been detected in the rat adrenal gland, the effects of antidepressant and MDMA treatment on adrenal SERT has also been determined. In order to achieve these goals the following main areas were investigated:

In chapter 2, SERT site directed antibodies are characterised as to their potential use in Western blots and immunocytochemical techniques to evaluate SERT abundance. These studies were extended to investigate the potential of these antibodies to recognise native SERT protein. This is an important prerequisite for the separation of purified 5-HT nerve terminals for other studies involved in the project from which this thesis was funded.

In chapter 4, the evaluation and pharmacological characterisation of serotonin and noradrenaline transporter (SERT & NET) radioligand binding assays are performed in rat brain and adrenal gland tissue. These are extended with the use of one of the antibodies in chapter 2, to investigate the distribution of adrenal gland SERT protein.

In chapter 5, the development and characterisation of radioligand binding assays for the 5-HT<sub>1A</sub>, 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors are performed. The conditions employed for identifying native rat 5-HT<sub>7</sub> receptors are autoradiographically used to determine the distribution of this receptor subtype in the rat brain.

In chapter 6, the antibodies characterised in chapter 2 and radioligand binding assays developed in chapters 4 & 5 are used to investigate the effects of chronic antidepressant treatments on the affinity and density of SERT, 5-HT<sub>1A</sub>, 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors in the rat brain. The effect of such treatments on SERT affinity and density is also assessed in the rat adrenal gland. Two SSRIs, which block 5-HT reuptake were used, namely citalopram (the most selective) and fluoxetine (the least selective) in comparison to the atypical antidepressant, tianeptine.

Finally in Chapter 7 the effect of repeated MDMA administration on 5-HT receptor and SERT affinity and density is investigated in rat brain and adrenal gland using the radioligand binding assays developed and characterised in previous chapters. These effects were also investigated in the presence of two potentially neuroprotective drugs, namely FK506 and FR122175.

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**CHAPTER 2**

**IMMUNOLOGICAL CHARACTERISATION OF SEROTONIN  
TRANSPORTER SITE DIRECTED ANTIBODIES**

Autoradiographic visualisation of neurotransmitter transporters/receptors is only possible with the availability of suitable high affinity radioligands. Molecular cloning has enabled researchers to exploit the specificity of the mammalian immune response (Burnet, 1957) to produce antibodies that are specific for the 5-HT transporter/receptors (Table 2.1). Some 5-HT receptors still lack suitable high affinity radioligands and so the anatomical localisation of 5-HT<sub>5A</sub> receptors, for example, has been achieved using techniques employing antibodies (Carson *et al.*, 1996; Table 2.1). The advent of molecular cloning has also permitted the use of oligonucleotide probes in conjunction with *in situ* hybridisation to identify cells that express transporter/receptor mRNA. However, in the case of neurotransmitter transporters, the mRNA tends to localise in the soma, thereby providing little information regarding the localisation of the transporter in the nerve terminals and dendrites, where the transporter protein is most likely to be expressed. Although, in the case of SERT, there are high affinity radioligands available, the production of anti-SERT antibodies can complement autoradiographic studies and provide a non-radioactive method of investigating the distribution and function of the transporter. Immunological techniques such as immunohistochemistry and Western blotting have been employed to study the role of this protein. Furthermore as an alternative to autoradiographic analysis, immunohistochemical techniques are very useful for studying specific brain areas, which may be too small for conventional membrane binding studies.

Antibodies are produced by immunisation of animals with synthetic antigenic peptides containing amino acid sequences that are unique to the protein of interest, as revealed by molecular cloning techniques. In the absence of methods to predict the immunogenicity (ability to elicit an immune response) of a given protein, peptide sequences must be selected on the basis of their antigenicity (ability to be recognised by cells of the immune system). This is often quoted as being regions of a protein that are easily accessible for antibody binding, such as extracellular loop sequences of membrane associated proteins on the cells surface. The antigenic index has been developed using weighted computer algorithms, showing that regions of a high

Receptor/ Transporter	Epitope	Reference
5-HT <sub>1A</sub> *	EL1 (96-111) EL2 (173-193)	Verdot <i>et al.</i> , 1994, 1995
5-HT <sub>1A</sub>	EL3 (170-186) IL3 (258-274)	Azmitia <i>et al.</i> , 1992
5-HT <sub>1A</sub>	IL3 (243-268)	El Mestikawy <i>et al.</i> , 1990; Hamon <i>et al.</i> , 1991; Gozlan <i>et al.</i> , 1993; Kia <i>et al.</i> , 1996a & b
5-HT <sub>1B</sub>	IL3 (23-287)	Langlois <i>et al.</i> , 1995; Sari <i>et al.</i> , 1997
5-HT <sub>2A</sub>	N-terminal (22-41)	Garlow <i>et al.</i> , 1993; Morilak <i>et al.</i> , 1993
5-HT <sub>2B</sub>	N-terminal (1-12)	Duxon <i>et al.</i> , 1997a
5-HT <sub>2C</sub>	N-terminal (1-10)	Sharma <i>et al.</i> , 1997
5-HT <sub>2C</sub>	IL3 (270-288)	Backstrom <i>et al.</i> , 1995
5-HT <sub>2C</sub> **	IL3 (239-257) COOH-tail (373-459)	Abramowski & Staufenbiel, 1995; Abramowski <i>et al.</i> , 1995
5-HT <sub>3</sub>	N-terminal (1-10)	Turton <i>et al.</i> , 1993
5-ht <sub>5A</sub>	IL3 (239-257) COOH-tail (343-357)	Carson <i>et al.</i> , 1996
5-ht <sub>6</sub>	COOH-tail (398-415)	Miquel <i>et al.</i> , 1996; Gérard <i>et al.</i> , 1997
SERT	EL4 (388-401) COOH-tail FP of last 34 amino acids	Qian <i>et al.</i> , 1995
SERT	EL4 (388-401)	Sur <i>et al.</i> , 1996
SERT	EL3 (315-325) N-terminal (55-68) N-terminal FP of first 71 amino acids	Zhou <i>et al.</i> , 1996

**Table 2.1: 5-HT Receptor/Transporter Site-Directed Antibodies.**

Antibodies were raised against rat receptors except \*human, and \*\* mouse. Amino acid sequences against which antibodies were produced are indicated in parentheses (numbering from the N-terminal end of the protein). EL & IL = numbered external and intracellular loops respectively. FP = fusion protein.

antigenic index correlate well with antigenic and immunogenic epitopes (Jameson & Wolf, 1988). This model integrates a variety of characteristics of the protein of interest including: hydrophilicity (Hopps & Woods, 1981), surface accessibility (Janin *et al.*, 1978), chain flexibility (Karplus & Schultz, 1985) and secondary structure (Chou & Fasman, 1978; Garnier *et al.*, 1978).

The optimal size for antigenic peptides is 10-15 amino acids, minimising the risk of cross-reactivity with the use of longer sequences, and the poor immunogenicity of shorter peptides (Harlow & Lane, 1988). To increase the immune response the synthetic peptide may be coupled to a phylogenetically removed protein, such as the invertebrate protein keyhole limpet haemocyanin (KLH). For larger peptides (> 30 amino acids) a fusion protein may be generated (see Miller *et al.*, 1998). These peptide-carrier conjugates are often given to the host in combination with an adjuvant, such as Freund's Complete Adjuvant, which prevents rapid dispersal from the injection site and stimulates lymphocyte infiltration and proliferation, prolonging and increasing exposure of the immunogen to cells of the immune system and enhancing the response (Merser *et al.*, 1975; Harlow & Lane, 1988). During the immunisation protocol, blood is taken at frequent intervals, the serum isolated and the immunoreactivity of the antisera determined by indirect Enzyme-Linked Immunosorbent Assays (ELISAs) against the synthetic peptide. This enables calculation of the antibody titre and the time at which the antibody production is most effective. Despite the highly specific nature of antibody-antigen binding, anti-peptide antibodies must be characterised to ensure that the protein is selectively recognised. The detection of a single band of an appropriate molecular weight following Western blotting is generally considered to indicate antibody specificity (Azmitia *et al.*, 1992). Specific labelling should be eliminated by pre-incubation of the immune serum with the antigenic peptide and absent following detection with pre-immune serum or in the absence of primary antibody. Antibodies generated in a natural immune response or after immunisation in laboratory animals, however, are a mixture of molecules of different specificities and affinities. These polyclonal antibodies therefore have the ability to bind to determinants other than the antigen of interest. To increase the specificity of polyclonal antibodies, the antisera may be purified by techniques such as affinity chromatography, which exploits the



specific binding of antibody to antigen held on a solid matrix. Confirmation that these purified antibodies are specific by Western blots enable their use to study the distribution of the protein of interest by immunohistochemical methods. However, it is possible that antibodies may vary in their specificity between techniques, and so a combination of these techniques is often used to determine the best antibody for such purposes. The procedures outlined above for the production of polyclonal antibodies (and in detail in Harlow & Lane, 1988) have been used to produce anti-peptide antibodies to the noradrenaline (Melikian *et al.*, 1994), dopamine (Vaughan *et al.*, 1992), and serotonin transporters (Qian *et al.*, 1995; Sur *et al.*, 1996; Zhou *et al.*, 1996).

In designing an immunisation protocol it is important to avoid protein regions which contain cysteine residues or potential glycosylation sites, as these regions may reduce the chance of antibody binding due to steric hindrance. However specific polyclonal antibodies for opioid receptors raised from antigenic peptide sequences in these regions has been successfully reported (Garzón *et al.*, 1994). The molecular cloning of SERT (Blakely *et al.*, 1991) has shown this protein to traverse the membrane twelve times, and be most closely related to NET and DAT with greatest sequence divergence present in the exposed intracellular NH<sub>2</sub> and COOH termini as well as in the accessible large TMD 3-4 extracellular loop (Amar & Kuhar, 1993). It is these regions which have been used to select appropriate antigenic sequences for anti-SERT antibody production (Table 2.1). Anti-SERT peptide antibodies have been used to study the anatomical localisation and SDS-polacrylamide gel electrophoresis (PAGE) mobility of the SERT protein (Table 2.1; Qian *et al.*, 1995; Sur *et al.*, 1996; Zhou *et al.*, 1996). These studies revealed that brain structures such as the cortex, hippocampus and caudate putamen contain high levels of SERT immunoreactivity which electrophoretically migrate at 76 kDa in denaturing Western blots. In contrast, low SERT density was evident in the cerebellum, which only receives a sparse serotonergic innervation (Steinbusch 1984; Takeuchi, 1988). Furthermore there is a high density of cell body staining in the midbrain in particular in the dorsal and midbrain raphé nuclei from which these projectional fields arise (Qian *et al.*, 1995; Sur *et al.*, 1996; Zhou *et al.*, 1996). This SERT positive immunoreactivity was substantially reduced by 5,7-dihydroxytryptamine (5,7-DHT) lesioning of the

serotonergic system, further confirming the specificity of these antibodies (Zhou *et al.*, 1996). Of particular importance is that these immunological studies show a close resemblance to autoradiographic analyses of SERT radioligand binding sites (D'Amato *et al.*, 1987; De Souza & Kuyatt, 1987; Hrdina *et al.*, 1990; Chen *et al.*, 1992; Duncan *et al.*, 1992) and the localisation of 5-HT immunoreactivity (Steinbusch, 1984; Takeuchi, 1988) suggesting specific labelling of serotonergic neurons.

Prior to the commercial availability of SERT antibodies a whole library of site-directed antibodies to SERT had been generated in our laboratory by Dr Jane Lawrence. Two of the affinity purified antibodies produced (1001 and 998; Table 2.2; Figure 2.1) have previously been shown to specifically recognise SERT in denaturing Western blots and fixed tissue immunohistochemical studies as reported in a short communication (Lawrence *et al.*, 1995a). Since these initial studies, commercially available site directed antibodies against SERT have also become available, including a monoclonal antibody (Table 2.2). The problems of specificity and reproducibility associated with traditional polyclonal antisera was originally resolved by the Nobel Prize winning work on the production of monoclonal antibodies (Kohler & Milstein, 1975). Monoclonal antibodies are usually produced by making hybrid antibody-forming cells from a fusion of myeloma cells with immune spleen cells. These cells are grown in culture (rather than in whole animals) and will secrete an antibody against only one antigenic determinant, thereby providing a longer-term source of the specific antibody.

The first aim of this chapter was therefore to assess the specificity of a variety of these antibodies in SDS (denaturing) Western blots and immunohistochemistry, for use after chronic antidepressant treatment studies (Chapter 6). The purpose of such antibody studies was to complement conventional binding experiments using a non-radioactive method, especially in brain regions too small for membrane binding, such as the raphé nuclei.

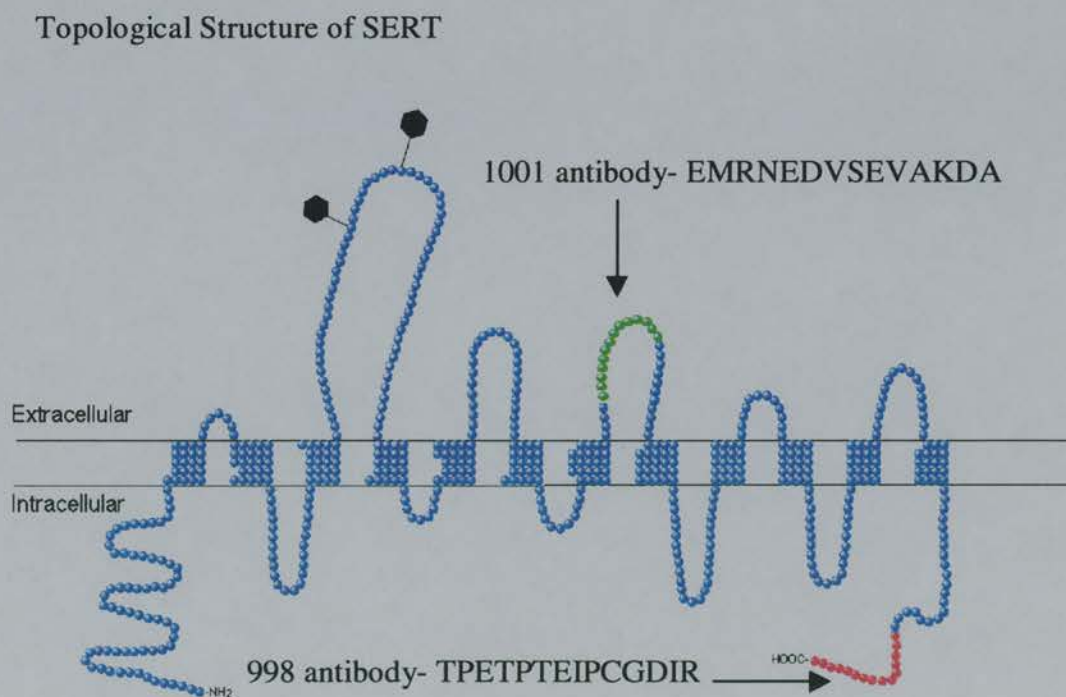
5-HT neurotransmission is known to be modulated not only by somatodendritic 5-HT<sub>1A</sub> autoreceptors, but also by 5-HT<sub>1B/1D</sub> autoreceptors located on 5-HT containing nerve terminals (Middlemiss & Hutson, 1990), where SERT is specifically found. However 5-HT<sub>1B/1D</sub> receptors are also located on

**Table 2.2: Summary of Affinity Purified SERT Antibodies.**

Source	Immunised Animal	Amino acids	SERT region	Code/catalogue number
<b>Polyclonal Antibodies</b>				
In-house	Rabbit	613-626	COOH-tail	998
In-house	Rabbit	388-401	4 <sup>th</sup> EL	1001
Chemicon	Rabbit	388-401	4 <sup>th</sup> EL	AB1594P
Chemicon	Guinea pig	634-653	COOH-tail	AB1772
Incstar (Diasorin)	Rabbit	579-599	COOH-tail	24330
<b>Monoclonal Antibodies</b>				
Chemicon	Mouse	1-85 (fusion protein)	N-terminal	MAB1564

EL = extracellular loop

**Figure 2.1: Sequences Used for In-House Antibody Production.**



non-serotonergic receptors acting as heteroreceptors (Molderings *et al.*, 1987; Bolaños & Fillion, 1989). SERT antibodies may therefore provide a useful tool to isolate pure 5-HT containing nerve terminals from the rest of the nerve terminals present in various brain structures (Lawrence *et al.*, 1995a & b). Such a technique would involve an immunomagnetic separation whereby synaptosomes are incubated with an anti-SERT antibody and magnetic microspheres recognising the SERT antibody, before being poured into a column influenced by a magnetic field. The eluted fraction would consist of the non-5-HT component, whereas the magnetically bound fraction would consist of isolated 5-HT containing nerve terminals, which can easily be recovered after removal of the magnet.

A long-term goal of this research group is to study the ionic conductances present at pure serotonergic nerve terminals and to investigate the influence of antidepressant treatments on the 5-HT receptor subtypes mediating these currents. Production of pure serotonergic synaptosomes using these antibodies, in combination with the formation of large enough structures (fusosomes) from which electrophysiologists can record would therefore aid such investigations. However the electrophysiological experiments, by virtue of their nature, require the constituents of nerve terminals to exist in their native form. Therefore, before immunomagnetic separation can commence, it was important to ascertain whether or not the anti-SERT antibodies that recognise SERT in denaturing Western blots or fixed tissue immunohistochemistry, could also recognise native SERT, where no conformational changes of the protein are induced. The final aim of this chapter was therefore to investigate the potential of a variety of the anti-SERT antibodies (Table 2.2) to recognise native SERT using a number of techniques, namely ELISA, non-denaturing Western blots and immunoprecipitations.

## **2.1 Methods**

All animals used in these studies were adult male Sprague Dawley rats (Charles River, 200-350g). All reagents were prepared in milliQ distilled water (d.H<sub>2</sub>O).

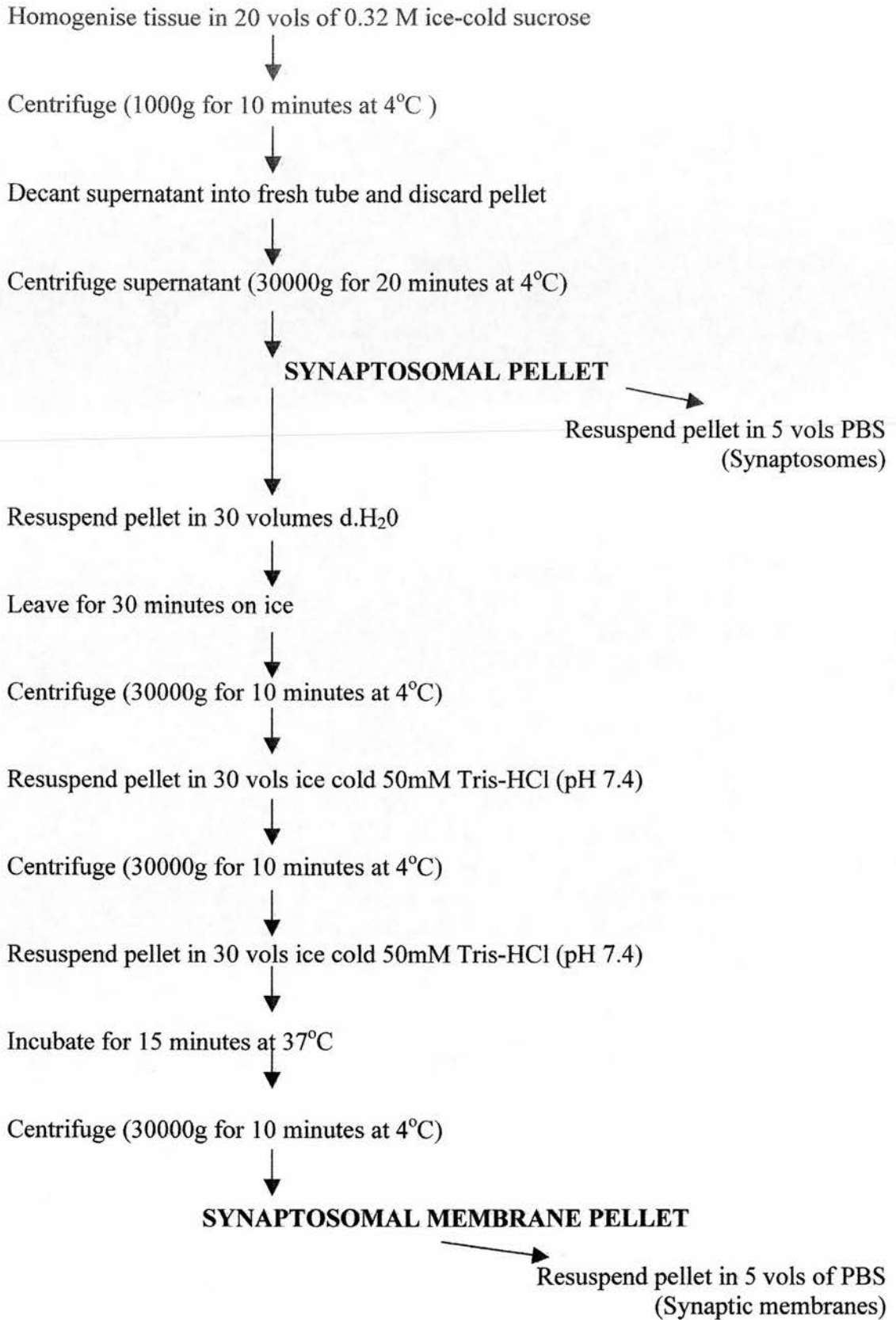
### **2.1.1 Preparation of Homogenates and Membranes**

Rat brain and liver homogenates, membranes, synaptosomes or synaptic plasma membranes were used in this chapter. Rats were stunned and killed by decapitation. Brains and liver were removed and immediately placed into ice-cold saline prior to dissection. Brains were dissected to reveal specific brain areas; whole cortex, hippocampus, striatum and/or cerebellum. Brain or liver tissue was rolled on filter paper to remove excess blood vessels and weighed. Homogenates were prepared by homogenisation (800 rpm) in 5 volumes of Phosphate Buffered Saline (PBS: Na<sub>2</sub>HPO<sub>4</sub> (40mM), Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub> (10mM), NaCl (150mM) at pH 8.0) containing protease inhibitors (Roche Diagnostics), using a glass-teflon homogenizer with 80µm clearance. Brain and liver membranes were prepared according to the methods outlined in chapter 3 (section 3.2.1.1). Following the final centrifugation membranes were resuspended in 5 volumes of PBS (containing protease inhibitors, Roche Diagnostics). Tissue synaptosomes or synaptic membranes were prepared according to the method in Figure 2.2.

Rat platelet homogenates or membranes were also used in this chapter. Rat platelets were obtained from blood as described in Chapter 3 (section 3.2.1.3). Platelet homogenates were obtained after the final PRP was centrifuged (30000g; 20 min, 4°C) prior to lyse. The resulting platelet pellet was resuspended in 0.5ml of 10mM Tris- HCl (pH 9.0 with; containing protease inhibitors, Roche Diagnostics) per 9mls of blood collected. Platelet membranes were prepared as described in chapter 3 (sections 3.2.1.3 and 3.2.2.5) and homogenised in 1ml of PBS (containing protease inhibitors, Roche Diagnostics) per 9mls of blood following the final centrifugation step. All tissue was stored at -20°C until required.

### **2.1.2 Storage of SERT Antibodies**

Affinity purified antibodies were initially diluted in PBS to 1mg/ml, unless otherwise stated. Following further dilution in PBS, antibodies were stored at 0.1mg/ml as 20-100µl aliquots at -20°C. The dilution of antibodies used in this thesis relates to dilutions from the original 1mg/ml stock. Therefore a 1 in 1000 dilution represents an antibody used at a concentration of 1µg/ml. Dilutions of all antibodies for the techniques used in this chapter are summarised in Table 2.3.



**Figure 2.2: Preparation of Synaptosomes and Synaptic Membranes.**

Primary antibody	1001		998		Incstar		Chemicon		5-HT		Chemicon		Chemicon	
	Primary antibody host	Secondary antibody host	Rabbit	Goat	Rabbit	Goat	Rabbit	Goat	Rabbit	Goat	Rabbit	Goat	Guinea pig	Chicken
<b>SDS westerns</b>														
Primary antibody dilution	1 in 200		1 in 200		1 in 500		1 in 200					1 in 2000		1 in 750
Secondary antibody dilution	1 in 1000		1 in 1000		1 in 1000		1 in 1000					1 in 5000		1 in 1000
<b>Immunohistochemistry</b>														
Primary antibody dilution	1 in 2000		1 in 2000		1 in 3000					1 in 20000				1 in 750
Secondary antibody dilution														
<b>ELISA assays</b>														
Primary antibody dilution	1 in 100		1 in 100		1 in 100		1 in 100					1 in 100		1 in 100
Secondary antibody dilution	1 in 1000		1 in 1000		1 in 1000		1 in 1000					1 in 1000		1 in 1000
Biotin. secondary dilution	1 in 1500*				1 in 1500*									1 in 250**
Avidin HRP dilution***	1 in 2500				1 in 2500									1 in 2500
<b>Native Westerns</b>														
Primary antibody dilution														1 in 750
Secondary antibody dilution														1 in 1000
<b>Immunoprecipitations</b>														
1 <sup>st</sup> Primary antibody dilution														1 in 60final
2 <sup>nd</sup> primary antibody dilution														
Secondary antibody dilution														1 in 2000
														1 in 5000

**Table 2.3: Antibody Dilutions Used in Immunological Techniques.**

Dilutions quoted are based on a 1mg/ml stock of antibody. \* represents a 1.5mg/ml stock; \*\* represents a 0.5mg/ml stock; \*\*\* represents a 5mg/ml stock. Biotin = biotinylated. All secondary antibodies are conjugated to HRP or alkaline phosphatase. Vectastain kits: Mouse IgG (PK-4002) & Rabbit IgG (PK-4001) from Vector Labs (Peterborough, U.K.).

## **2.1.3 Denaturing Western Blots**

### **2.1.3.1 Preparation of Samples**

Tissue or brain homogenates were thawed on ice and protein assayed as detailed in Chapter 3 (section 3.2.1.6). Following centrifugation (30000g; 20 min, 4°C), the resulting pellets were resuspended in 10% SDS to give a concentration of 10mg/ml protein. These were left for at least two hours at room temperature to allow full denaturation, with intermittent vortexing to aid the process. Samples were then diluted 1:1 with x2 Laemmli buffer so as to give a final concentration of 5mg/ml protein. A 100ml stock of Laemmli buffer consisted of 10% SDS (20ml), 50% glycerol (20ml), d.H<sub>2</sub>O (35ml), 0.5M Tris-HCl (pH 6.8; 25ml) and 0.02g of bromophenol blue to enable visualisation of the running of the samples. 250µl mercaptoethanol per 5ml of Laemmli buffer was added to act as a reducing agent prior to use. This solution was prepared fresh for each new set of samples, whereas the Laemmli stock was used for periods up to 6 months. Prior to their use in SDS-polyacrylamide gel electrophoresis (PAGE), all tissue samples diluted in reduced Laemmli buffer were heated for 20 mins at 37°C, then centrifuged briefly (approximately 2 mins) at 20g to remove any particulate debris. Molecular weight standards (colour wide range markers; Sigma; C3437), stored as 20µl aliquots at -20°C were also diluted 1:1 with x2 Laemmli buffer after thawing at room temperature and then boiled (84°C) for 20 mins prior to their use.

### **2.1.3.2 Polyacrylamide Gel Electrophoresis (PAGE)**

Resolving gels (7.5% containing 40% acrylamide (3.75ml), 2% bisacrylamide (2ml), 3M Tris (pH8.8, 2.5ml), 10% SDS (0.2ml), 1.5% ammonium persulfate (1ml), d.H<sub>2</sub>O; (10.55ml) and TEMED (10µl) to initiate polymerisation) were poured between fixed glass and aluminium plates (10x10cm) separated by 0.5mm spacers, leaving a 3cm gap from the top, layered with 400µl saturated butan-1-ol and covered until polymerised. Following polymerisation, the butan-1-ol was washed off several times with d.H<sub>2</sub>O. Combs were inserted 5mm from the resolving gel surface, and the stacking gel poured (7.5% containing 40% acrylamide (0.9ml), 2% bisacrylamide (0.45ml), 0.5M Tris (pH6.8, 2.5ml), 10% SDS (0.1ml), 1.5% ammonium persulfate



(0.5ml), mQ d.H<sub>2</sub>O (5.55ml) and TEMED (7.5µl)). After the stacking gel had set, the combs were removed, the plates transferred onto a Hoefer electrophoresis unit and the wells and electrodes immersed in running buffer (containing 25mM Tris, 192mM glycine and 1% SDS). Samples were loaded (usually 10µl) and electrophoresed with a non-limiting voltage at 30mA at room temperature until the dye front was <0.5cm from the bottom of the plate (1-2 hours).

The stacking portion of the gel was cut away and the resolving portion of the gel laid protein side up on PVDF membranes (Hybond™-P, Amersham; equilibrated in 100% methanol for 30 seconds) and the dye fronts marked on the membrane. Following immersion in running buffer (25mM Tris, 192mM glycine and 20% methanol), the gel and PVDF membranes were surrounded by filter paper and air bubbles gently rolled out. These membrane/gel 'sandwiches' were locked in cassettes containing thick sponges and immersed in running buffer in a Hoefer wet transfer system. The proteins were then transferred to the blotting paper overnight at 4°C using a non-limiting current at approximately 17V.

### **2.1.3.3 Detection of SERT Immunoreactivity**

The blots from PAGE were then processed for SERT immunoreactivity at room temperature. They were washed briefly (2 x 30 seconds) in wash buffer (PBS containing 0.1% Tween-20) before non-specific sites were blocked in 5% Blotto (or 3% BSA) and 0.1% Tween-20 in PBS for two hours. The primary anti-SERT antibody to be investigated was diluted in antibody diluent (PBS containing 0.5% Blotto (or 0.3% BSA) and 0.01% Tween-20; see Table 2.3) and incubated with PVDF membranes for one hour. Following 3 x 10 min and 2 x 5 min washes of PVDF membranes, the secondary antibody conjugated to HRP (horseradish peroxidase) diluted in antibody diluent (see Table 2.3) was incubated with PVDF membranes for 45 mins. Following 2 x 5 min and 7 x 3 min washes in wash buffer, the blots were finally washed in PBS only (2 x 30 seconds). For a full size gel, wash volumes generally were 20-30ml, and antibody incubation volumes were 10-15ml. After each addition, blots were gently incubated on orbital shakers to allow even antibody binding and thorough washing.

Immunoreactivity was finally detected using the ECL plus™ (Amersham) system before exposure on Hyperfilm™ (Amersham) according to the manufacturer's instructions. Films were immersed in developer (Ilford Multigrade, U.K.; catalogue number 757855; diluted 10 fold with water) for 4 min, dipped in water before two 4 min washes in fixative (Ilford Multigrade, U.K.; catalogue number 758285; diluted 10 fold with water) and finally dipped in water containing detergent (0.1%). Films were then rinsed under cold running tap water for 30 min, dried in a cabinet at 30°C, covered and stored at room temperature prior to analysis.

As the rainbow molecular weight markers used do not transfer onto PVDF membranes, the original blot, once dried was overlaid over the film and the position of the molecular weight markers marked.  $R_f$  values for molecular weight markers and protein samples were calculated according to their distance travelled with respect to the distance travelled by the tracking dye. A standard curve of  $\log_{10}$  molecular weight against  $R_f$  was produced for marker proteins, and molecular weights of the unknown sample proteins were then calculated by interpolation from the curve generated by computerised linear regression analysis.

#### **2.1.4 Immunohistochemistry**

Rats were anaesthetised with sodium pentobarbitone (60mg/kg; i.p.). Following cessation of reflex responses, the heart was exposed and via a trans-cardial perfusion route, sterile saline (0.9% w/v containing 2% w/v heparin) was delivered for 1 min followed immediately by ice-cold fixative (4% paraformaldehyde, 0.05% glutaraldehyde) for a further 9 mins at a flow rate of 20ml min<sup>-1</sup>. Brains were then removed and post-fixed in the same fixative for 2 hours at 4°C, followed by overnight with 20% sucrose at 4°C.

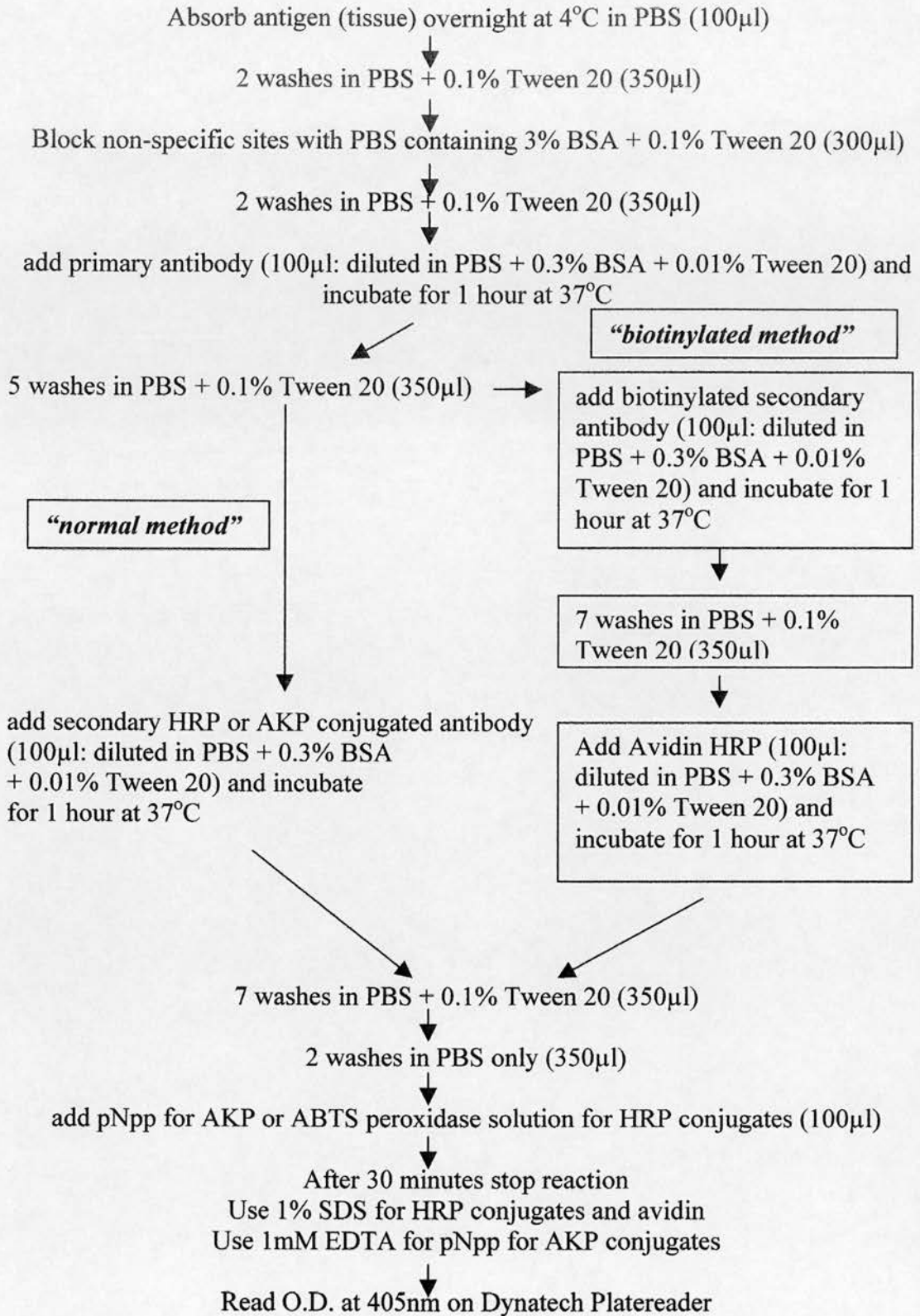
Brains sections (20µm) were cut on a Bright cryostat in a similar fashion to those used in autoradiographic studies (chapter 3 section 3.3.2 for general method) at the level of the raphé nuclei, the septum and the medial geniculate. SERT immunohistochemical detection was carried out on free-floating sections in 24-well culture plates at room temperature (unless stated). For each well, 1-3 sections were processed with wash volumes being 1ml, and antibody additions 0.5ml. After each addition plates were gently incubated on orbital shakers to allow even and thorough

washing. Sections were initially incubated for 30 mins in PBS in 0.3% H<sub>2</sub>O<sub>2</sub> (containing 0.3% Triton X-100) to remove endogenous peroxidase activity. Following washing in PBS (5 x 5 mins), non-specific binding sites were blocked for 2 hours with 20% normal goat serum (NGS) in PBS. After washing (3 x 1 min) in PBS, the primary antibody was diluted in antibody diluent (PBS containing 2% NGS, 0.01% NaN<sub>3</sub> and 0.3% Triton X-100) and incubated overnight at 4°C according to dilutions in Table 2.3. After 3 x 1 min and then 2 x 10 min washes in PBS, sections were processed using VECTASTAIN ABC kits. Sections were incubated with the biotinylated antibody required for 1 hour, followed by 5 washes (as before) and incubation (1 hour) in the avidin-biotin-peroxidase complex according to the manufacturers instructions. Following another set of 5 washes, the peroxidase reaction was observed using DAB-H<sub>2</sub>O<sub>2</sub> (3,3'-diaminobenzine tetrahydrochloride-hydrogen peroxide). Sections were rinsed in PBS, laid on microscope slides (subbed as for autoradiographic experiments outlined in Chapter 3, section 3.3.1), dried overnight, dehydrated by placing in increasing concentrations of ethanol (70-100%) then xylene, and coverslips finally attached using styrolite mounting medium.

## **2.1.5 ELISAs**

### **2.1.5.1 Plate ELISAs**

A 96 well plate ELISA was used as the initial attempt to identify native SERT protein. Maxisorb™ (Nunc) 96 well plates were used for these experiments. The methodology is outlined in Figure 2.3. An indirect ELISA was used, whereby the antigen (protein) was absorbed in the well, and the remaining sites blocked. Subsequent incubation with specific antibodies, which bind to the antigen, was followed by incubation with an enzyme-conjugated secondary antibody. Two different enzyme-conjugated secondary antibodies were used. Alkaline phosphate conjugated antibodies were detected using the pNpp substrate (SIGMA FAST™ p-Nitrophenyl Phosphate), whose reaction was stopped after 30 mins with an equal volume of 1mM EDTA. Horseradish peroxidase (HRP) conjugated antibodies were also used, in keeping with the immunohistochemical and Western blot experiments. The biotinylated method outlined in Figure 2.3, makes use of the high affinity of avidin for biotinylated secondary antibodies to potentially increase signal size (avidin



**Figure 2.3: ELISA (96 Well Plate) Methodology.**

Washes were performed by inverting the plate and “flicking”, followed by pressing on paper absorbent towels. HRP = horseradish peroxidase. AKP = alkaline phosphatase. O.D. = optical density

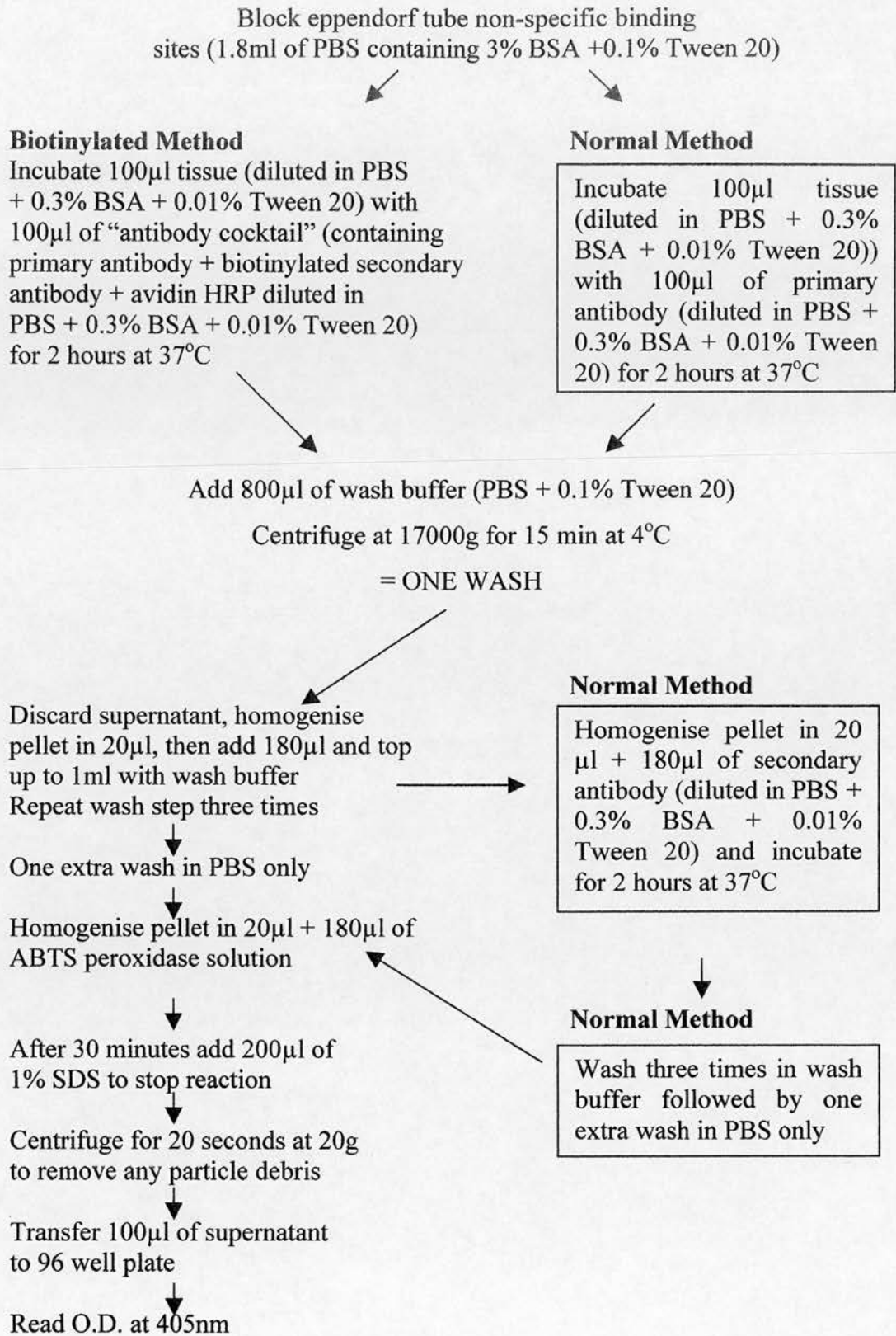
binds noncovalently to 4 molecules of biotin with high affinity;  $K_D = 10^{-15}$  M; Lillehoj, 1994). These HRP antibodies were detected using the ABTS peroxidase substrate whose reaction was stopped after 30 mins with 1% SDS. Using a Dynatech plate reader, samples were mixed by shaking for 20 seconds and absorbance measured at an optical density of 405nm ( $OD_{405}$ ).

### **2.1.5.2 Centrifugation ELISAs**

To facilitate the use of larger amounts of protein compared to the 96 well plate ELISAs, a centrifugation ELISA style protocol was adopted which incorporated the steps of the 96 well plate assay. Initial experiments revealed that centrifuging samples in eppendorf tubes at 17000g (max.) for 2 mins in a lab top centrifuge, caused a 70% decrease in protein pelleted compared to the original sample. Increasing the centrifugation time to 15 mins only caused a 1-5% decrease in protein after each centrifugation. However to run centrifugation ELISAs as per the 96 well plate ELISA methodology (centrifugation replacing conventional washes; Figure 2.3) long experiments and more importantly a 13-51% and 19-66% loss in protein in the normal and biotinylated methods respectively would result. Therefore an alternative approach was attempted, to reduce the number of washes required as outlined in Figure 2.4. Eppendorf non-specific binding sites were blocked and then tissue and antibodies incubated in one step. In the biotinylated method, this involved the formation of a primary antibody, secondary biotinylated antibody and avidin HRP complex. These HRP antibodies were detected using the ABTS peroxidase substrate whose reaction was stopped after 30 mins with 1% SDS. Samples were mixed and transferred to a 96 well plate, shook for 20 seconds and absorbance measured at an optical density of 405nm ( $OD_{405}$ ) using a Dynatech plate reader.

### **2.1.6 Native Western Blotting**

Native Western blotting followed a similar protocol to SDS-PAGE Western blotting, except that the protein was extracted from samples using the non-ionic detergent, Triton X-100. For native gel electrophoresis, protein was extracted in 20mM Tris-Glycine pH9.0 containing 2% Triton X-100 and a cocktail of protease and phosphatase inhibitors. Protein was separated on a native polyacrylamide gel (3-10% gradient) (running buffer: 100mM Tris-HCl-Glycine pH 9.0 containing 0.1%



**Figure 2.4: Generalised Methodology for Centrifugation ELISAs.**  
 HRP = horseradish peroxidase. O.D. = optical density.

Triton X-100) and transferred to PVDF membranes (transfer buffer: 100mM Tris-Glycine pH 9.0). These experiments were performed in collaboration with Dr. Jane Matthews (Department of Neuroscience)

### **2.1.7 Immunoprecipitations**

For immunoprecipitations, protein was extracted in 50mM Tris-HCl (pH 8.0; containing 120mM NaCl, 1% Triton X-100 and a cocktail of protease and phosphatase inhibitors). SERT was immunoprecipitated overnight at 4°C with one primary antibody (mouse monoclonal), isolated following binding of antibody to Protein A-Sepharose and denatured in RIPA buffer (containing 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS).

Protein was separated by SDS-PAGE on a 7.5% resolving gel, transferred to PVDF membranes and immunoblotted using another primary antibody (guinea pig polyclonal) as outlined above in the denaturing Western blot methodology (section 2.1.3) and in Table 2.3. Typically 60µl of an initial 1 in 10 dilution of the mouse monoclonal antibody was added to 300µl (1.5mg) of tissue and 30µl of 50% Protein A-Sepharose. These experiments were conducted by Dr Jane Matthews and are included in this thesis for the purpose of completion.

### **2.1.8 Materials**

Sodium pentobarbitone was purchased from Rhône Mérieux (Harlow, Essex, UK). 24-well culture plates were obtained from Costar (Cambridge, MA, U.S.A.). ABTS peroxidase solution was purchased from Kirkegaard & Perry Laboratories, Inc. (Maryland, U.S.A.). Vectastain ABC peroxidase kits and DAB were purchased from Vector Laboratories (Peterborough, U.K.). The alkaline phosphatase substrate pNpp (p-nitrophenyl phosphate) was purchased from Sigma (Poole, Dorset, U.K.). All secondary antibodies and normal serum were purchased from Chemicon International (Harrow, U.K.). The 5-HT antibody was purchased from Incstar (now Diasorin, Berkshire, U.K.). All other chemicals were obtained from regular commercial sources, and all reagents used were of the highest analytical grade.

## 2.2 Results

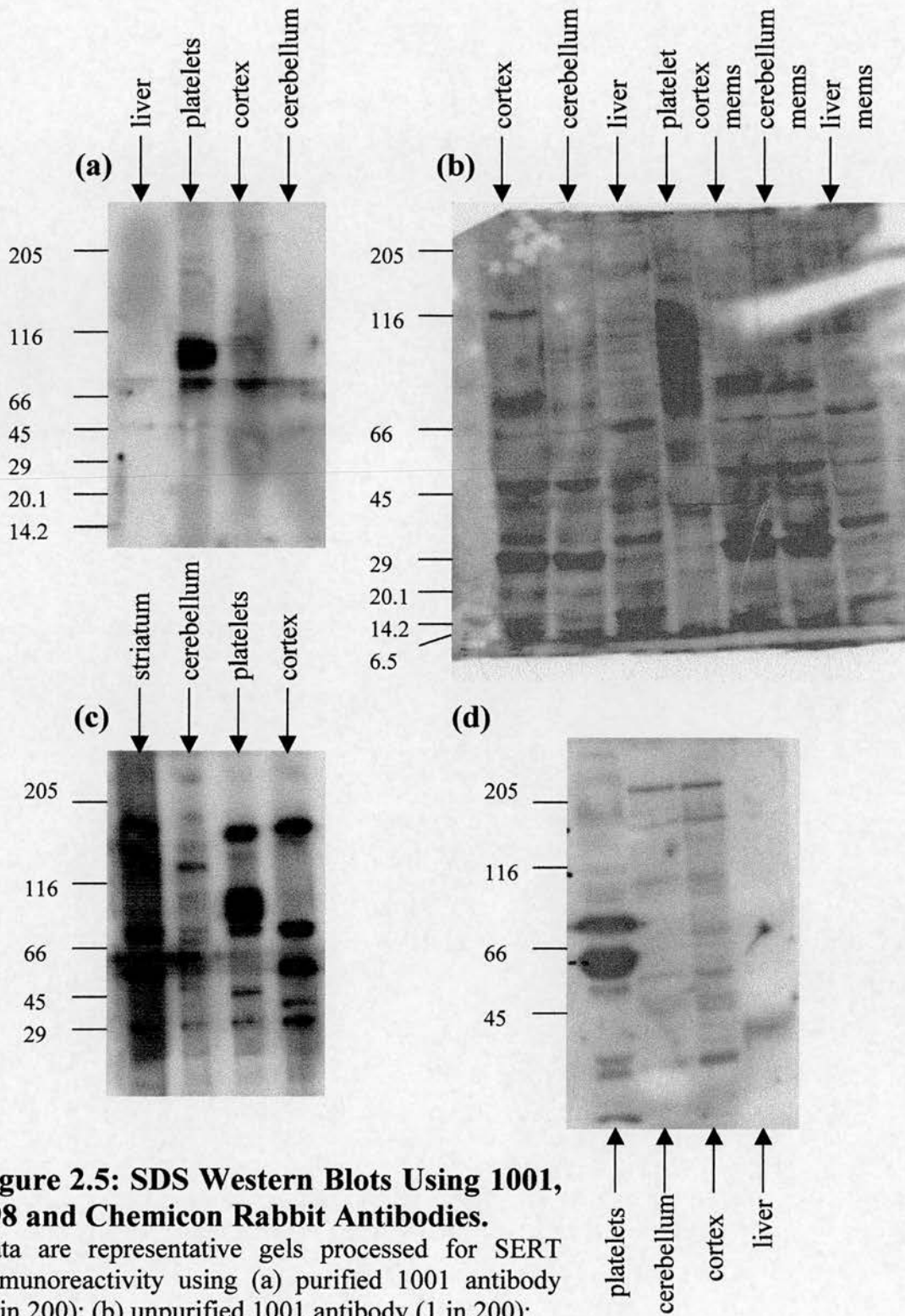
### 2.2.1 Denaturing Western Blots

The 1001 (4<sup>th</sup> extracellular loop) affinity purified antibody recognises SERT, with a molecular weight of 94kDa in platelets and 76kDa in brain homogenates, with much lower intensity in cerebellum homogenates compared to cortex homogenates (Figure 2.5a). No immunoreactivity was detected in liver homogenates (Figure 2.5a). Preincubation of the 1001 antibody with the immunising peptide removed all immunoreactivity, as did the absence of the primary antibody (Dr Jane Lawrence-personal communication; Lawrence *et al.*, 1995a; data not shown). Unpurified 1001 antibody, recognised multiple bands in all tissues, whether homogenates or membranes, but did recognise SERT immunoreactivity at 94kDa and 76kDa in platelet and cortical tissues respectively (Figure 2.5b). Immunoreactivity was also apparent in cerebellum tissue but to a much lesser extent than in cortical and platelet tissue (Figure 2.5b). The disappearance of non-specific banding confirms the affinity purification method conducted by Dr Jane Lawrence.

The 998 affinity purified antibody, which was generated to a peptide sequence from the carboxyl tail rather than in the 4<sup>th</sup> extracellular loop (Table 2.2) recognised the specific elements that the other in-house antibody recognised (1001) with prominent platelet immunoreactivity at 94kDa and strong cortical and striatal immunoreactivity at 76kDa with much less cerebellum immunoreactivity at this migration size (Figure 2.5c). This specific banding was removed on preincubation with the antibody to the antigenic peptide (Dr Jane Lawrence-personal communication; Lawrence *et al.*, 1995a; data not shown). However this purified antibody did, unlike 1001, display immunoreactivity to more than one species in all tissues (Figure 2.5c) which was not removed by preincubation with the antigenic peptide (Dr Jane Lawrence-personal communication; data not shown).

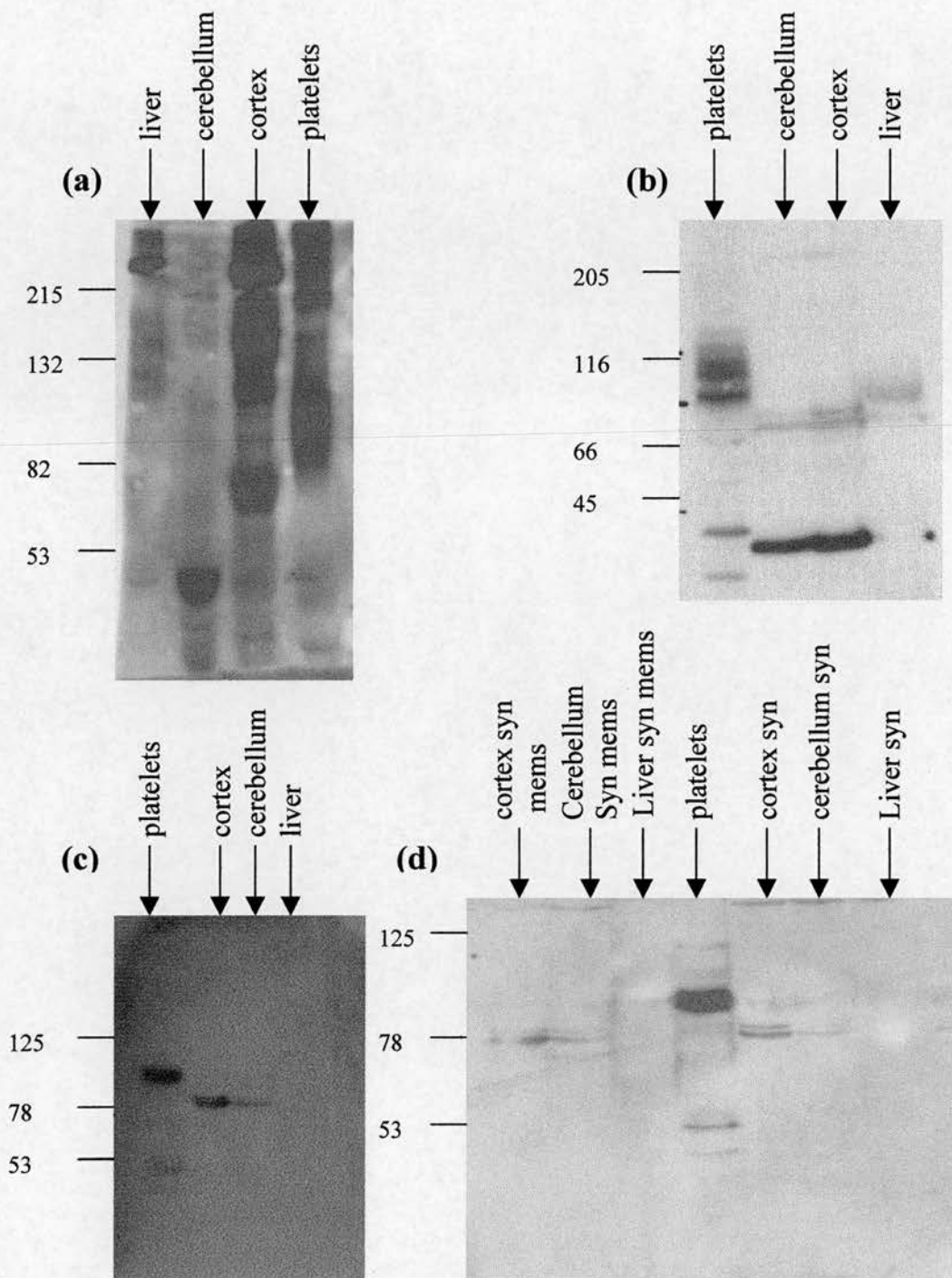
The Chemicon rabbit antibody raised to the same sequence as 1001 recognised multiple immunoreactive bands in all tissues, that did not include SERT at 94kDa in platelets or 76kDa in cortical membranes (Figure 2.5d). Furthermore this immunoreactivity was not abolished on preincubation of the antibody with the antigenic peptide (data not shown). Of the other antibodies tested, both the Chemicon





**Figure 2.5: SDS Western Blots Using 1001, 998 and Chemicon Rabbit Antibodies.**

Data are representative gels processed for SERT immunoreactivity using (a) purified 1001 antibody (1 in 200); (b) unpurified 1001 antibody (1 in 200); (c) purified 998 (1 in 200); (d) chemicon rabbit (1 in 200). Dilutions are as detailed in brackets. The corresponding secondary antibody dilutions are in Table 2.3. The molecular weight is to the left of the gel (in kDa) according to the standards used. 50µg protein loaded in each lane. All lanes are for homogenate tissue, unless stated otherwise. Mem = membranes.



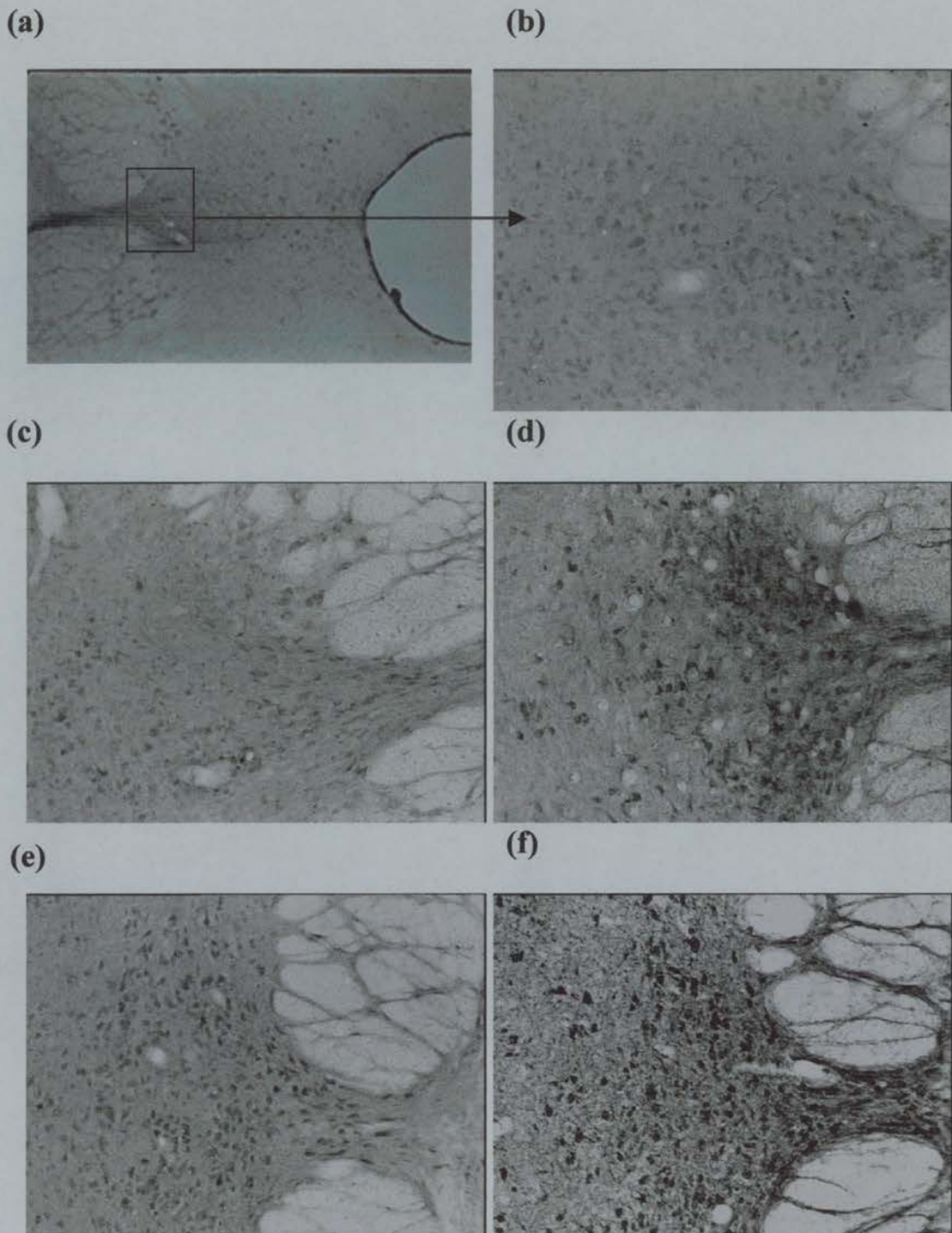
**Figure 2.6: SDS Western Blots Using Guinea Pig, Incstar and Monoclonal Antibodies.**

Data are representative gels processed for SERT immunoreactivity using (a) guinea pig (1 in 2000); (b) Incstar (1 in 500); (c) & (d) monoclonal (1 in 750) antibodies. Dilutions are as detailed in brackets. The corresponding secondary antibody dilutions are in Table 2.3. The molecular weight is to the left of the gel (in kDa) according to the standards used. 50 $\mu$ g protein loaded in each lane. All lanes are for homogenate tissue, unless stated otherwise. Syn Mems = synaptic membranes. Syn = synaptosomes.

guinea pig and Incstar rabbit antibodies recognised multiple immunoreactivity with prominent bands at 94kDa in platelets and 76kDa in cortical tissue with limited or no immunoreactivity at these molecular weights in cerebellum or liver homogenates (Figure 2.6a & b). In contrast the mouse monoclonal antibody, recognised one immunoreactive band in brain tissue at 76kDa, and prominent immunoreactivity at 94kDa in platelets (Figure 2.6c) with no difference in the intensity of immunoreactivity between synaptosomes and synaptosomal membranes (Figure 2.6d). For all of the antibodies tested (excluding the Chemicon rabbit polyclonal), those that recognised immunoreactive bands at 94kDa in platelets, also showed some weaker immunoreactivity between 40 and 76kDa (Figures 2.5 & 2.6) and bands at 76kDa in brain tissue. Furthermore the rank order of intensity of antibody labelling (1001, 998, Chemicon guinea pig, Incstar and mouse monoclonal) at these calculated molecular weights for these tissues was platelets >> cortex >> cerebellum >= liver (Figures 2.5 & 2.6). The antigenic peptides for all commercial antibodies were not available and therefore prevented experiments whereby the specificity of labelling was determined in the presence of antibody absorbed by competing peptide. However exclusion of primary antibody from the detection process (section 2.1.3.3) revealed no immunoreactivity (data not shown). All of the figures are representative examples of experiments that had been repeated at least twice.

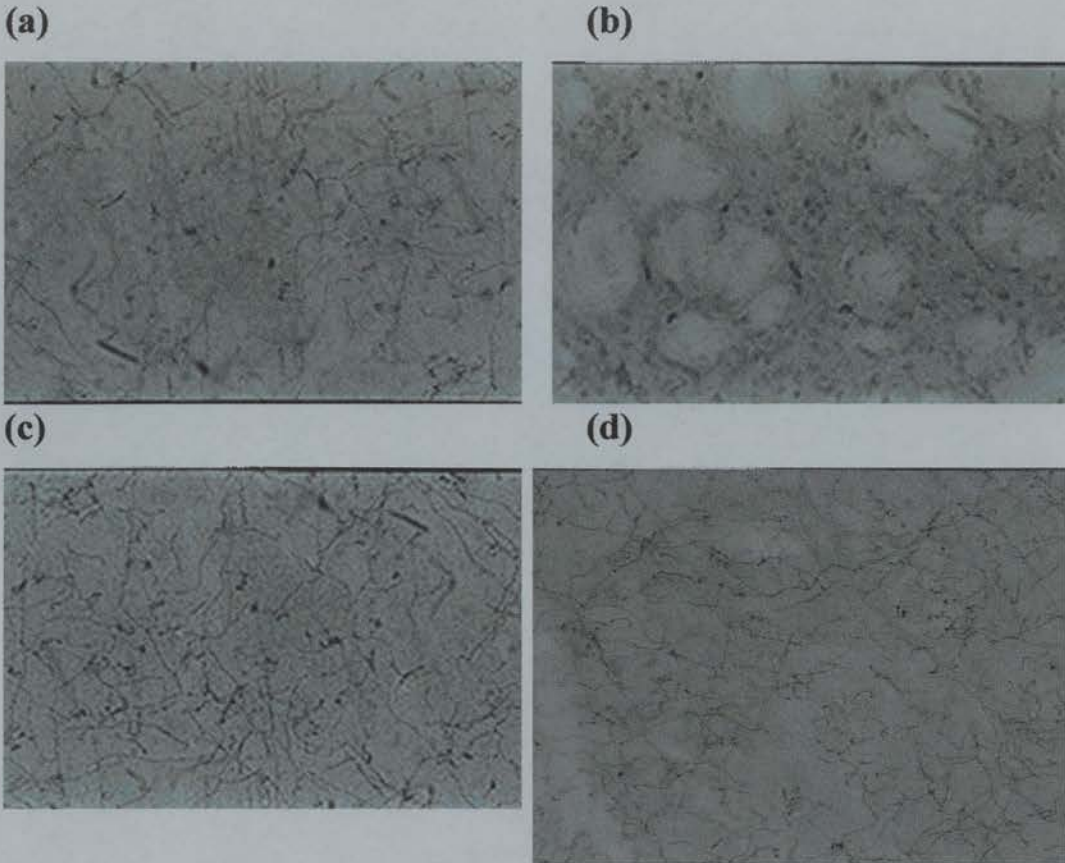
## **2.2.2 Immunohistochemistry**

All of the antibodies tested, 1001, 998, Incstar and mouse monoclonal at the dilutions specified in Table 2.3, labelled the cell bodies in the dorsal raphé nucleus (Figure 2.7). Furthermore all SERT immunoreactivity overlapped 5-HT immunoreactivity (Figure 2.7). In the terminal fields of the hippocampus and the striatum, good specific staining was obtained with the 998 antibody and also the mouse monoclonal, whereas poor staining was obtained for the 1001 antibody and more so for the Incstar antibody (Figure 2.8). Absence of the primary antibody in each case revealed negligible staining (data not shown). Previous studies have demonstrated that the 998 antibody staining is specific, whereby 5,7-DHT lesions significantly depleted the immunoreactivity observed (Dr Jane Lawrence,



**Figure 2.7: Immunohistochemical Localisation of SERT Protein In Raphé Complex.**

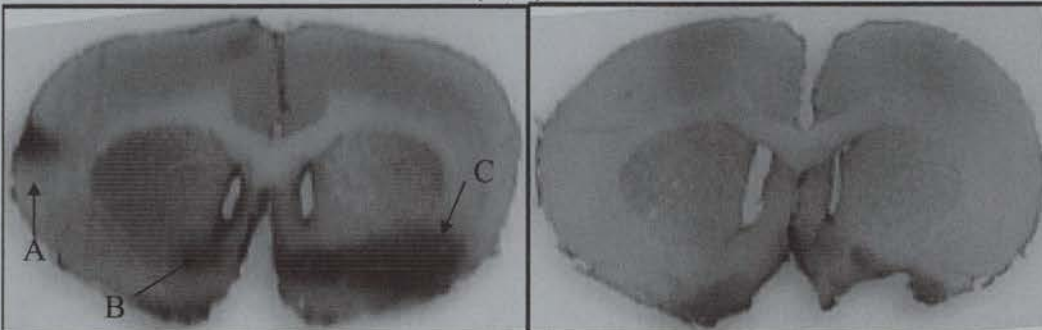
Data are representative immunohistological rat brain sections processed for SERT immunoreactivity using different antibodies as outlined in Table 2.3. Images represent; 998 antibody in (a) & (b); 1001 antibody in (c); Incstar antibody in (d); mouse monoclonal antibody in (e); 5-HT antibody in (f). All images are displayed at a magnification of x100 except (a), which is at x16.



**Figure 2.8: Immunohistochemical Localisation of SERT Protein In 5-HT Nerve Terminal Containing Projection Areas.**

Data are representative immunohistological rat brain sections processed for SERT immunoreactivity using different antibodies as outlined in Table 2.3 in the hippocampus (a) & (c) and in the striatum (b) & (d). Images represent; 1001 antibody in (a); Incstar antibody in (b); mouse monoclonal in (c); 998 antibody in (d). All images are displayed at a magnification of x200.

(e) and (f) below are figures courtesy of Dr Jane Lawrence showing examples of reduced SERT density as observed on an anatomical level following 5,7- DHT lesions. Immunoreactivity is visualized with 998 antibody (1:1000 dilution). Areas shown are approximately 0.9-1mm lateral with respect to bregma. Most profound reductions are seen in cortical areas (A,B) and the anterior commissural nucleus (C).



(e)

(f)

unpublished findings, Figure 2.8e & f). All of the figures are representative examples of experiments that had been repeated at least twice.

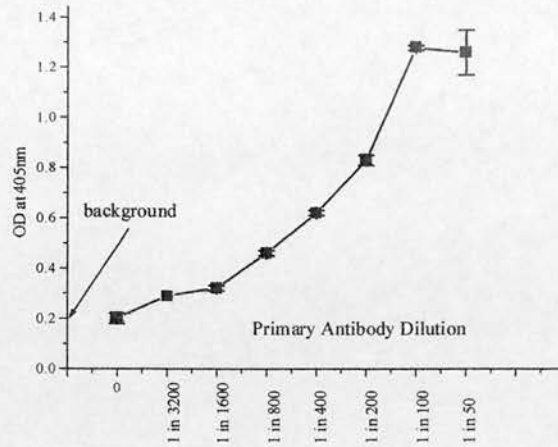
### **2.2.3 ELISAs (96 well plate)**

The 96 well plate ELISA was initially established with the unpurified 1001 antibody, in order to conserve affinity purified material on the basis of expense and time to purify the antibody. Initial titre of the antibody against cortical synaptosomes at 400ng (near to the binding capacity of the plate, 400-800ng of protein) gave a working dilution of 1 in 100 (Figure 2.9a), and showed a rank order of reactivity of liver > cerebellum > cortex > platelets (Figure 2.9b). No differences were observed between synaptosomes or membranes (Figure 2.9b). There was no difference in the rank order of reactivity using either alkaline phosphatase or horseradish peroxidase conjugated antibodies (data not shown). The tissue showed no endogenous alkaline phosphatase or peroxidase activity in experiments with only tissue and no antibodies (data not shown). Use of purified antibodies at dilution of up to 1 in 50, gave no signal (data not shown). In an attempt to increase the signal, biotinylated secondary antibodies were used in conjunction with the unpurified 1001 antibody and found to increase the response, as shown by the shift to the left in Figure 2.9c. However, the biotinylated method still did not give a signal when using the affinity purified antibodies (998, 1001 and all commercial antibodies) at up to dilutions of 1 in 50. Carrying out incubations at 4°C instead of room temperature, to prevent any potential capping or internalisation of the transporter protein, did not solve the signal problem. Replacing PBS with 50mM Tris-HCl (pH 7.4; containing NaCl (120mM), and KCl 5mM) the assay buffer used for used in SERT radioligand binding assays (Chapter 4), similarly made no difference.

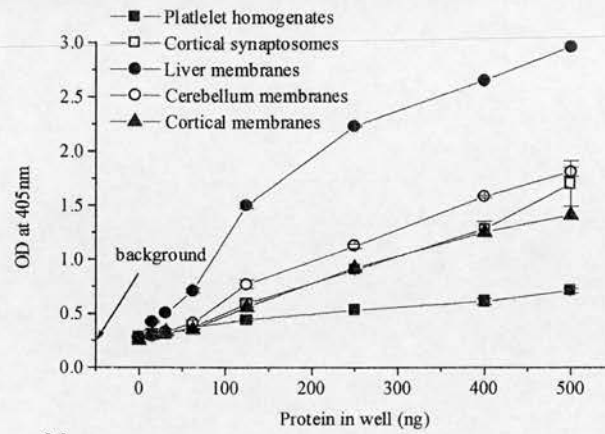
### **2.2.4 Centrifugation ELISAs**

To increase the amount of protein that could be assayed, which would be more comparable to Western blots (50µg) where immunoreactivity is detected with the purified antibodies, a centrifugation ELISA was attempted. This was only tested using the mouse monoclonal antibody to save on wastage of the in-house purified antibody in developing the assay. Similar “specific” signals for both cortical and liver membranes were obtained using the biotinylated approach to increase the

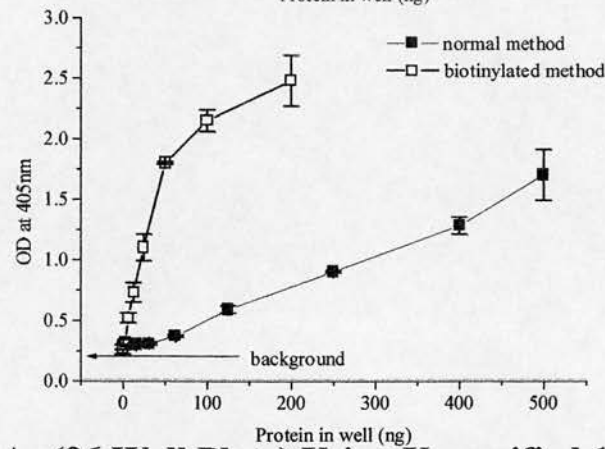
(a)



(b)



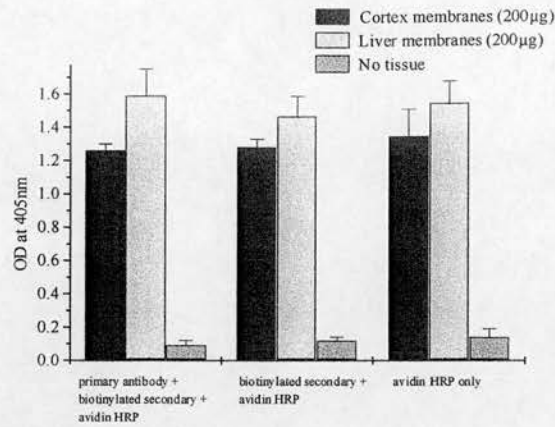
(c)



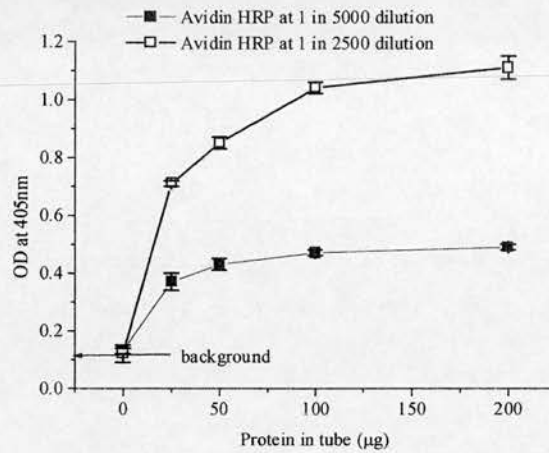
**Figure 2.9: ELISAs (96 Well Plate) Using Unpurified 1001 Antibody.**

Data represent: (a) titre of 1001 unpurified antibody against 400ng per well cortical synaptosomes; (b) 1 in 100 dilution of 1001 unpurified antibody against increasing protein; (c) comparison of biotinylated method against normal method using 1 in 100 dilution of 1001 unpurified antibody in cortical membranes. In each case the secondary antibody (anti-rabbit IgG conjugated to HRP) was diluted 1 in 1000. For the biotinylated method in (c), the biotinylated anti-rabbit secondary antibody (1.5mg/ml stock; Vector) was diluted 1 in 1500, whereas avidin HRP (5mg/ml stock; Vector) was diluted 1 in 2500. Each point represents the mean of duplicate points with each experiment repeated at least three times. The background (no tissue or antibodies) is shown in each case. OD = optical density measured at 405nm.

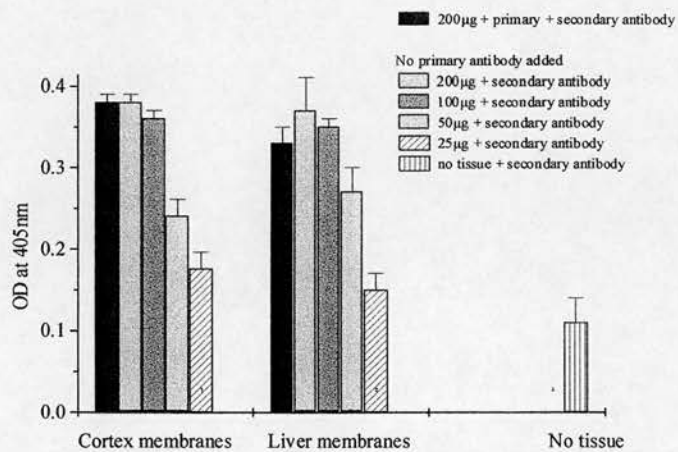
(a)



(b)



(c)



### Figure 2.10: Centrifugation ELISA using Monoclonal Antibody.

Data represent: (a) membranes in the presence of Avidin HRP (1 in 2500 dilution of 5mg/ml stock; Vector)  $\pm$  biotinylated secondary antibody (1 in 250 of 2.5mg/ml stock)  $\pm$  1 in 100 dilution of mouse monoclonal antibody; (b) cortical synaptic membranes (200µg) in the presence of Avidin HRP only (1 in 2500 dilution of 5mg/ml stock; Vector); (c) non-biotinylated approach using 1 in 1000 anti-mouse IgG secondary antibody conjugated to HRP  $\pm$  1 in 100 dilution of mouse monoclonal antibody and increasing concentrations of membranes. Each point represents the mean of duplicate points with each experiment repeated at least three times. The background (no tissue or antibodies) is shown in (b) and is no different to any permutation of antibodies with no tissue. OD = optical density measured at 405nm.



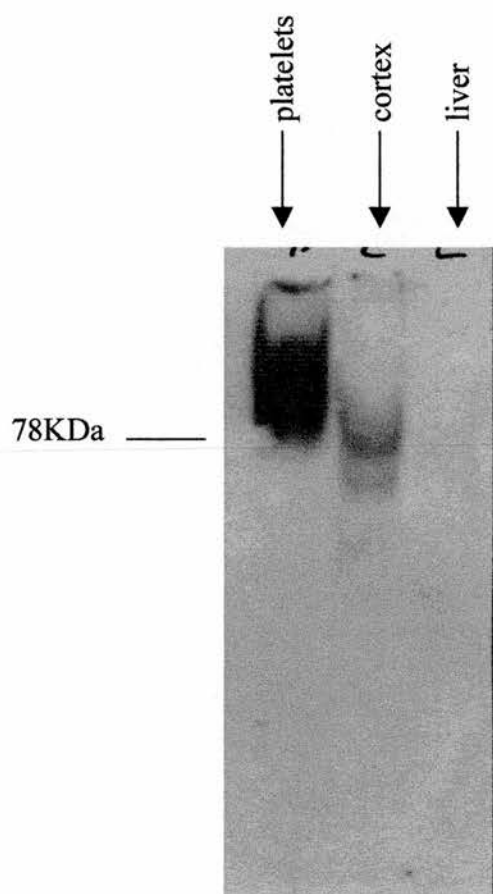
sensitivity of the assay (Figure 2.10a). However this signal was also detected in the absence of the antibody and also in the presence of avidin HRP alone, but not in the absence of tissue (Figure 2.10a). This suggested that centrifugation resulted in avidin HRP becoming incorporated into the pellet. Adding two more washes decreased this non-specific signal only slightly (probably due to loss of protein; data not shown). In the presence of avidin HRP alone and increasing concentrations of protein in the tube, there was an increase in signal (Figure 2.10b), irrespective of any tissue used (data not shown). This confirmed that avidin HRP was centrifuged down with the protein. Using the non-biotinylated method (as used originally in the 96 well plate ELISA), an identical story was apparent. A similar signal was obtained in cortical and liver membranes, which remained in the absence of primary antibody and gradually decreased with decreasing protein (Figure 2.10c).

### **2.2.5 Native Western Blots**

Extraction of native SERT using the non-ionising detergent, Triton X-100, followed by electrophoresis on a non-denaturing gel (Triton X-100 replacing SDS), allowed the migration of native protein from the wells into the gel. Larger banding was observed, probably due to the appreciable sample volume (10-20 $\mu$ l) loaded onto a gel lacking the focussing capabilities of a stacking gel. Specific immunoreactivity was observed in both platelet homogenates (94kDa determined from Ferguson plots; data not shown) and cortex membranes with no immunodetection in liver membranes using the Chemicon guinea pig antibody (Figure 2.11). The mouse monoclonal antibody has also been tested with a similar immunoreactive profile (data not shown; Dr Jane Matthews).

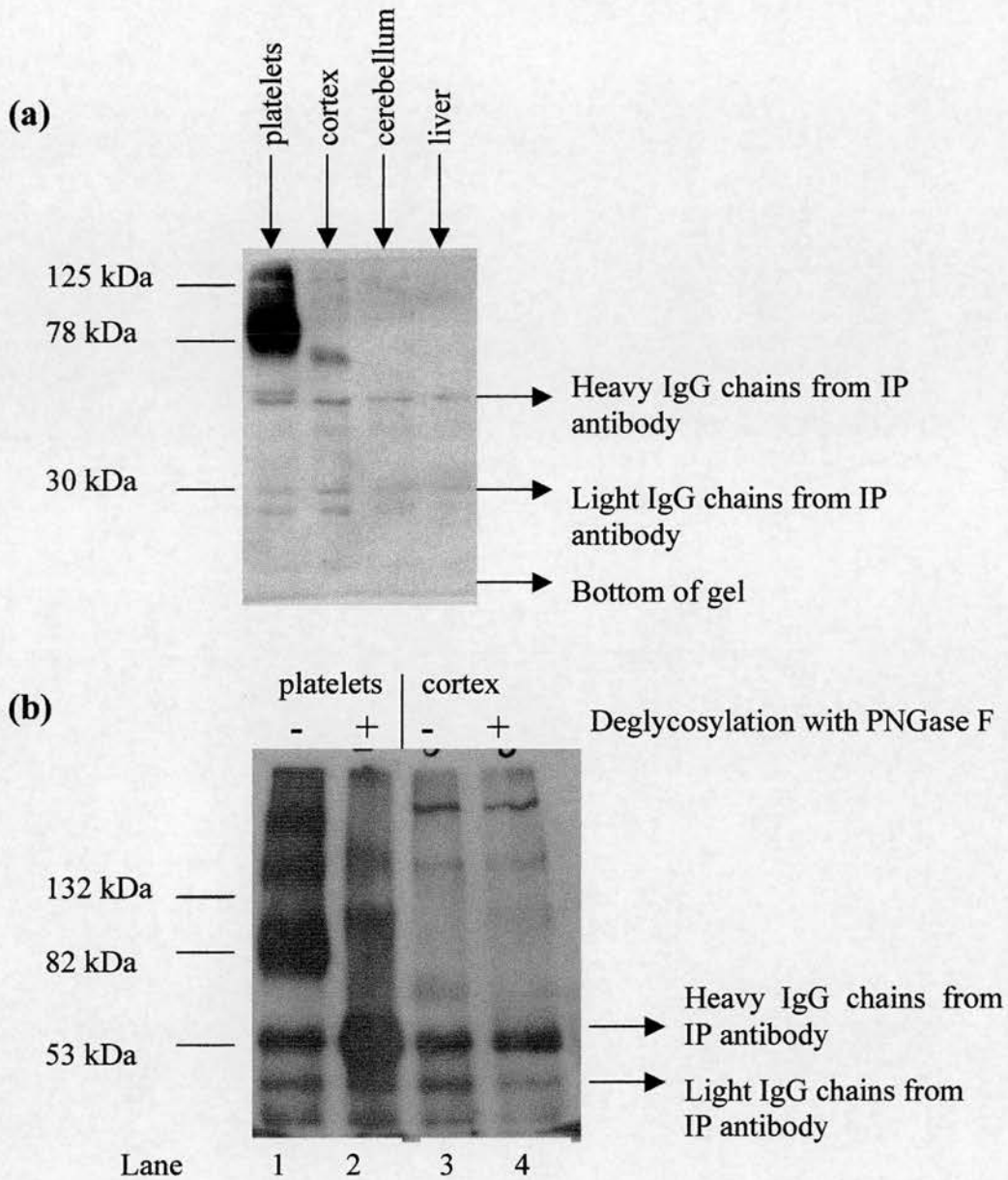
### **2.2.6 Immunoprecipitation of Native SERT**

Native protein extracts were immunoprecipitated with the mouse monoclonal antibody and then denatured. Following SDS-PAGE, immunodetection with the guinea pig polyclonal antibody, revealed a prominent platelet homogenate band (94 kDa) and a weaker band (76 kDa) in cortex (Figure 2.12a). However no such immunoreactive band was present in cerebellum membranes (Figure 2.12a). Species cross-reactivity of the secondary antibody in the immunodetection was also apparent, since both heavy and light chains of the immunoprecipitating monoclonal antibody



**Figure 2.11: Native Western Blotting of SERT.**

Native protein was triton extracted from platelet homogenates, cortex and liver membranes. Following separation of protein by native gel electrophoresis and transfer to PVDF membrane, SERT was immunodetected using Chemicon guinea pig antibody (1 in 2000 dilution). These results are representative of four separate experiments.



**Figure 2.12: Immunoprecipitation of Native SERT.**

Data represent typical (a) immunoprecipitation of native SERT and (b) immunoprecipitation of native SERT in the presence and absence of the endoglycosidase PNGase F. In (a) native protein was triton extracted from cortex and cerebellum membranes and platelet homogenates. SERT was immunodetected using Chemicon guinea-pig polyclonal SERT antibody (1 in 2000 dilution) following immunoprecipitation with Chemicon monoclonal SERT antibody (1 in 750 dilution), separation on SDS-PAGE and transfer to PVDF membrane. In (b) native protein was triton extracted from cortex synaptosomes and platelet homogenates and  $\pm$  deglycosylated. SERT was immunodetected as in (a). These experiments are representative of at least 3 independent experiments in each case. IP antibody = immunoprecipitation antibody.

were detected in all samples (Figure 2.12a), including in the absence of protein (data not shown). Using this method SERT has also been detected in cortical synaptosomes (76 kDa), albeit with low abundance, which clearly migrates further on deglycosylation with PNGase F (Figure 2.12b; compare lane 4 to lane 3). SERT from platelets also migrate to the same position (54kDa) following treatment with endoglycosidase PNGase F, which in the case of the cortical synaptosomes is masked by the presence of the heavy IgG chains (Figure 2.12b).

### 2.3 Discussion

The aim of this chapter was to firstly characterise the library of site-directed SERT antibodies (Table 2.2) as to their use in Western blot and immunohistochemical studies. The purpose of such antibodies was to qualitatively assess the effect of chronic antidepressant treatment on the abundance of SERT. It is important to use an antibody, which specifically recognises SERT in the raphé nuclei in the brain, a brain area that is too small for the conventional membrane binding studies used in Chapter 6. Due to the existence of 5-HT receptors on non-serotonergic nerve terminals (Maura & Raiteri, 1986; Molderings *et al.*, 1987; Bolaños & Fillion, 1989; Cassel *et al.*, 1995), SERT directed antibodies may provide a useful tool for the isolation of pure serotonergic terminals (Lawrence *et al.*, 1995a & b). The use of such an antibody that can recognise the native form of SERT is a prerequisite for immunomagnetically isolating functionally viable 5-HT nerve terminals to allow subsequent electrophysiological analysis. The second aim of this chapter was to use ELISA techniques in an attempt to identify a site-directed SERT antibody that was capable of recognising SERT in its native form. The development of such an ELISA assay would also enable the quantification of SERT abundance after chronic antidepressant treatments to complement membrane binding experiments and to possibly replace them with a non-radioactive method.

SDS denaturing Western blots, for all the antibodies tested (except the chemicon rabbit polyclonal antibody) revealed prominent immunoreactivity at 94 kDa in platelets and 76 kDa in brain tissue (Figures 2.5 & 2.6). This is consistent with SERT recognition in previous studies using site-directed SERT antibodies (Qian *et al.*, 1995; Sur *et al.*, 1996; Zhou *et al.*, 1996). Despite the identical primary amino

acid sequence of human brain and platelet SERT (Lesch *et al.*, 1993b &c) and the presence of a single SERT genomic locus (Ramamoorthy *et al.*, 1993) there is an obvious difference in the size of these brain and peripheral SERTs. Previous experiments have revealed that this difference in size, reflected in a reduced electrophoretic mobility, is due to differential N-linked glycosylation caused by post-translational modifications (Qian *et al.*, 1995). This glycosylation is required for the optimal stability of SERT but not for 5-HT transport or ligand binding *per se* (Tate & Blakely, 1994). In addition to the 94 kDa immunoreactive band in platelets there was also other banding for all antibodies between 76 and 40 kDa in this tissue (Figures 2.5 & 2.6). Though this extra banding could represent non-specific binding, it may also represent the differential extent of platelet SERT glycosylation, caused by their susceptibility to degradation during preparation, where full deglycosylated SERT migrates to approximately 54 kDa (Qian *et al.*, 1995). The rank order of the intensity of immunoreactive bands was: platelets >> cortex >> cerebellum >= liver (Figures 2.5 & 2.6). This is consistent with the known distribution of SERT (Plenge & Mellerup, 1991; Qian *et al.*, 1995) and agrees well with [<sup>3</sup>H]citalopram binding studies in chapter 4. Though the detection of cerebellum immunoreactivity is always less than corresponding cortical tissue, there are discrepancies in the amount detected in cerebellum. The cerebellum receives only a sparse serotonergic innervation (Steinbusch, 1984; Takeuchi, 1988), and therefore it likely that the detection of SERT in this brain region is due to the contamination of midbrain at the dissection level. Despite this, liver membranes served as a consistent negative control as in previous reports (Qian *et al.*, 1995). The most specific antibodies tested, as determined by the presence of only one brain immunoreactive species, were the mouse monoclonal and 1001 antibodies. The specificity of the latter had previously been confirmed by the disappearance of immunoreactivity following absorption of the antibody with the antigenic peptide prior to immunoblotting (Lawrence *et al.*, 1995a). Corresponding work with the antigenic peptide used for the generation of the mouse monoclonal antibody were not performed, due to the lack of commercially available peptide. The commercial rabbit polyclonal antibody raised to the same sequence as the 1001 antibody did not reveal specific immunodetection. The

possibility of batch variations was not addressed as other antibodies served the role required.

The immunohistochemical localisation of SERT was assessed in rat brain using those antibodies that recognised the appropriate SERT immunoreactive bands in platelets and brain tissue in denaturing Western blots. All antibodies tested (1001, 998, Chemicon mouse monoclonal and Incstar) revealed specific staining of cell bodies in the raphé nuclei (Figure 2.7a-e). This was virtually identical to the pattern of 5-HT specific staining (Figure 2.7f), suggesting that SERT had been specifically detected on 5-HT containing neurons. This is comparable to previous reports for other SERT site directed antibodies (Qian *et al.*, 1995; Sur *et al.*, 1995) in a manner virtually identical to that observed for rSERT gene expression (Blakely *et al.*, 1991; Fujita *et al.*, 1993). Though all these antibodies revealed appropriate SERT staining in the raphé nuclei, there was a mixed quality of staining in the projectional areas. In the hippocampus and striatum, only the mouse monoclonal and 998 antibodies revealed good staining of dendrites and terminal bulbs (Figure 2.8). Overall, immunohistochemical studies demonstrated a high correspondence in the cellular and axonal distribution of SERT and 5-HT immunoreactivity, with high levels in the raphé and hippocampus (Figures 2.7 & 2.8). Negligible levels of SERT immunoreactivity were detected in the cerebellum for all antibodies (data not shown) consistent with the sparse 5-HT innervation of this structure in the rat brain (Steinbusch, 1984; Takeuchi, 1988). The specificity of the 998 antibody has been demonstrated previously firstly using absorption of the antibody with the antigenic peptide prior to immunodetection and secondly with diminished immunoreactivity following 5, 7 DHT lesioning of the serotonergic system (Dr Jane Lawrence, unpublished findings; Figures 2.8e & f).

5-HT reuptake activity has been reported in glial cells in particular those derived from primary cultures of cortical astrocytes (Dave & Kimelberg *et al.*, 1994; Bal *et al.*, 1997). Although SERT mRNA has been detected in these cultures, there has yet to be any reports of SERT mRNA by *in situ* hybridisation or SERT protein by immunohistochemical techniques *in vivo* (Qian *et al.*, 1995; Sur *et al.*, 1996; Hansson *et al.*, 1998). In this chapter there was no evidence of glial staining. Furthermore previous studies in this laboratory have demonstrated a non-overlapping

of SERT immunoreactivity (998 antibody) and immunoreactivity of the glial marker GFAP (glial fibrillary acidic protein; Dr. Jane Lawrence-personal communication).

I identified the antibodies that could be used for Western blotting and immunohistochemistry studies, but these techniques are for denatured/fixed tissue. The second part of the work was designed to identify whether these antibodies could recognise the native form of SERT. This was an important first step leading up to the immunomagnetic separation of serotonergic nerve terminals. The detection and quantification of native protein was initially attempted using ELISA techniques. The methodologies for a 96 well plate ELISA assay was developed using the unpurified 1001 antibody (Figure 2.9a). This method revealed a specific signal in cortical membranes and synaptosomes but also revealed a larger signal in cerebellum and liver membranes (Figure 2.9b). These latter tissues, however, display low or no SERT immunoreactivity (Qian *et al.*, 1995). Their detection was also apparent in denaturing Western blots using the same unpurified antibody (Figure 2.5) suggesting the non-specific binding of the unpurified antibody. This was confirmed using the corresponding purified antibody which gave no signal in any tissue and only displayed single species immunoreactivity in known 5-HT containing tissue (Figure 2.5). This lack of ELISA signal was also apparent for all the other affinity-purified antibodies tested, irrespective of the detection method employed. The use of the biotin/avidin system increased sensitivity (Figure 2.9c), but still failed to detect any signal using the affinity-purified antibodies.

The explanation may be that there were insufficient quantities of SERT present that would allow detection. 96 well plates by virtue of their size generally have a binding capacity of 400ng of protein. Maxisorb plates used in this chapter are considered to have a slightly higher binding capacity of up to 800ng). In chapter 4, radioligand binding assays for SERT reveal a binding site density of approximately  $1700 \text{ fmoles mg}^{-1}$  protein in cortical membranes. This is equivalent to  $1.7 \times 10^{-18}$  moles  $\text{ng}^{-1}$  of protein. In ELISA assays, using 100ng (up to 1000ng) of membrane protein (where no signal was detected) there would be  $1.7 \times 10^{-16}$  moles of SERT present. Using Avogadro's rule (one mole, molecular weight or gram of any substance is equivalent to  $6 \times 10^{23}$  molecules), the number of molecules of SERT present would be  $1 \times 10^8$ . In indirect ELISAs, where the antigenic peptide is

recognised by the produced affinity purified antibody (1001; as performed by Dr Jane Lawrence; data not shown) rather than membranes, 100ng of protein produces a specific signal. The antigenic peptide is 15 amino acids long and is conjugated to an extra cysteine residue to allow coupling to KLH, with a molecular weight of approximately 2000 (EMRNEDVSEVAKDA-C). The number of moles in 100ng of this peptide would be  $5 \times 10^{-11}$ , which corresponds to  $3 \times 10^{13}$  molecules. Therefore there are  $3 \times 10^5$  fold more molecules of peptide compared to SERT protein in membrane fractions in each well. This would mean that only 333fg ( $3.33 \times 10^{-4}$  ng) of the peptide sequence within the 100ng of membranes would be recognised by the 1001 antibody. This is approximately 300 times below the lower limit of detection of the ELISA assay of the antigenic peptide (0.1ng; Dr Jane Lawrence-personal communication). Even increasing protein concentration to the maximum capacity of the plate (~800ng), the amount of SERT present would still be below the detection limit. This would explain why no signal was obtained even when using the avidin/biotin system in conjunction with the purified antibodies.

In order to increase the sensitivity of the ELISA assay, the amount of protein was increased to an upper limit of 200 $\mu$ g by the use of a centrifugation ELISA. This would theoretically allow the detection of SERT using an ELISA approach, as now approximately 0.67ng of peptide sequence protein would be available for detection. Furthermore 50 $\mu$ g of total protein has already been shown to elicit a signal in denaturing Western blots. This assay employed the use of eppendorf tubes for antibody-antigen binding. The principle of such an assay was to separate the bound from free antibody via centrifugation wash steps. A similar signal was obtained from different tissue sources at 200 $\mu$ g of protein (Figure 2.10). Any specific signal could not be detected due to the incorporation of the secondary antibody or avidin HRP into the pellet after centrifugation (Figure 2.10), which could not be removed with repeated washes. Therefore the detection and quantification of native SERT in ELISA assays was unsuccessful.

Cell based ELISA assays may have alleviated this problem. Cells transfected with the SERT gene (if available) could have been centrifuged down at only 800g, which may have prevented incorporation of the secondary antibodies into the pellet.



Furthermore over expression of SERT in these cells could have enabled their use in a conventional 96 well plate ELISA assays.

It was therefore not possible to investigate whether the antibodies that recognised SERT in its denatured form also recognised it in its native form using ELISA techniques. Native Western blots and immunoprecipitations were subsequently used to attempt to immunodetect native SERT, although these methods are purely qualitative in our laboratory. Following protein extraction and gradient PAGE under non-denaturing conditions, a distinct immunoreactive band was detected in platelet and cortex but not liver membrane extracts using the Chemicon guinea pig and mouse monoclonal antibodies (Figure 2.11). This was expected since platelets and cortex, but not liver, express SERT at the plasma membrane (Qian *et al.*, 1995). Subsequently, Ferguson plots were constructed to determine the corresponding molecular weight for the distinct band from platelets, which was found to be consistent with immunodetection of SERT (94 kDa in platelets).

In addition the method for immunoprecipitation of native SERT with subsequent immunodetection following denaturation and SDS-PAGE was defined. The mouse monoclonal antibody was used for immunoprecipitation and the Chemicon guinea pig polyclonal antibody used for immunodetection. These antibodies were selected not only on their specificity in SDS and native PAGE, but because they were also raised against different epitopes of SERT and therefore would not compete for the same binding domain. Distinct bands corresponding to SERT were obtained from extracts of cortex membranes/synaptosomes and platelet homogenates (76 kDa and 94 kDa respectively; Figure 2.12). Pre-treatment with the endoglycosidase, PNGase F, increased the mobility on SDS-PAGE of SERT immunoprecipitated from cortex and platelet extracts. Subsequent immunodetection indicated SERT from both sources had migrated equally, consistent with deglycosylated SERT at a molecular weight of 54 kDa (Figure 2.12b).

Native PAGE has shown that both the guinea pig and monoclonal antibodies recognise SERT in its native form, the latter being confirmed in the immunoprecipitation experiments described above. Therefore it is possible that the two antibodies (Chemicon mouse monoclonal and guinea pig polyclonal) will be suitable for the first attempts of immunomagnetically isolating pure 5-HT nerve

terminals. The in-house antibodies have yet to be tested for their ability to recognise native SERT. However in the longer term the stocks of the in-house antibodies are limited, and it is more likely that the commercial monoclonal antibody will be used instead. The main advantage of this already affinity purified antibody is that it is from a stable cell line, which will require only limited analysis each time a new stock is ordered.

The immunomagnetic separation method involves the binding of the primary antibody to the native SERT protein in non-denatured/fixed tissue as a first step followed by incubation with a secondary magnetic antibody. The first step in the binding of primary antibody is identical to in the successful immunoprecipitation experiments. SERT containing nerve terminals may then be separated in theory by means of a magnetic column, whereby the negative elute contains non-serotonergic nerve terminals. Removal of the magnetic and washing of the column should then reveal the pure serotonergic population, though it is appreciated that this final process may have to be repeated many times to increase the purity. To assess the purity of the immunomagnetically separated nerve terminals, an appropriate detection system must be sought. Unfortunately immunoprecipitation experiments have only ready shown some degree of species cross reactivity when one antibody is used for SERT extraction and the other for detection (Figure 2.12). These antibodies however, do not inhibit [<sup>3</sup>H]citalopram binding (a selective marker for SERT; method in Chapter 3), at the concentrations that would be used for immunomagnetic separation (data not shown). Therefore it is possible that the purity and yield of separation may be monitored with quantitative radioligand binding studies. [<sup>3</sup>H]citalopram binding would be expected to be present in the positive elute (retained by magnet) and not in the negative elute, whereas [<sup>3</sup>H]nisoxetine (a selective marker for noradrenergic terminals but not 5-HT terminals) binding would be expected to be in the negative but not positive elute. The ratio of [<sup>3</sup>H]citalopram:[<sup>3</sup>H]nisoxetine binding would therefore give a good estimate of purity.

## 2.4 Summary

Western blot analysis revealed two site-directed antibodies, that specifically recognised denatured SERT; in-house 1001 and mouse monoclonal antibodies. These antibodies are used in the following chapters to investigate the effect of chronic antidepressant treatments on SERT abundance in the rat brain and also in platelets.

Immunohistochemical analysis demonstrated two site-directed antibodies that specifically recognised SERT in the cell bodies of the raphé nuclei and also in the dendrites and terminals, namely the in-house 998 and the mouse monoclonal antibody. These antibodies are used in the following chapters to determine the effect of chronic antidepressant treatments on SERT abundance specifically in the raphé nuclei, which is too small for conventional membrane binding studies.

Attempts to recognise the native form of SERT proved unsuccessful with ELISA techniques, due to the insufficient amount of protein that could be assayed in 96 well plates and also due to the inability of centrifugation methods to separate unbound and bound secondary antibodies.

Native Western blotting with the mouse monoclonal and guinea pig antibodies recognised one immunoreactive species consistent with SERT. This native detection was also confirmed using immunoprecipitation techniques. The mouse monoclonal antibody was firstly used for immunoprecipitation, followed immunodetection after SDS-PAGE by the guinea pig antibody, recognising a different epitope. These antibodies will provide a useful tool in immunomagnetically isolating pure serotonergic nerve terminals.

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## **CHAPTER 3**

### **METHODOLOGY FOR RADIOLIGAND BINDING ASSAYS**

Studies described within this thesis used two types of radioligand binding assays. For the purpose of receptor/transporter affinity and density determinations, membrane radioligand binding assays were used. For the purpose of determining the localisation and density of a variety of these receptors/transporters, radioligand autoradiography was used. These two methods are described in detail below, as is the statistical analysis of the generated results used throughout this thesis.

### **3.1 Animals**

Unless otherwise stated, animals used in these studies were male Wistar Cobb rats (200-300g; bred in-house; Department of Neuroscience, University of Edinburgh). Animals were given free access to food and water and maintained on a 12hr light/12hr dark cycle.

### **3.2 *In Vitro* Membrane Receptor Binding Assays**

#### **3.2.1 Preparation of Membranes for Receptor Binding**

##### **3.2.1.1 Brain Membrane Preparations**

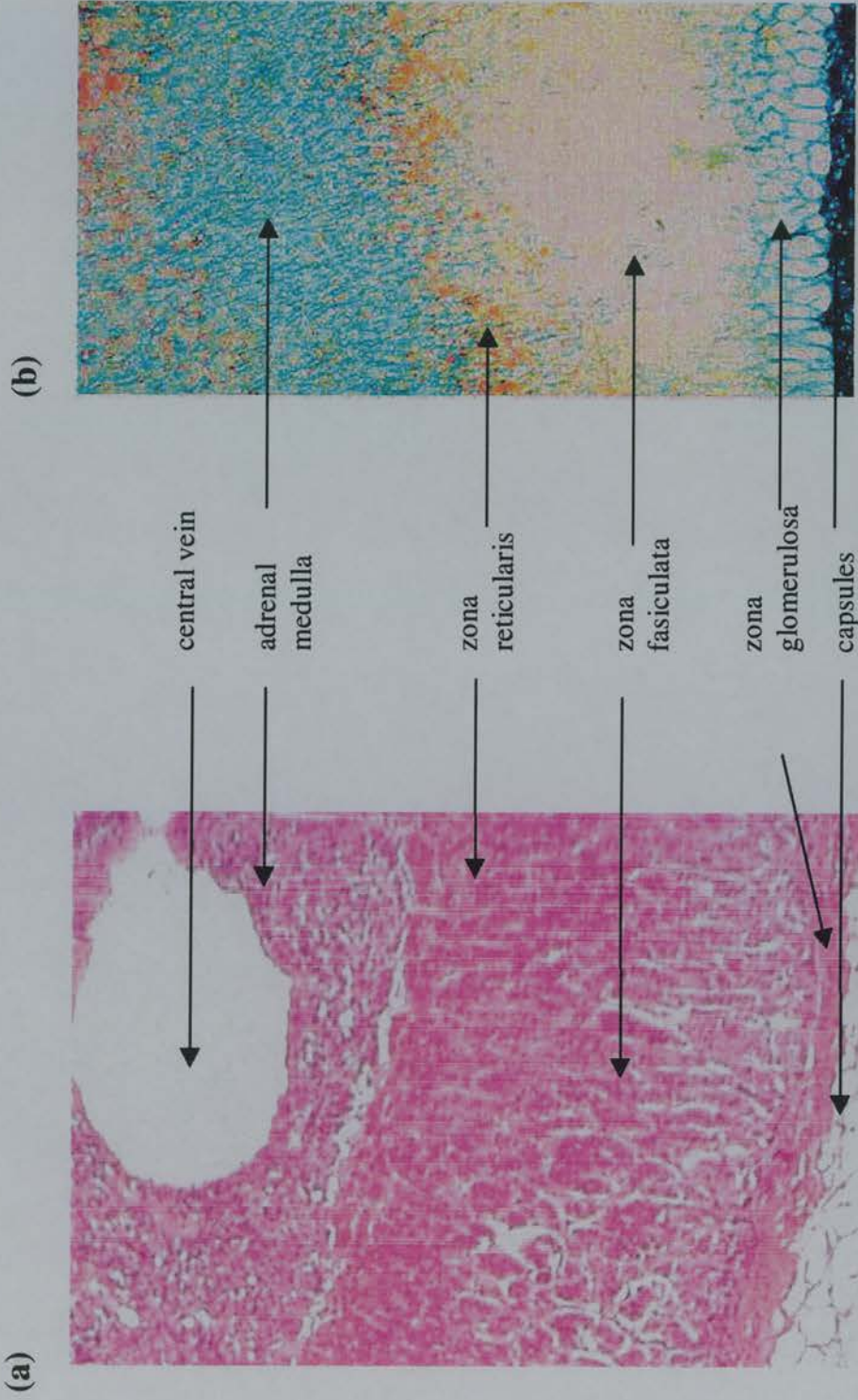
Rats were stunned and killed by decapitation. Brains were removed and immediately placed into ice-cold saline prior to dissection. Brains were dissected to reveal specific brain areas; whole cortex, frontal cortex, caudal cortex, hippocampus, and/or striatum. Tissue was rolled on filter paper to remove excess blood vessels, specific brain regions were pooled, weighed and homogenised (800 rpm) in 10 vols. of ice-cold 50mM Tris-HCl (pH 7.4) using a glass-teflon homogenizer with 80 $\mu$ m clearance. Homogenates were further diluted to 50 vols. with ice-cold 50mM Tris-HCl, then centrifuged at 30000g for 10 min at 4°C. The resulting membrane pellet was resuspended (50mM Tris-HCl) and centrifuged (30000g; 10min, 4°C). The pellet was resuspended as before and incubated for 15 min at 37°C to facilitate dissociation of endogenous 5-HT, then centrifuged (30000g; 10min, 4°C). The final pellet was resuspended in 25 vols. ice-cold 50mM Tris-HCl (pH 7.4) and aliquoted if necessary before freezing at -20°C. Tissues were kept frozen for no longer than 4 weeks before binding assays were carried out.

### **3.2.1.2 Adrenal Gland Membrane Preparations**

Rats were stunned and killed by decapitation. Whole adrenal glands were removed and immediately placed in ice-cold saline prior to dissection. Adrenal glands (Figure 3.1) were essentially decapsulated, by removing the capsular (including five cell thick zona glomerulosa layer) region to reveal the medullary portion (including regions of the inner cortex, particularly the zona reticularis). Tissue from several adrenals were pooled as required. Adrenal capsules and/or adrenal medulla tissue was homogenised in 10 vols. ice-cold 50mM Tris HCl (pH 7.4) by 2x10 second bursts using a polytron homogeniser (setting 5) on ice. Homogenates were further diluted to 50 vols. with ice-cold 50mM Tris HCl (pH 7.4), and the preparation of adrenal capsule and/or medulla membranes carried out as described above for the brain membrane preparation.

### **3.2.1.3 Platelet Membrane Preparations**

An adaptation of the method by Gordon & Olverman (1978) was used to obtain platelet rich plasma (PRP). Rats were anaesthetised with 4% halothane in oxygen and nitrous oxide (30:70 v:v). Blood was taken by puncture of the vena cava and the animals subsequently sacrificed, by cutting of the diaphragm and cervical dislocation of the neck. 9 parts of blood was transferred into a tube containing 1 part 3.1% Tri sodium citrate to act as anti-coagulant. After gentle mixing, the samples were centrifuged for 2 minutes at 800g at room temperature. The upper layer (PRP) was collected carefully at the interface using a 1ml syringe and the lower layer centrifuged as before. PRP was once again collected and the lower layer centrifuged as before. PRP was pooled after the final collection, and the final lower layer, containing the red blood cells, discarded. Typically between 7 and 9ml of blood was obtained from each rat, resulting in 4-5ml of PRP. The PRP was then centrifuged at 30000g for 20 min at 4°C. The resulting platelet pellet was resuspended in 300-500µl d.H<sub>2</sub>O. Each pellet was allowed to sit on ice for 1 hour to lyse the platelets, and then frozen overnight at -20°C to make sure of full lysis. The following day, platelets were allowed to thaw on ice, further diluted to 10mls with ice-cold 50mM Tris HCl (pH 7.4) and incubated for 30 min at 37°C. Platelet membranes were prepared as described above for brain membranes, except that the incubation period at 37°C was



**Figure 3.1: Histological Sections Showing Various Regions of the Adrenal Gland.**

Sections are stained with (a) H & E and (b) Maltcry-Azan. These pictures were adjusted for representation purposes from the Loyola University Medical Center Web Page (<http://www.meddean.luc.edu/lumen/MedEd/Histo>). In (b) The capsule is dark blue. The zona glomerulosa is narrow and has pale cells lying in loops and arches, with fine blue reticular fibers in between the cords. The zona fasciculata is quite wide and pale pinkish yellow. Cells in these layers are often pale and "foamy" looking because of dissolved lipid of their steroid secretions. The zona reticularis shades from red to blue and merges indistinctly here with the medulla.

extended to 30 minutes. At each step the platelet membranes derived from one rat, were homogenised in 5ml of ice-cold 50mM Tris HCl (pH 7.4) and further diluted to 10ml. After the final centrifugation, the resultant pellets were homogenised and stored at  $-20^{\circ}\text{C}$  in 1ml of ice-cold 50mM Tris HCl (pH 7.4) per 7-9mls of rat blood initially used.

#### **3.2.1.4 Membrane Preparations After Drug Treatments**

The methodology for membrane preparations after *in vivo* drug treatments was essentially the same as described above. However, tissue derived from drug treatment studies were not pooled. Instead dissected specific brain or adrenal areas from each rat were individually treated. Brain tissue was weighed and homogenised (800 rpm) in the centrifuge tube using a loose fitting teflon pestle with  $80\mu\text{m}$  clearance. Adrenal tissue was weighed and homogenised in the centrifuge tube using a polytron homogeniser as described above. Following the initial centrifugation step, further homogenisations of all tissue were performed in the centrifuge tube using the appropriate loose fitting teflon pestle. Table 3.1a shows the typical conditions used for preparing individual brain area (frontal cortex, caudal cortex, hippocampus or striatum) and adrenal medulla membranes. The speed and ease of handling multiple tissue samples was maintained by using constant homogenisation and centrifugation volumes for each specific tissue area group that were appropriate to the typical weight of the tissue. Homogenisation volumes were adjusted to between 10 and 25 vols. Centrifugation volumes were adjusted to between 25 and 50 vols. The final pellets were resuspended in between 10 and 30 vols. A varying number of small aliquots were stored at  $-20^{\circ}\text{C}$  to maximise the number of binding assays that could be performed on one brain region without damaging the tissue through multiple freeze-thaw cycles.

#### **3.2.1.5 Treatment of Membranes on Day of Assay**

Pooled (non-drug treatment) membranes stored at  $-20^{\circ}\text{C}$  were allowed to thaw on ice, mixed if necessary and further diluted in the appropriate ice-cold assay buffer. Table 3.2 outlines the diluted number of vols. of the original wet weight of tissue used, to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding. Protein linearity experiments,



**(a)**

Membranes	Typical Weight (mgs)	Homogenisation Volume (mls)	Centrifuged Volume (mls)	Final Resuspension Volume (mls)	Equivalent Volumes	Aliquots
Whole cortex	500-750	10	25	12	15-25	8 x 1.5ml
Frontal cortex	300-500	7.5	15	7.5	15-25	5 x 1.5ml
Caudal cortex	200-250	5	10	6	25-30	4 x 1.5 ml
Hippocampus	100-120	2	4	3	25-30	2 x 1.5 ml
Striatum	60-100	1.5	2.5	1.5	15-25	1 x 1.5 ml
Adrenal Medulla	50-75	1	2	0.75	10-15	1 x 0.75 ml

**(b)**

Membranes	<sup>3</sup> H]citalopram Chapter 4		<sup>3</sup> H]8-OH DPAT Chapter 5		<sup>3</sup> H]5-CT Chapter 5		<sup>3</sup> H]GR125,743 Chapter 5	
	Single	Pair	Single	Pair	Single	Pair	Single	Pair
Frontal cortex	-	1	-	1	2	-	2	-
Caudal cortex	-	1	-	1	2	-	2	-
Hippocampus	-	1	-	1	1	-	1	-
Striatum	-	1	-	1	1	-	1	-
Adrenal Medulla	1	1	-	-	-	-	-	-

**Table 3.1: Preparation of Rat Brain and Adrenal Medulla Membranes from Individual Animals, and use in****Binding Assays After Drug Treatments.**

(a) shows typical weights of individual structures, centrifuges in ~ 25-50 vols., finally resuspended in 10 - 30 vols., and aliquoted into 1.5ml aliquots before freezing at -20°C. (b) shows use of these aliquots in binding assays used throughout this thesis in either single (12 tube) or duplicate (pair; 24 tube) point experiments. The chapter for the characterisation of each ligand used is given.

Binding Assay	Chapter	Membranes (Vols.)	
		Brain	Adrenal Medulla
[ <sup>3</sup> H]citalopram	4	150-300	100-200
[ <sup>3</sup> H]nisoxetine	4	40-50	25-50
[ <sup>3</sup> H]8-OH DPAT	5	100-200	-
[ <sup>3</sup> H]5-CT	5	40-300*	-
[ <sup>3</sup> H]GR125,743	5	40-80	-

**Table 3.2: Diluted Volumes of Membranes for use in Binding Assays**

For each radioligand membrane binding assay, the range of the diluted number of vols. of membranes are given, prior to addition to assay tubes, to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding. On addition to assay tubes these are further diluted two fold. Hence in the case of [<sup>3</sup>H]citalopram binding in brain, membrane vol. in the assay is actually 300-600 vols. These vols. relate to standard pooled tissue membrane preparations from multiple animals stored at 25 vols. One volume relates to 1g of original wet weight tissue in 1ml of assay buffer. The protein concentration ranges used to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding are stated in the relevant chapters (chapters 4 & 5) determined from protein linearity experiments. \* = The large range of diluted membrane suspension used for [<sup>3</sup>H]5-CT binding relating to binding in the presence or absence of various masking drugs (see chapter 5). As components of [<sup>3</sup>H]5-CT binding are blocked, the amount of membrane suspension used has to be increased in order to maintain a workable amount of specific binding (see chapter 5).

whereby the amount of specific binding is monitored with increasing concentrations of membrane protein, enabled the calculation of the optimal range of membrane protein concentrations to be used (see Chapters 4 & 5).

Drug treatment membrane aliquots were thawed as above and used in either single point or duplicate point assays to allow multiple binding assays to be performed from the same source of tissue. In table 3.1b the number of aliquots used for single point or duplicate point assays are shown. The total aliquot volume was further diluted to 7mls for single point assays or 12.5ml for duplicate point assays in the appropriate assay buffer to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

For pooled and drug treatment membranes, two separate 250 $\mu$ l samples were taken for protein content determination. These two protein samples were assayed one week apart to ensure correct protein concentration use for subsequent calculations.

### **3.2.1.6 Protein Assay**

The amount of protein was determined using a Bradford protein assay (Bradford, 1976). Standard curves were constructed using serial dilutions of bovine serum albumin (BSA) ranging from 10 to 200  $\mu$ g ml<sup>-1</sup> of protein. 50 $\mu$ l aliquots of standards or test sample were added to Corning 96 well plates followed by 250 $\mu$ l of Bradford reagent (0.1% coomassie blue (w/v), 5% of 95% ethanol (v/v), 10% of 85% orthophosphoric acid (v/v) in d.H<sub>2</sub>O; filtered twice through Whatman filter paper and stored at 4°C). Using a Dynatech plate reader, samples were mixed by shaking for 20 seconds and absorbance measured at an optical density of 595nm (OD<sub>595</sub>) after 20 min. (OD<sub>595</sub>) measurements for BSA samples were fitted to a straight line through the linear portion of the curve, and the amount of protein in each sample determined by extrapolation.

## **3.2.2 *In Vitro* Membrane Receptor Binding Assay Methods and Analysis**

### **3.2.2.1 Generalised Receptor Binding Experiments**

All drugs used in the study of receptor/transporter binding were dissolved in either d.H<sub>2</sub>O or the appropriate solvent. Where drugs were diluted in DMSO, methanol or ethanol, the final concentration of the solvent in the assay did not exceed 1%. Drugs and membrane preparations were stored for up to 3 months at -20°C, unless stated otherwise. Table 3.3 outlines the details of each radioligand membrane binding assay used in this thesis. Buffers were prepared in d.H<sub>2</sub>O and stored at 4°C. Radioligands were diluted in d.H<sub>2</sub>O to appropriate concentrations and stored at -70°C under liquid N<sub>2</sub> in aliquots sufficient for one experiment.

Binding assays were conducted in 5ml round bottomed polypropylene tubes (Sterilin (RT35), UK). Radioligand membrane binding assays were typically carried out by preincubating 100µl of buffer or test drug, 300µl of assay buffer with 100µl of [<sup>3</sup>H]ligand for 2 min at 25°C unless stated otherwise. Non-specific binding was determined in the presence of a maximal concentration of drug that was at least 100 fold its affinity for the receptor/transporter of interest. Binding was initiated by addition of 0.5ml of diluted membrane suspension to give a final volume of 1ml. Incubations were continued until binding was at equilibrium (except for time courses) as outlined in Table 3.3. Bound and free ligand were then separated over pre-wetted Whatman GF/B filters (assay buffer ± polyethyleneimine; PEI) using a Brandel cell harvester. Following rapid assay buffer washes (Table 3.3), the resultant filter disks were transferred to scintillation vials and incubated with 100µl of 100% formic acid for at least 10 min. Emulsifier safe scintillant (2.5ml) was then added and equilibrated overnight. For each binding assay eight samples of the radioactivity added to the assay were used as standards to calculate the concentration of the ligand added. Radioactivity was determined in a Packard 2500TR scintillation counter with automatic quench correction (counting efficiency approximately 50%), with samples counted for 4 minutes and results expressed as disintegrations per minute (dpm) per sample.

[ <sup>3</sup> H] Ligand		Assay Buffer		NSB	Incubation		Harvesting		
Name	Source	Ci/mmol	Labels	[Final] (nM)	Chap	50mM Tris (pH 7.4) containing	°C	Time	Filters
<b>Citalopram</b>	N.E.N.	82-86	SERT	0.25	4	NaCl (120mM) + KCl (5mM)	25	1 hour	(+ % PEI) Washes 0.05 2 x 2ml
<b>Nisoxetine</b>	Amers.	86	NET	0.50	4	NaCl (300mM) + KCl (5mM)	25	1 hour	No PEI 3 x 3ml
<b>8-OH DPAT</b>	N.E.N.	127	5-HT <sub>1A</sub>	0.25	5	MgSO <sub>4</sub> (5mM)	37	30 min	No PEI 2 x 2ml
<b>5-CT</b>	Amers.	80-90	5-HT <sub>7</sub> *	0.25	5	Ascorbic acid (0.1% wt. / vol.)+ pargyline (10µM) + MgCl <sub>2</sub> (4mM)	25	1 hour	0.05 2 x 2ml
<b>GR125,743</b>	Amers.	47	5-HT <sub>1B/1D</sub>	0.25	5		25	1 hour	0.10 3 x 3ml

**Table 3.3: Specific Details of Radioligand Membrane Binding Assays.**

For each radioligand membrane binding assay, the [<sup>3</sup>H]ligand used, along with the assay buffer constituents, NSB (non-specific binding), incubation and harvesting details are given. N.E.N. =New England Nuclear. Amers. = Amersham. PEI = Polyethyleneimine, used to reduce non-specific binding. Chap = Chapter ligand characterised. \* = 5-HT<sub>7</sub> receptor identified in the present of various masking drugs (see chapter 5). SERT = Serotonin transporter. NET = Noradrenaline transporter. Ci/mmol = specific activity.

### 3.2.2.2 Competition Experiments

Competition experiments ('cold' saturations) are carried out under equilibrium conditions with a fixed incubation time, membrane (receptor/transporter) concentration and radioligand concentration. The concentration of competing drug is varied. As the concentration of competing drug is increased the amount of bound radioligand decreases. Competition binding curves generally comprised of 2 duplicate tubes for both total and non-specific binding and 10 duplicate concentrations of competing drug. Stock drugs were generally diluted 1 in 10 and then 1 in 3, followed by serial 10 fold dilutions of both to give a series of concentrations one half of a log unit apart. Such curves enable the calculation of the affinity and density of labelled binding sites and also the affinity of inhibitors for that binding site.

Data were initially analysed using an iterative, non-linear least squares curve fitting programme (Microcal Origin) to a one site logistic model (Barlow, 1983);

$$y = ((A1-A2) / (1 + x / x_0)^P) + A2$$

where A1 = the initial binding maximum, A2 = the binding minimum,  $x_0$  = the concentration of competing drug which inhibits 50% of specific radioligand binding ( $IC_{50}$ ) and P = the slope. The calculated specific binding (A1-A2) and non-specific binding (A2) were compared with experimentally determined values. Values were accepted if within 10%.

The slope of competition binding curves from the logistic equation above, known as the "Hill Slope" or "Hill coefficient" (nH), may indicate the type of binding model appropriate for data analysis. If nH=1, the data are consistent with a simple one site model and increasing the concentration of competing drug from one tenth to 10 times the  $IC_{50}$  value, should reduce the specific binding of the radioligand from 90.9% to 9.1%. A Hill slope of less than unity indicates binding is not consistent with a single population of non-interacting binding sites and a negative co-operative or more likely two site model may be more appropriate. The data with Hill slopes of less than unity were analysed using a two site model (where stated). In

determining the proportions and affinity of these two states or different receptor subtypes, data was fitted to a two site hyperbola model using the following equation (in Graphpad Prism: Barlow, 1983):

$$y = \left( \frac{A1-A2}{1+x/x_0} + A2 \right) + \left( \frac{A3-A4}{1+x/x_1} + A4 \right)$$

where A1 = the initial binding maximum of the high affinity site, A2 = the binding minimum of the high affinity site,  $x_0$  = IC<sub>50</sub> of high affinity site, A3 = the initial binding maximum of the low affinity site, A4 = the binding minimum of the low affinity site,  $x_1$  = IC<sub>50</sub> of the low affinity site.

The results of the fit were statistically compared to those of the one site fit by the use of a partial F test defined as below:

$$F = \frac{(SS_1 - SS_2) / (df_1 - df_2)}{SS_2 / df_2}$$

where SS<sub>1</sub> is the sum of the squares error for the single site, SS<sub>2</sub> is the sum of squares error for the two site model, and df<sub>1</sub> and df<sub>2</sub> are the degrees of freedom for the one and two site fit models respectively. A two site fit was assumed to be significantly better than a single site fit if the determined F value was significant (p < 0.05) (De Lean *et al.*, 1981).

The range of inhibitor concentrations used in the assay were altered in accordance with the IC<sub>50</sub> of each compound. Where appropriate, concentration ranges were further adjusted to fit more tightly around the IC<sub>50</sub>, to reduce the experimental error, especially in drug treatment studies. For two site models, experiments were repeated with 20 different concentrations of the inhibitor using at least three concentrations per log cycle (where stated), in an attempt to provide a clearer definition of the high and low affinity sites.

When the unlabelled form of the [<sup>3</sup>H]ligand is used as the inhibitor (a 'cold' saturation experiment), it is possible to derive the equilibrium dissociation constant (affinity; K<sub>D</sub>) and the number of binding sites (density; B<sub>max</sub>) using the following equations (Bylund & Yamamura, 1990).

$$K_D = IC_{50} - [^3H]ligand$$

where  $IC_{50}$  (M) is that of the unlabelled ligand and  $[^3H]ligand$  (M) is the concentration in the assay as determined from the standards.

$B_{max}$  values ( $mol\ mg^{-1}$  protein) were calculated by converting the specific binding (DPM) of the radioligand bound, into the number of moles bound using the specific activity and the following equation;

$$B_{max} = (b \times IC_{50}) / ([^3H]ligand \times Pr)$$

where  $b$  is the specific binding (moles) at the  $[^3H]ligand$  concentration used in the assay in the absence of inhibitor,  $IC_{50}$  (M) is that of the unlabelled ligand,  $[^3H]ligand$  (M) is the concentration in the assay and  $Pr$  is the amount of protein added to assay tube (mg).  $B_{max}$  was expressed in  $mol\ mg^{-1}$  protein by determining the amount of protein used in the assay (mg) of each sample.

'Cold' saturation experiments were used after drug treatments to determine possible changes in the density or affinity of the receptor/transporter under investigation. Dilutions of the unlabelled ligand were prepared in significant quantities to use for all the individual membrane preparations from each study and stored at  $-20^{\circ}C$  until required. Prior to their use, these dilutions were thawed at room temperature and the affinity and tightness of the data points checked in naïve membranes binding assays.

As the  $IC_{50}$  is an experimentally defined parameter, an inhibition constant ( $K_i$ ), which is dependent of radioligand concentration, was calculated according to the Cheng Prussoff approximation for all inhibitors other than the cold form of the  $[^3H]ligand$  (Cheng & Prussoff, 1973);

$$K_i = IC_{50} / (1 + ([^3H]ligand / K_D))$$

where  $IC_{50}$  (M) is that of the compound being tested,  $[^3H]ligand$  (M) is the concentration in the assay and the  $K_D$  is the affinity of the radioligand.



### 3.2.2.3 'Hot' Saturation Experiments

As for competition experiments, binding is carried out under equilibrium conditions. Receptor/transporter concentration and time are kept constant and the amount of bound radioligand is measured as a function of the free radioligand concentration at various increasing concentrations of the radioligand. 'Hot' saturation experiments, like competition experiments, allows the determination of  $K_D$  and  $B_{max}$  values. Both methods should give the same values and Hill slopes are again indicative of the type of receptor binding..

Radioligands were diluted to give a range of concentrations approximately 10 fold above (depending on the amount of ligand available) and below the expected  $K_D$ . Generally 8 concentrations in duplicate were used to determine total binding and 8 single concentrations used to determine non-specific binding. Samples from each radioligand concentration was used as standards to calculate ligand concentrations. Specific binding (bound [ $^3H$ ]ligand) was determined for each point by subtracting non-specific binding from total binding. The free [ $^3H$ ]ligand at equilibrium was calculated by subtracting specifically bound [ $^3H$ ]ligand from [ $^3H$ ]ligand added. Using the specific activity of the radioligand the concentration (M) of both free and bound [ $^3H$ ]ligand was calculated. The data were fitted using the iterative, non-linear least squares curve fitting programme (Microcal Origin) to a hyperbolic model and the  $K_D$  and  $B_{max}$  values estimated (Bylund & Yamamura, 1990);

$$b = (B_{max} \times L) / (L + K_D)$$

where b: is the specifically bound [ $^3H$ ]ligand (M), L: is the free [ $^3H$ ]ligand (M) concentration,  $K_D$  (M) the affinity of the ligand and  $B_{max}$  (M) the density of the receptor.

### 3.2.2.4 Time Course Experiments

Time course experiments allow us to determine when equilibrium has been reached, and can also provide us with the affinity of the radioligand. In association and dissociation experiments, receptor/transporter and radioligand concentration are constant, with radioligand bound measured as a function of time. Samples of the

radioligand added were counted as standards to calculate the radioligand concentration.

In association experiments the specific binding at each time point was determined by subtracting non-specific binding from total binding and data fitted to a single exponential function (Bylund & Yamamura, 1990);

$$B_t = B_e - (B_e / \exp(k_{\text{obs}} \cdot t))$$

where  $t$ : is time (min),  $B_t$ : is bound [ $^3\text{H}$ ]ligand (dpm) at time  $t$ ,  $B_e$ : total specific binding (dpm) at equilibrium and  $k_{\text{obs}}$  ( $\text{min}^{-1}$ ) is the observed constant. Alternatively semilogarithmic conversion of the data, where  $\text{Ln}(B_e / B_e - B_t)$  is plotted as a function of time, produces a straight line with a slope of  $k_{\text{obs}}$ . The  $t_{1/2}$  time may be calculated which is the time at which  $B_t$  is equal to one-half of  $B_e$ .

The association rate constant ( $k_{+1}$ ) was determined by estimating the free [ $^3\text{H}$ ]ligand concentration ( $M$ ) when maximum binding is observed and calculated using the following equation;

$$k_{+1} = (k_{\text{obs}} - k_{-1}) / L$$

where:  $k_{\text{obs}}$  ( $\text{min}^{-1}$ ) is the observed constant,  $k_{-1}$  ( $\text{min}^{-1}$ )(see below) is the dissociation rate constant and  $L$ : is the free concentration of [ $^3\text{H}$ ]ligand ( $M$ ).

In dissociation experiments, radioligand and receptor are incubated until equilibrium is achieved. Further association of the radioligand is prevented by the addition of excess unlabelled drug (usually at least one hundred times the  $K_D$ ) so the dissociation of the radioligand from the receptor can be monitored and the  $k_{-1}$  measured.

The dissociation rate constant ( $k_{-1}$ ) was calculated from a dissociation time course experiment. Specific binding at each time point was determined by subtracting non-specific binding from total binding and data fitted to the following equation (Bylund & Yamamura, 1990);

$$B_t = (B_0 / \exp(k_{-1} \cdot t))$$

where  $t$ : is time (min),  $B_t$ : is bound [ $^3\text{H}$ ]ligand (dpm) at time  $t$ ,  $B_0$ : total specific binding (dpm) at time 0 and  $k_{-1}$  ( $\text{min}^{-1}$ ) is the dissociation rate constant. Alternatively semilogarithmic conversion of the data, where  $\text{Ln}(B_t/B_0)$  is plotted as a function of time, produces a straight line with a slope of  $k_{-1}$ . The  $t_{1/2}$  time may be calculated which is the time at which  $B_t$  is equal to one-half of  $B_0$ .

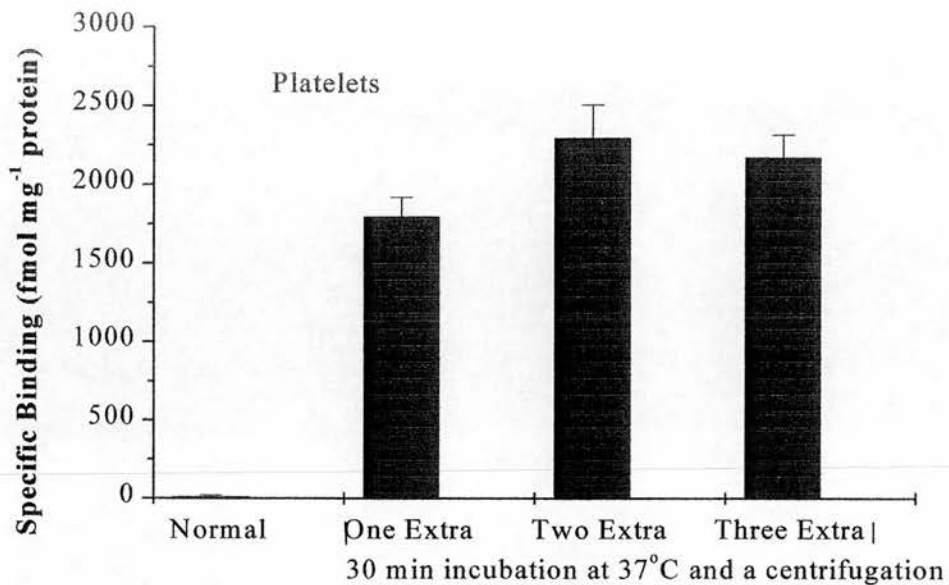
Time course experiments provide an independent estimate of the  $K_D$  which should agree with the  $K_D$  from competition and 'hot' saturation studies. The  $K_D$  (M) was calculated from the time course data using the following equation;

$$K_D = k_{-1} / k_{+1}$$

where  $k_{-1}$  ( $\text{min}^{-1}$ ) and  $k_{+1}$  ( $\text{min}^{-1} \text{M}^{-1}$ ) are the dissociation and association rate constants respectively.

### **3.2.2.5 [ $^3\text{H}$ ]Citalopram Binding in Platelet Membranes**

Platelet membrane 1ml aliquots (prepared in section 3.2.1.3) were diluted in 10-200ml of assay buffer for use in binding assays. However initial experiments revealed no specific binding (data not shown) despite the reported high abundance of both 5-HT and SERT (Rudnick, 1977). On the day of the assay, platelet membranes were subjected to further washes and incubations at  $37^\circ\text{C}$  in an attempt to completely remove the high levels of endogenous 5-HT found in platelets which may inhibit [ $^3\text{H}$ ]citalopram binding. Platelet membrane 1ml aliquots were thawed on ice and further diluted to 10ml in 50mM Tris-HCl (pH 7.4). Membranes were incubated for a further 30 min at  $37^\circ\text{C}$  and centrifuged (30000g; 10min,  $4^\circ\text{C}$ ). This was repeated three times and at the end of each centrifugation step, the amount of specific [ $^3\text{H}$ ]citalopram binding assessed. In comparison to the standard brain membrane preparation (see section 3.2.1.1), the addition of two extra incubation and centrifugation steps were sufficient to give maximal binding in platelet membranes (Figure 3.2), presumably due to the complete dissociation of the high levels of endogenous 5-HT. The final pellet from a 1ml aliquot after these two extra wash steps was further diluted in 100-200ml assay buffer for subsequent use in



**Membrane Preparation Method**

**Figure 3.2: Effect of Platelet Membrane Preparation Method on [<sup>3</sup>H]Citalopram Membrane Binding.**

Data represent single experiments in platelet membranes. Data represents the amount of specific [<sup>3</sup>H]citalopram binding after increasing incubation and centrifugation steps of platelet membranes. Each data point represents the mean of triplicate tubes.

[<sup>3</sup>H]citalopram platelet membrane binding assays (Chapter 4) to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

### **3.3 *In Vitro* Autoradiographic Receptor Binding Assays**

#### **3.3.1 Preparation of Slides for *In Vitro* Autoradiography**

Double frosted glass microscope slides for *in vitro* autoradiography were cleaned in running tap water and dried in an oven at 60°C. A subbing solution (10 g gelatin, 0.5 g chromic potassium sulphate in 1L d.H<sub>2</sub>O) was heat stirred to a temperature of 60-70°C and filtered through Whatman 91 filter paper. The solution was allowed to cool to below 40°C and racks containing clean slides were dipped in subbing solution. Slides were dried overnight at 60°C in an oven containing a tray of copper sulphate desiccant. The subbing solution could be used to coat several batches of slides at once but was discarded after use.

#### **3.3.2 Preparation of Sections for *In Vitro* Autoradiography**

For *in vitro* autoradiographic studies, rats were killed by stunning and then decapitation. Brain or adrenal glands were immediately removed and frozen in isopentane at -45°C (temperature was maintained by cooling in a dry ice/acetone mixture). Brain or adrenal glands were frozen onto orientating microtome chucks with Tissue tek, dipped in Lipshaw Embedding Matrix, sprayed with cryospray and equilibrated for 30 min at -20°C. Coronal sections (20µm) were cut on a Bright cryostat and thaw mounted onto gelatin subbed slides. Sections were taken in the caudal to rostral direction in a plane related to Bregma as described by Paxinos & Watson (1982). Sections were taken continuously at the levels required, and the unwanted levels discarded. Generally 3 sections per slide were used. Adjacent sections on separate slides were used to determine total binding and non-specific binding. Rat adrenal sections were cut until both the adrenal medulla and cortical levels were apparent. Sections were allowed to dry at room temperature and then stored at -70°C for at least 3 days before use, to facilitate adhesion to the slide.

### 3.3.3 *In Vitro* Autoradiographic Method and Analysis

For *in vitro* autoradiographic studies, tissue sections were removed from the freezer and left at room temperature for 60 min. All autoradiographic procedures were carried out at room temperature in 50 ml assay buffer in Coplin jars. The procedure for each autoradiographic study is outlined in Table 3.4. Briefly sections were pre-incubated in assay buffer, then incubated in the presence of [<sup>3</sup>H]ligand ( $\pm$  non-specific determinant in adjacent sections on separate slides), and washed in separate coplin jars containing fresh ice-cold assay buffer. Following dipping in d.H<sub>2</sub>O and blotting, excess liquid was carefully removed under vacuum with a fine tipped pasteur pipette and dried under a hot or cold stream of air. For autoradiographical analysis, sections were apposed together with tritium standards ([<sup>3</sup>H] microscales, Amersham) to [<sup>3</sup>H]-sensitive Hyperfilm in x-ray cassettes for varying periods at  $-70^{\circ}\text{C}$ , as outlined in Table 3.4.

Cassettes were left at room temperature for 60 min, after the stated period, before removal of the [<sup>3</sup>H]-Hyperfilm and development in a dark room. Films were bathed in Kodak D19 developer (diluted 5 fold in tap water) for 4 min, dipped in water before two 4 min washes in Kodak fixative (diluted 4 fold in tap water) and finally dipped in water containing detergent (0.1%). Films were then rinsed under cold running tap water for 30 min and dried in a cabinet at  $30^{\circ}\text{C}$ . Films were then covered and stored at room temperature prior to analysis.

Autoradiograms were analysed using a computer based image analyser (MCID, Imaging Research Inc.). After background subtraction by a matrix shading correction facility, optical density (O.D.) values were converted to binding density ( $\text{fmol mg}^{-1}$  tissue equivalent) using tritium standards and the specific activity of the ligand. Standard curves were calculated as O.D. measurements versus radioactivity in  $\text{fmol mg}^{-1}$  tissue. O.D. measurements were performed by selecting a measuring box of appropriate dimension, overlaying the structure in a total binding section and measuring the optical density of that area four times for all sections on each slide. The same dimension overlay was then used for adjacent non-specific binding and total binding plus masking drugs (if present) slides. Specific binding was then determined by automatic subtraction of non-specific binding from the respective totals.

Name / Reference	Source	Ci/mmol	Labels	[Final]	Chap	Assay Buffer and Non-Specific Binding (NSB) Determinant	Procedure: Pre-Incubation + Incubation	
							with [ <sup>3</sup> H]ligand + Washes + Drying +	Length of exposure to <sup>3</sup> H Film
<b>Paroxetine</b> / Battaglia <i>et al.</i> , (1987).	N.E.N.	25	SERT	250pM	4 & 7	50mM Tris containing NaCl (120mM) + KCl (5mM), pH 7.7. 4μM Citalopram = NSB.	2 x 5 min + 2 hour + 4 x 30 min + hot air dried + 6 weeks	
<b>5-CT</b> / adapted from Gustafson <i>et al.</i> , 1996	Amers.	80-90	<b>5-HT<sub>7</sub>*</b>	0.50nM	5	50mM Tris containing Ascorbic acid (0.1% wt. / vol.)+ pargyline (10μM) + MgCl <sub>2</sub> (4mM), pH 7.4. 10μM 5-HT = NSB.	2 x 5 min + 1 hour + 2 x 5 min + hot air dried + 12 weeks	

**Table 3.4: Specific Details of Radioligand Autoradiography Methods.**

For each radioligand membrane binding assay, the [<sup>3</sup>H]ligand used, along with the assay buffer constituents, NSB (non-specific binding), and methodology details are given. N.E.N. = New England Nuclear. Amers. = Amersham. Chap = Chapter ligand used. \* = 5-HT<sub>7</sub> receptor identified in the present of various masking drugs (see chapter 5). SERT = Serotonin transporter. Ci/mmol = specific activity.

### **3.4 Statistical Analysis**

The majority of figures displayed in the thesis are a representation of one experiment, all of which have been carried out at least three times, unless otherwise stated. Data in tables and text are shown as the mean  $\pm$  the standard error (s.e.m.) for at least three separate determinations/animals. For comparisons of drug affinities in different membrane preparations, the Students t-test was used (Welch corrected; assuming that each group may have different standard deviations). This same test was used for comparison between 2 drug treated groups. For the comparison of 3 drug treated groups or more a one-way ANOVA test was performed with Dunnett's multiple comparison post-testing against controls. In all cases, conformity to normality and equality of variance was checked prior to parametric analysis. If data did not conform to an equality of variance then non-parametric testing was carried out with the Dunn's method of Post-hoc analyses. All statistical analyses were carried out using Graphpad Instat® and the level of significance set as  $P < 0.05$ .

### **3.5 Materials**

For studies in this thesis the majority of drugs used were obtained from regular commercial sources, and all reagents used were of the highest analytical grade. The following drugs, however, were kindly donated as gifts from their pharmaceutical company of origin: citalopram hydrobromide (H.Lundbeck A.S., Denmark), fluoxetine hydrochloride (Lilly, UK), tianeptine hydrochloride (Servier, France), DP-5-CT (Novartis, Switzerland), paroxetine hydrochloride, SB216641 and BRL15572 (Smithkline Beecham, UK), FK506 and FR122175 (Dr J. Sharkey, Fujisawa Institute of Neuroscience, Edinburgh) and GR127,935, GR125,743, GR85548 and sumatriptan (Glaxo Wellcome, UK).



## **CHAPTER 4**

### **PHARMACOLOGICAL CHARACTERISATION OF THE SEROTONIN AND NORADRENALINE TRANSPORTERS IN THE RAT ADRENAL MEDULLA**

5-HT has also been immunocytochemically and neurochemically detected in the rat adrenal medulla (Verhofstad & Jonsson, 1983; Holzwarth *et al.*, 1984; Brownfield *et al.*, 1985; Holzwarth & Brownfield, 1985). Although 5-HT affects adrenal physiology (see Haning *et al.*, 1970; Furman & Waton, 1989; Aguilera *et al.*, 1993; Vijayaraghavan *et al.*, 1993) its source is unclear at present. Tryptophan loading demonstrated serotonin synthesis in adrenochromaffin cells (Delarue *et al.*, 1992), suggesting in part that the majority of enzymes for 5-HT synthesis are present. However, despite significant quantities of 5-HT in chromaffin granules there is low or insignificant levels of *de novo* 5-HT synthesis (Holzwarth *et al.*, 1984; Vandenberg *et al.*, 1991). It has long been known that, the adrenal medulla of both rats and mice take up and store exogenous 5-HT as well as its precursor 5-hydroxytryptophan as shown by autoradiographic experiments (Gershon and Ross, 1966a; Gershon and Ross 1966b; Csaba and Baráth, 1974; Csaba and Sudár, 1978). This active accumulation of 5-HT in adrenal chromaffin cells is antidepressant-sensitive (Gershon & Ross, 1966b; Baráth & Csaba, 1973; Trost & Muller, 1976; Kent & Coupland, 1984). It has been hypothesised that 5-HT in the rat adrenal gland is derived from circulating 5-HT (Verhofstad & Jonsson, 1983). The proteins responsible for adrenal 5-HT accumulation have been suggested as those responsible for noradrenaline transport (noradrenaline transporter; NET) since they are also expressed by chromaffin cells (Yoffe & Borchardt, 1982; Michael-Hepp *et al.*, 1992; Role & Perlman, 1993; Cubells *et al.*, 1995). These proteins will transport 5-HT albeit with low affinity (Thoa *et al.*, 1969). Although 5-HT transporter (SERT) gene expression appears to be exclusively neuronal in the CNS, SERTs are known to be expressed in several peripheral tissues, including lung, placenta (Balkovetz *et al.*, 1989), platelets (Rudnick, 1977) and lymphocytes (Marazzitti *et al.*, 1998). In the adrenal gland, an alternative transport mechanism for 5-HT uptake has been suggested since the discovery of high levels of SERT mRNA expression (Blakely *et al.*, 1991; Hoffman *et al.*, 1991). This expression is confined to medullary chromaffin cells of rodents (Blakely *et al.*, 1994; Chang *et al.*, 1996; Schroeter & Blakely, 1996). This suggests that adrenal chromaffin cell 5-HT may be captured from circulating blood, via a mechanism analogous to SERT-mediated accumulation of 5-HT in platelets (Rudnick, 1977; Gillis & Pitt, 1982; Stoltz, 1985).

In this chapter the potential of SERT protein expression was investigated in the rat adrenal gland. Citalopram, a selective serotonin re-uptake inhibitor (SSRI; Hyttel, 1994), was used to label SERT. [ $^3\text{H}$ ]Citalopram binding was characterised in rat brain and adrenal gland membranes and the pharmacology of these two distinct CNS and peripheral tissues directly compared. The pharmacology of platelet [ $^3\text{H}$ ]citalopram binding was also included, as it is known that the pharmacology of this SERT and brain SERT is identical (Plenge & Mellerup, 1991). Another SSRI, [ $^3\text{H}$ ]paroxetine, has previously been reported to autoradiographically quantify SERT in the rat brain (Chen *et al.*, 1992). This radiolabelled ligand was used to investigate the autoradiographic distribution of SERT in the adrenal gland. The cloning of SERT (Blakely *et al.*, 1991; Hoffman *et al.*, 1991), has enabled the production of specific site-directed anti-SERT antibodies (described in Chapter 2). One such antibody (as identified in Chapter 2) was used to further assess the distribution of SERT using brain, adrenal and platelet tissue in SDS-PAGE Western blots.

NET is a marker for noradrenergic terminals in the brain and is also present in adrenal chromaffin cells (Yoffe & Borchardt, 1982; Michael-Hepp *et al.*, 1992; Role & Perlman, 1993; Cubells *et al.*, 1995). This chapter also pharmacologically characterises a NET radioligand binding assays in both the rat adrenal gland and brain membranes, using the selective ligand [ $^3\text{H}$ ]nisoxetine (Tejani-Butt *et al.*, 1990; Tejani-Butt, 1992; Cheetham *et al.*, 1996).

#### **4.1 Methods**

[ $^3\text{H}$ ]Citalopram and [ $^3\text{H}$ ]nisoxetine binding assays, to label SERT and NET respectively, were conducted as described in chapter 3. [ $^3\text{H}$ ]Paroxetine autoradiography experiments, to assess the distribution of SERT, were conducted as described in chapter 3. Western blot experiments were conducted using the mouse monoclonal antibody as described in chapter 2 using tissue homogenates. Denatured protein was prepared at 5mg/ml as described in chapter 2. In order to load greater than 50 $\mu\text{g}$  of protein per lane, the volume of denatured sample added was accordingly adjusted. To obtain 200 $\mu\text{g}$  of protein per lane for example, 40 $\mu\text{l}$  of denatured protein was added per lane rather than 10 $\mu\text{l}$  to obtain 50 $\mu\text{g}$  of denatured protein.

## 4.2 Results

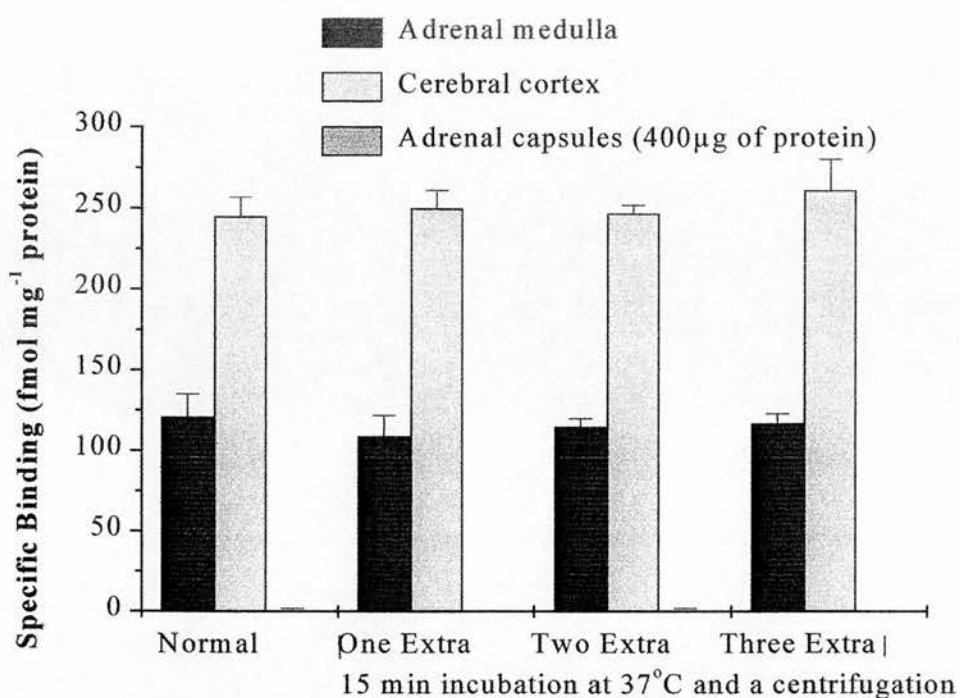
### 4.2.1 [<sup>3</sup>H]Citalopram Binding Assays

Initial experiments using a one hour incubation period, with membranes prepared from rat adrenal medulla (including the inner cortex), adrenal capsule (including the zona glomerulosa), brain cortex and platelet membranes revealed differences in the amount of specific [<sup>3</sup>H]citalopram binding. Though specific binding could be detected in adrenal medulla, brain and platelet membranes, no specific binding could be detected in adrenal capsule membranes (even using ten times more protein than in the adrenal medulla and neocortex membranes). The inclusion of further incubation and wash steps, as described for platelet membrane [<sup>3</sup>H]citalopram binding (section 3.2.2.5 described in Chapter 3), did not alter the amount of specific binding in brain, adrenal medulla or adrenal capsule membranes (Figure 4.1).

Adrenal medulla and cerebral cortex membranes were therefore used to characterise the [<sup>3</sup>H]citalopram binding assay and to directly compare the pharmacology of the binding sites in these two tissues and in rat blood platelets.

#### 4.2.1.1 Time Course of [<sup>3</sup>H]Citalopram Binding to Rat Adrenal Medulla and Cerebral Cortex Membranes

A time course (0-120 min) of [<sup>3</sup>H]citalopram (0.25nM) binding to rat adrenal medulla and cerebral cortex membranes was carried out at 25°C. Equilibrium was attained by 50 min, and was stable for at least another 70 min. Curve fitting using non linear regression gave an observed constant ( $k_{obs}$ ) of  $0.076 \pm 0.004 \text{ min}^{-1}$  ( $n=3$ ) with a  $t_{1/2}$  of  $8.712 \pm 0.018 \text{ min}$  ( $n=3$ ) in adrenal medulla membranes and an observed constant ( $k_{obs}$ ) of  $0.073 \pm 0.003 \text{ min}^{-1}$  ( $n=3$ ) with a  $t_{1/2}$  of  $8.725 \pm 0.091 \text{ min}$  ( $n=3$ ) in cerebral cortical membranes (Figure 4.2a). Dissociation was initiated after addition of 10 $\mu$ M citalopram following incubation for 60 min at 25°C, and followed first order kinetics with a  $t_{1/2}$  of  $11.747 \pm 0.946 \text{ min}$  and a dissociation rate constant ( $k_{-1}$ ) of  $0.0633 \pm 0.005 \text{ min}^{-1}$  in adrenal medulla membranes and a  $t_{1/2}$  of  $11.162 \pm 0.954 \text{ min}$  and a dissociation rate constant ( $k_{-1}$ ) of  $0.0609 \pm 0.005 \text{ min}^{-1}$  in cerebral cortical membranes (Figure 4.2b). Subsequent calculation gave association rate constants

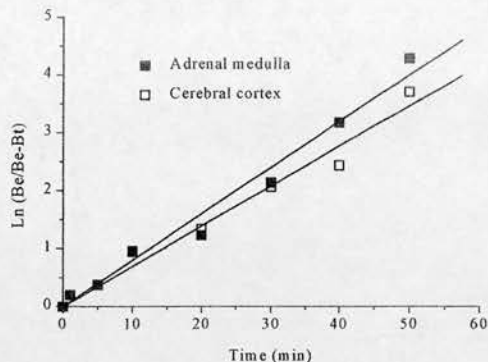
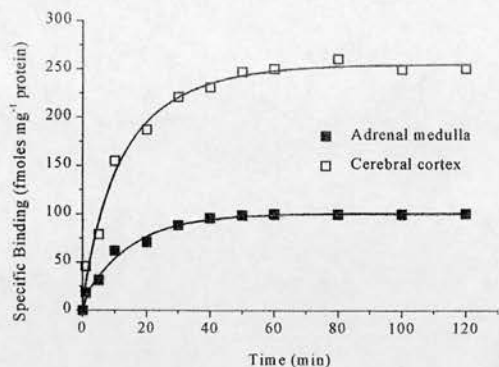


#### Membrane Preparation Method

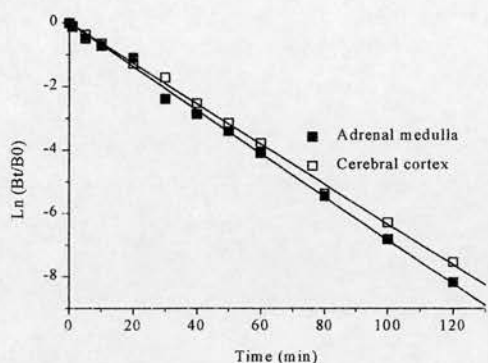
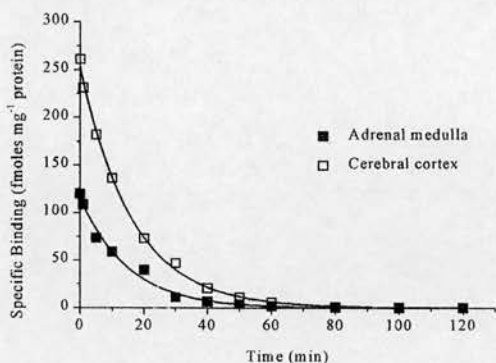
**Figure 4.1: Effect of Increasing the Number of Incubations at 37°C and Washes on [<sup>3</sup>H]Citalopram Membrane Binding.**

Data represent single experiments in adrenal medulla, adrenal capsule and cerebral cortex membranes. Membranes were subjected to further incubations at 37°C and washes as described in section 3.2.2.5. for [<sup>3</sup>H]citalopram binding in platelet membranes. Each data point represents the mean of triplicate tubes.

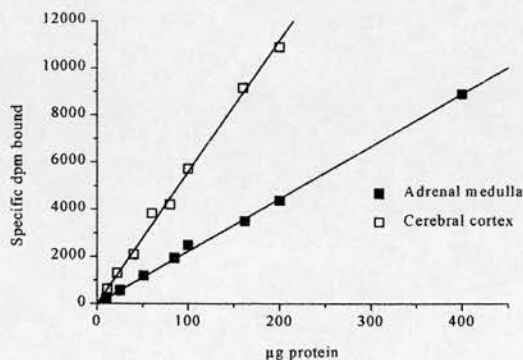
(a)



(b)



(c)



**Figure 4.2: Time Course and Protein Linearity of [<sup>3</sup>H]Citalopram Binding.**

The data represent typical (a) time course, and (b) dissociation experiments, and (c) a single protein linearity experiment. Graphs on the right hand side represent the semilogarithmic transformations of the data. Experiments were performed as described in the text, with mean data obtained from three experiments also in text.

( $k_{+1}$ )  $0.0474 \pm 0.008$  and of  $0.0434 \pm 0.004 \text{ min}^{-1}\text{M}^{-1}$  in adrenal medulla and cerebral cortex membranes respectively. The data from these time course experiments gave equilibrium constants ( $K_D$ ) of  $1.413 \pm 0.290$  and  $1.447 \pm 0.227 \text{ nM}$  in adrenal medulla and cerebral cortex membranes respectively. In the following competition experiments [ $^3\text{H}$ ]citalopram ( $0.25\text{nM}$ ) binding to both rat adrenal medulla and cerebral cortical membranes was carried out at equilibrium at  $25^\circ\text{C}$  for 60 min.

#### **4.2.1.2 Effect of Membrane Protein Concentration on [ $^3\text{H}$ ]Citalopram Binding to Rat Adrenal Medulla and Cerebral Cortical Membranes**

Specific [ $^3\text{H}$ ]citalopram binding was dependent on membrane protein (Figure 4.2c) and was linear over the concentrations tested ( $\sim 20\text{-}400\mu\text{g}$  protein/assay) for both adrenal medulla and cerebral cortex membranes. Subsequent experiments were conducted with  $40\text{-}160\mu\text{g}$  adrenal medulla membrane protein and  $20\text{-}60\mu\text{g}$  of cerebral cortex membrane protein to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

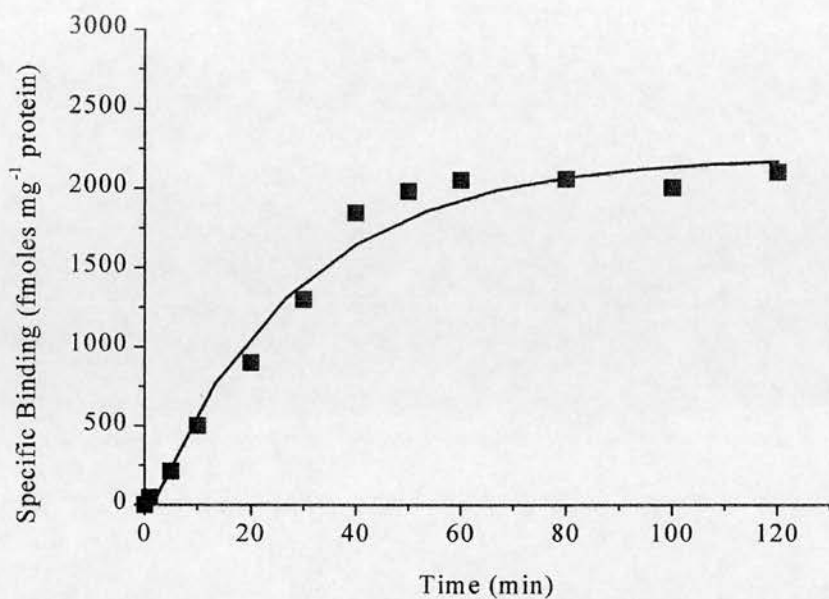
#### **4.2.1.3 Time Course and Concentration Dependence of [ $^3\text{H}$ ]Citalopram Binding in Rat Platelet Membranes**

As in rat adrenal medulla and cerebral cortical membranes, the time course of [ $^3\text{H}$ ]citalopram binding to rat platelet membranes reached equilibrium at 50 min, and was stable for at least another 70 min (Fig 4.3a). Competition binding studies using  $0.25\text{nM}$  [ $^3\text{H}$ ]citalopram and increasing concentrations of unlabelled citalopram ( $0.01\text{nM}\text{-}1\mu\text{M}$ ) (Figure 3.4b), gave a  $K_D$  of  $1.36 \pm 0.09 \text{ nM}$ , a  $B_{\text{max}}$  of  $16.05 \pm 2.64 \text{ pmoles mg}^{-1} \text{ protein}$  and a Hill slope of  $0.97 \pm 0.02$  ( $n=3$ ).

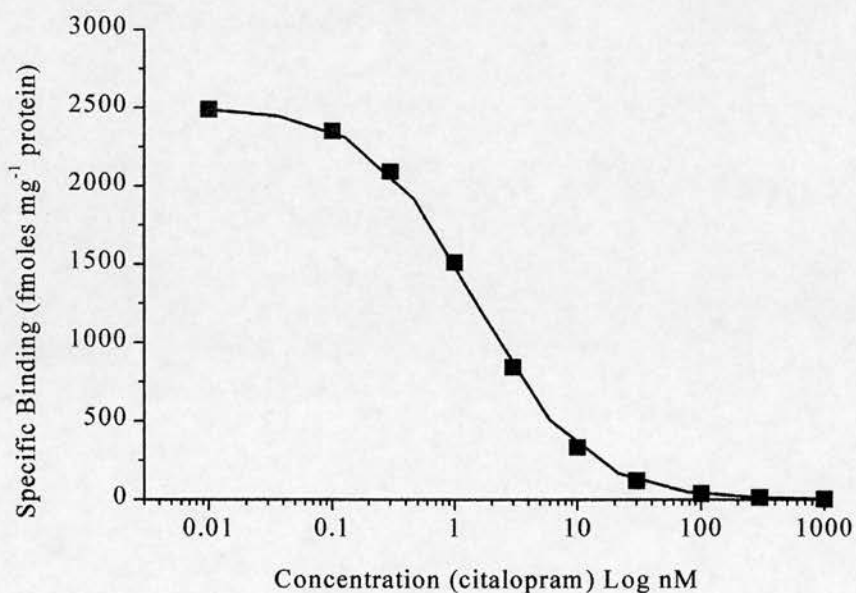
#### **4.2.1.4 Concentration Dependence of [ $^3\text{H}$ ]Citalopram Binding to Rat Adrenal Medulla and Cerebral Cortical Membranes**

Hot saturation analysis of [ $^3\text{H}$ ]citalopram binding to both adrenal medulla and cerebral cortex membranes was carried out using increasing concentrations of [ $^3\text{H}$ ]citalopram (Figure 4.4a). Curve fitting using a logistic model gave an equilibrium dissociation constant ( $K_D$ ) of  $1.28 \pm 0.07$  and  $1.28 \pm 0.08 \text{ nM}$  and a

(a)



(b)

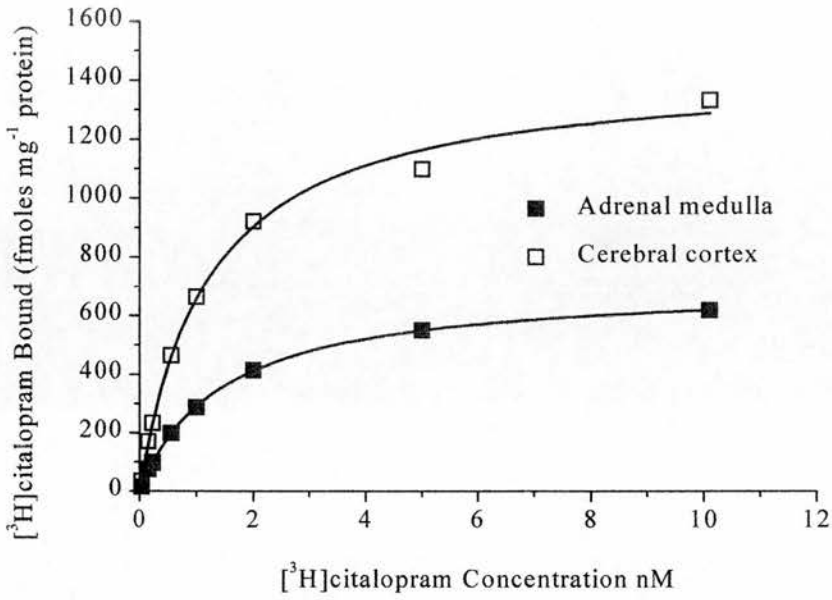


**Figure 4.3: [<sup>3</sup>H]Citalopram Binding in Rat Platelet Membranes.**

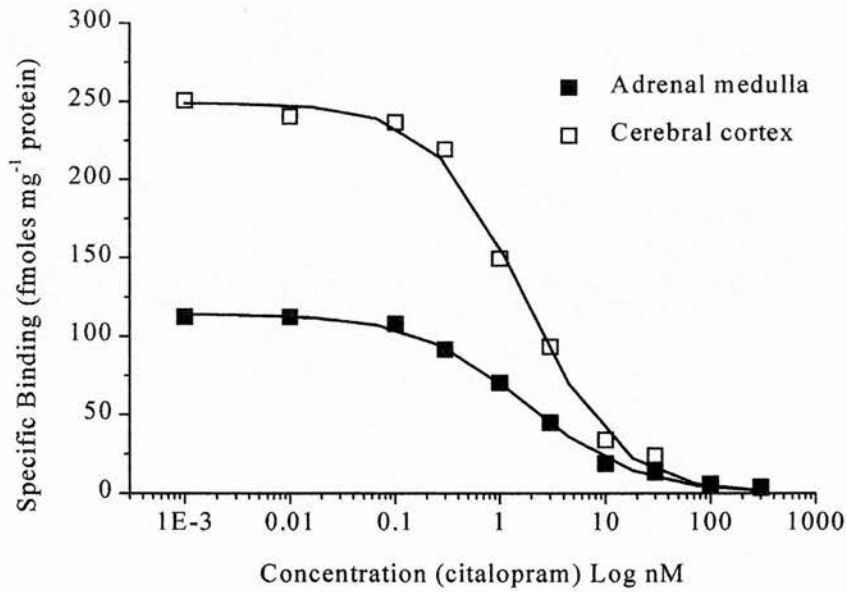
The data represent (a) a single time course and (b) a typical cold saturation. Experiments were performed as described in the text, with mean data obtained from the cold saturation experiments also in text.



(a)



(b)



**Figure 4.4: Concentration Dependence of Specific  $[^3\text{H}]$ Citalopram Binding.**

The results represent (a) hot saturation and (b) cold saturation experiments with each point performed in duplicate. See text for mean values.

binding site density ( $B_{\max}$ ) of  $0.69 \pm 0.02$  and  $1.63 \pm 0.10$  pmol  $\text{mg}^{-1}$  protein ( $n=3$ ) in adrenal medulla and cerebral cortex membranes respectively. Hill slopes of  $0.98 \pm 0.07$  and  $1.02 \pm 0.03$  ( $n=3$ ) in adrenal medulla and cerebral cortex membranes respectively were observed.

Competition binding studies using 0.25nM [ $^3\text{H}$ ]citalopram and increasing concentrations of unlabelled citalopram (0.01nM-1 $\mu\text{M}$ ) (Figure 4.4b), gave a  $K_D$  of  $1.41 \pm 0.02$  and  $1.58 \pm 0.20$  nM, a  $B_{\max}$  of  $0.66 \pm 0.03$  and  $1.66 \pm 0.03$  pmol  $\text{mg}^{-1}$  protein, and a Hill slope of  $0.94 \pm 0.10$  and  $1.03 \pm 0.18$  ( $n=3$ ) in adrenal medulla and cerebral cortex membranes respectively. This is in very close agreement with data obtained from 'hot'saturation and time course experiments. No significant differences were observed in the  $B_{\max}$  or  $pK_D$  values between these studies in Wistar Cobb or in Sprague Dawley rats (data not shown).

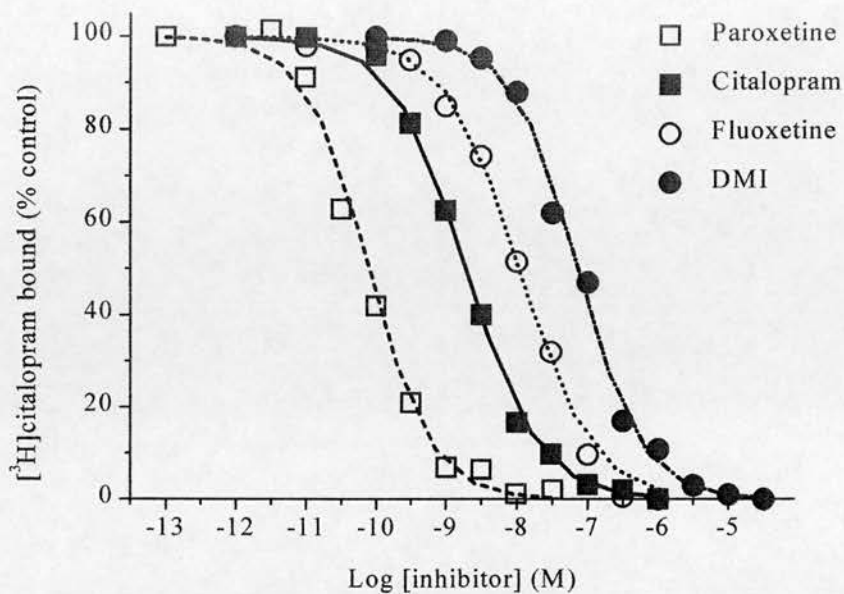
#### **4.2.1.5 Pharmacology of [ $^3\text{H}$ ]Citalopram Binding Sites in Rat Adrenal Medulla, Cerebral Cortical and Platelet Membranes**

To pharmacologically characterise [ $^3\text{H}$ ]citalopram binding sites, the affinity of a number of amine uptake inhibitors (paroxetine, fluoxetine, desmethylimipramine (DMI), nisoxetine, and nomifensine) and amine substrates (5-HT, dopamine, noradrenaline and MDMA) was investigated. Experiments with adrenal medulla and neocortical membranes were performed in parallel to allow direct comparison.

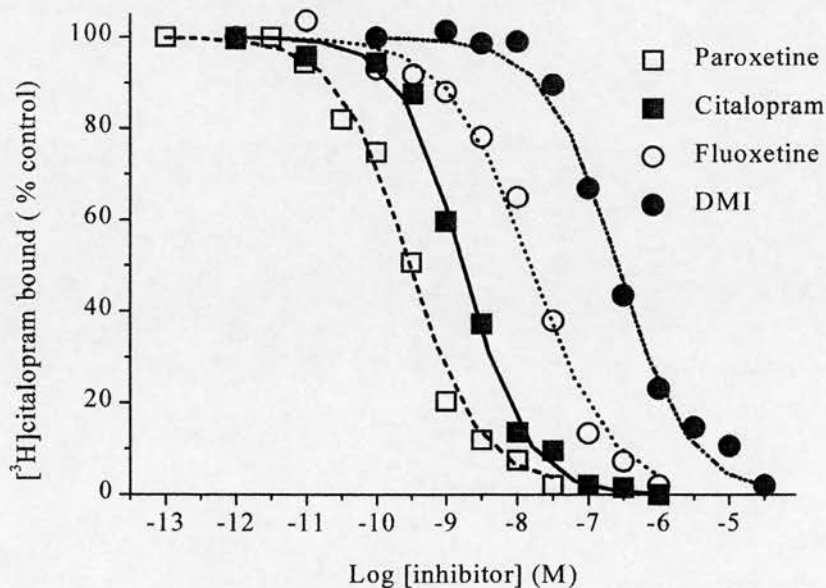
The serotonin specific re-uptake inhibitors (SSRIs; paroxetine, citalopram and fluoxetine) had the highest affinity in the adrenal medulla and neocortical membranes with  $pK_i$  values of 9.44, 8.85 and 8.04, and 9.58, 8.80 and 8.17 respectively (Table 4.1). The same pattern was observed with platelet membranes (Table 4.1). DMI which has affinity for both noradrenergic and serotonergic transporters, had a lower affinity than the SSRIs in both adrenal medulla and neocortical membranes with  $pK_i$  values of 6.83 and 6.81 respectively (Figure 4.5).

Nisoxetine (a selective noradrenergic uptake inhibitor) and nomifensine (a selective dopaminergic uptake inhibitor) also had a much lower affinity than the SSRIs in both adrenal medulla and neocortical membranes (Table 4.1) and also in rat platelet membranes.

(a)



(b)



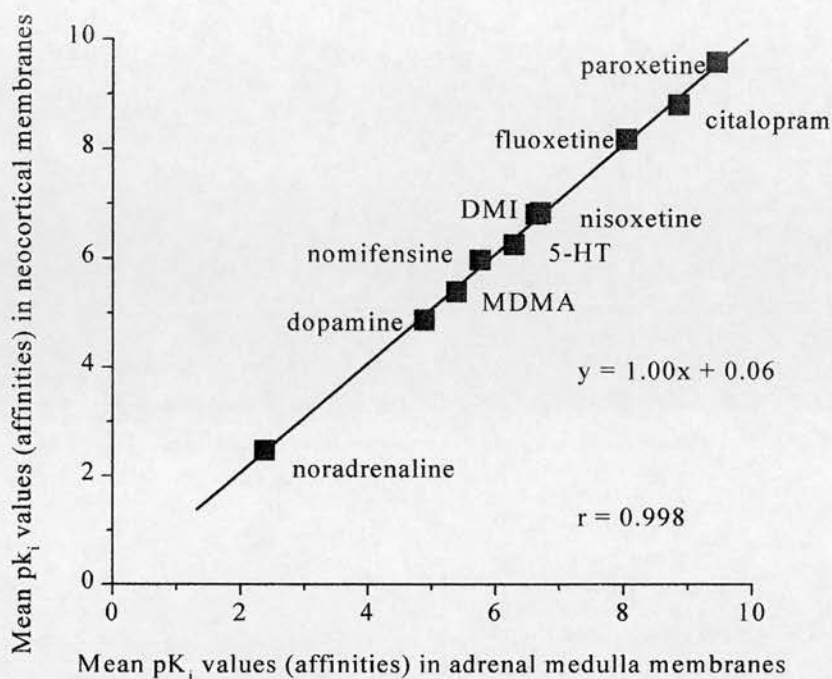
**Figure 4.5: Inhibition of  $[^3\text{H}]$ Citalopram Binding by Amine Uptake Inhibitors.**

The data represent a typical experiment in (a) adrenal medulla and (b) cerebral cortical membranes.  $\text{pK}_i$  values for competing drugs were determined from at least three independent experiments (Table 4.1).

<u>Inhibitors</u>	Adrenal Medulla Membranes		Cerebral Cortex Membranes		Platelet Membranes	
	pK <sub>i</sub>	nH	pK <sub>i</sub>	nH	pK <sub>i</sub>	nH
Paroxetine	9.44 ± 0.06	0.93 ± 0.07	9.58 ± 0.18	1.13 ± 0.05	9.45 ± 0.15	1.02 ± 0.06
Citalopram	8.85 ± 0.02	0.94 ± 0.10	8.80 ± 0.05	1.03 ± 0.18	8.87 ± 0.03	0.97 ± 0.02
Fluoxetine	8.04 ± 0.07	1.21 ± 0.38	8.17 ± 0.04	0.91 ± 0.06	nd	nd
Nisoxetine	6.70 ± 0.04	0.90 ± 0.05	6.84 ± 0.03	0.88 ± 0.17	6.78 ± 0.18	0.89 ± 0.16
DMI	6.63 ± 0.13	0.87 ± 0.09	6.81 ± 0.04	0.87 ± 0.10	nd	nd
Nomifensine	5.76 ± 0.09	0.92 ± 0.07	5.97 ± 0.02	0.94 ± 0.09	5.99 ± 0.12	0.95 ± 0.07
<u>Substrates</u>						
5-HT	6.29 ± 0.02	1.09 ± 0.13	6.25 ± 0.10	1.05 ± 0.10	6.26 ± 0.08	1.02 ± 0.05
MDMA	5.39 ± 0.04	0.99 ± 0.12	5.39 ± 0.02	0.94 ± 0.09	nd	nd
Dopamine	4.88 ± 0.02	1.00 ± 0.77	4.87 ± 0.05	1.12 ± 0.08	4.88 ± 0.06	1.15 ± 0.06
Noradrenaline	2.38 ± 0.14	0.97 ± 0.26	2.47 ± 0.02	0.96 ± 0.10	nd	nd

**Table 4.1: Pharmacology of [<sup>3</sup>H]citalopram Binding.**

The affinity values were determined from the inhibition of 0.25nM [<sup>3</sup>H]citalopram binding to rat adrenal medulla, cerebral cortex and platelet membranes. The results are expressed as pK<sub>i</sub> values and Hill slope (nH) for a single site logistic fit. Values shown are the mean ± s.e.mean. Data represents the results from at least three independent experiments. nd represents those compounds that were not determined.



**Figure 4.6: Correlation of Affinities of [<sup>3</sup>H]Citalopram Binding in Rat Adrenal Medulla and Cerebral Cortical Membranes.**

Affinities for inhibition of [<sup>3</sup>H]citalopram binding were determined from at least three experiments (Table 4.1). The correlation coefficient ( $r$ ) obtained by linear regression analyses of the data was 0.998 ( $p < 0.0001$ , degrees of freedom = 9) with a slope of  $1.00 \pm 0.02$  which is not significantly different from 1 (Student's  $t$ -test).

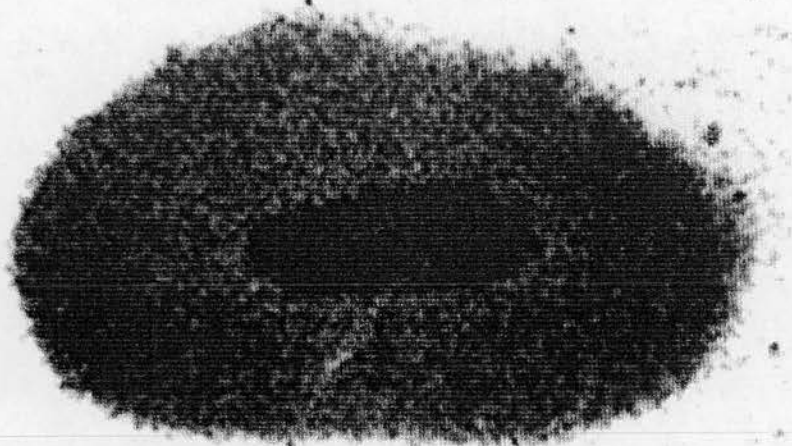
Of the amine substrates tested (5-HT, dopamine and noradrenaline), 5-HT was the most potent with a  $pK_i$  value of 6.29 in adrenal medulla membranes, 6.25 in neocortical membranes and 6.26 in platelet membranes. MDMA also had lower affinity than 5-HT, but was not significantly different between adrenal medulla and cerebral cortex membranes (Table 4.1). In all tissues, the atypical antidepressant, tianeptine, had no effect on [ $^3$ H]citalopram binding, when tested up to 1mM (data not shown).

Hill coefficients of all amine re-uptake inhibitors and substrates were close to unity (Table 4.1) in all membrane preparations, indicative of a single population of [ $^3$ H]citalopram binding sites. The rank order of potency of the drugs tested against [ $^3$ H]citalopram binding in adrenal medulla and neocortical membranes was paroxetine > citalopram > fluoxetine > nisoxetine > desmethylimpramine (DMI) > 5-HT > nomifensine > MDMA > dopamine > noradrenaline (Table 4.1). Although a limited range of drugs were tested in platelet membranes the same rank order was observed (Table 4.1). Comparison of drug affinities between adrenal medulla and neocortical membranes, show a very good correlation ( $r = 0.998$ , Figure 4.6), with no one drug being significantly different in affinity between the two membrane preparations ( $P > 0.05$ , Table 4.1). Likewise, there was no significant difference in the affinity of drugs tested between platelet and/or adrenal medulla or cerebral cortical membranes ( $P > 0.05$ , Table 4.1).

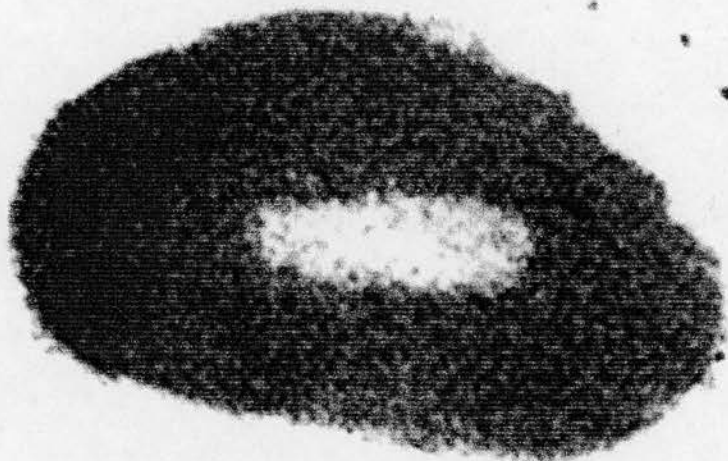
#### **4.2.2 [ $^3$ H]Paroxetine Autoradiography in the Rat Adrenal Gland**

The distribution of [ $^3$ H]paroxetine binding sites was assessed in the rat adrenal gland. In the presence of 250pM [ $^3$ H]paroxetine, total binding was found at a high density centrally located to the rat adrenal medulla with fewer sites in the adrenal cortex (Figure 4.7a). In the presence of 250pM [ $^3$ H]paroxetine and 4 $\mu$ M citalopram, non-specific binding was apparent in the cortex but not the centrally located adrenal medulla (Figure 4.7b). Comparing the autoradiograms (Figures 4.7a & b), a high degree of specific binding can be seen after subtraction between total and non-specific binding in the centrally located adrenal medulla, with little or no specific binding in the surrounding cortical areas. The density of [ $^3$ H]paroxetine binding sites was calculated to be  $220 \pm 20$  fmoles  $mg^{-1}$  of tissue in the adrenal

(a)



(b)



**Figure 4.7: [<sup>3</sup>H]Paroxetine Autoradiograms of Rat Adrenal Glands.**

Total binding was defined using 250pM [<sup>3</sup>H]paroxetine (a) and non-specific binding defined in adjacent sections using 250pM [<sup>3</sup>H]paroxetine in the presence of 4μM citalopram (b). Tissue isotope concentrations were measured, relative to appropriate [<sup>3</sup>H]standards, and specific binding determined by subtraction of image densities from total and non-specific binding images. Data shown are representative autoradiograms. [<sup>3</sup>H]Paroxetine binding densities were determined from at least three independent experiments.

medulla and  $7 \pm 6$  fmoles  $\text{mg}^{-1}$  tissue in the cortex ( $n=6$ ). No specific [ $^3\text{H}$ ]paroxetine binding could be detected in the capsular region, which includes the 5 cell thick layer of the zona glomerulosa ( $n=6$ ).

### 4.2.3 Western Blots

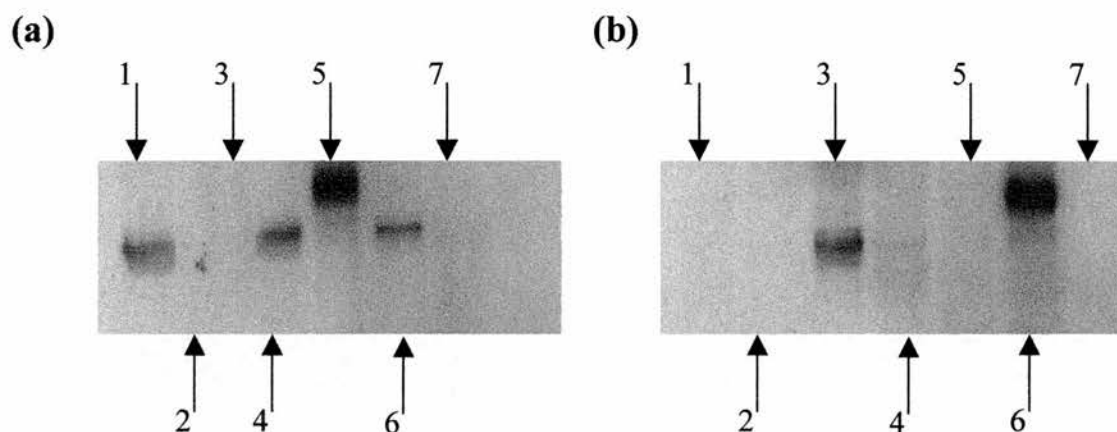
Western blot analysis (Figures 4.8 a & b) using a mouse monoclonal antibody raised against SERT, showed the presence of a single immunoreactive species at 76 kDa in the adrenal medulla (including inner cortex; lane 6 of Figure 4.8a). Doubling the concentration loaded, to 100 $\mu\text{g}$  of protein of adrenal medulla (including inner cortex) also showed the presence of a single immunoreactive species with increased band intensity at 76 kDa (lane 4 of Figure 4.8a and lane 3 of Figure 4.8b). No immunoreactivity could be detected in the adrenal capsule (including the zona glomerulosa) at 50 $\mu\text{g}$  of protein (lane 3 of Figure 4.8a and lane 7 of Figure 4.8b), 100 $\mu\text{g}$  of protein (lane 2 of Figure 4.8b) or at the even higher concentration of 200 $\mu\text{g}$  of protein (lane 5 of Figure 4.8b). Neocortical homogenates at 50 $\mu\text{g}$  of protein, as in the adrenal medulla homogenates, gave a single immunoreactive species at 76 kDa (lane 1 of Figure 4.8a), with a higher band intensity when compared to adrenal medulla homogenates also loaded at 50 $\mu\text{g}$ . No immunoreactivity was detected in liver (lane 7 of Figure 4.8a and lane 1 of Figure 4.8b) or cerebellum homogenates (lane 2 of Figure 4.8a and lane 4 of Figure 4.8b), both loaded at 50 $\mu\text{g}$ . Prominent immunoreactivity was also detected in platelets at 94 kDa loaded at 50 $\mu\text{g}$  (lanes 5 of Figure 4.8a and lane 6 of Figure 4.8b). No immunoreactivity was detected in the absence of primary antibody (data not shown).

### 4.2.4 [ $^3\text{H}$ ]Nisoxetine Binding

#### 4.2.4.1 Time Course of [ $^3\text{H}$ ]Nisoxetine Binding to Rat Adrenal Medulla and Cerebral Cortex Membranes

A time course (0-90 min) of [ $^3\text{H}$ ]nisoxetine (0.50nM) binding to rat adrenal medulla and cerebral cortex membranes was carried out at 25°C. Equilibrium was attained by 15 min and was stable for at least another 65 min. Curve fitting using non linear regression gave an observed constant ( $k_{\text{obs}}$ ) of  $0.277 \pm 0.018 \text{ min}^{-1}$  ( $n=3$ ) with a





**Figure 4.8: Western Blot Analysis of Rat Adrenal Glands, Cerebral Cortex and Platelets.**

SDS-PAGE western blot analysis using a 1 in 500 dilution of the anti-SERT monoclonal mouse antibody. Protein from each sample was loaded onto a 7.5% polyacrylamide gel. Rainbow markers (Sigma) were run on the gel and the transfer overlaid over the exposure to give the appropriate size markers (not shown) in kDa.

Immunodetection of SERT in 50µg adrenal medulla at 76 kDa (lane 6a) and 100µg of adrenal medulla (lane 4a and lane 3b).

No immunodetection of 50µg (lane 3a and lane 7b), 100µg (lane 2b) or 200µg (lane 5b) of adrenal capsule protein was detected.

50µg of neocortex was detected at 76 kDa (lane 1a).

50µg of liver (lane 7a and lane 1b) and cerebellum was loaded (lane 2a and lane 4b).

50µg platelets was immunodetected with a molecular weight of 94 kDa (lanes 5a and lane 6b).

$t_{1/2}$  of  $3.669 \pm 0.155$  min ( $n=3$ ) in adrenal medulla membranes and an observed constant ( $k_{obs}$ ) of  $0.274 \pm 0.015$  min<sup>-1</sup> ( $n=3$ ) with a  $t_{1/2}$  of  $3.762 \pm 0.091$  min ( $n=3$ ) in cerebral cortical membranes (Figure 4.9a). Dissociation was initiated after addition of 10 $\mu$ M nisoxetine following incubation for 60 min at 25°C, and followed first order kinetics with a  $t_{1/2}$  of  $2.448 \pm 0.131$  min and a dissociation rate constant ( $k_{-1}$ ) of  $0.236 \pm 0.005$  min<sup>-1</sup> in adrenal medulla membranes and a  $t_{1/2}$  of  $2.667 \pm 0.114$  min and a dissociation rate constant ( $k_{-1}$ ) of  $0.247 \pm 0.012$  min<sup>-1</sup> in cerebral cortical membranes (Figure 4.9b). Subsequent calculation gave association rate constants ( $k_{+1}$ ) of  $0.082 \pm 0.026$  and of  $0.053 \pm 0.015$  min<sup>-1</sup>M<sup>-1</sup> in adrenal medulla and cerebral cortex membranes respectively. The data from these time course experiments gave equilibrium constants ( $K_D$ ) of  $3.753 \pm 1.44$  and  $5.688 \pm 1.757$  nM in adrenal medulla and cerebral cortex membranes respectively. In the following competition experiments [<sup>3</sup>H]nisoxetine (0.50nM) binding to both rat cerebral cortical and adrenal medulla membranes was carried out at equilibrium at 25°C for 60 min.

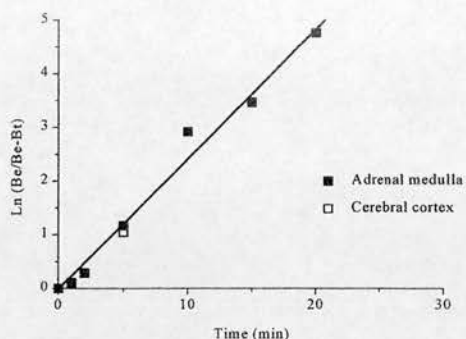
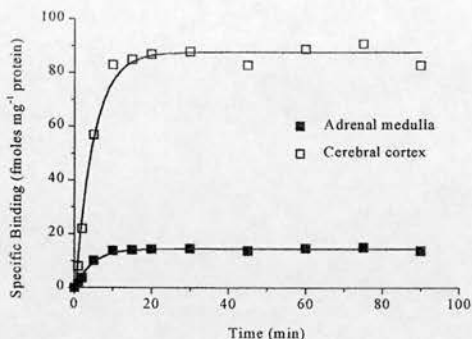
#### **4.2.4.2 Effect of Membrane Protein Concentration on [<sup>3</sup>H]Nisoxetine Binding to Rat Adrenal Medulla and Cerebral Cortical Membranes**

Specific [<sup>3</sup>H]citalopram binding was dependent on membrane protein (Figure 4.9c) and was linear over the concentrations tested (~20-400 $\mu$ g protein/assay) for both adrenal medulla and cerebral cortex membranes. Subsequent experiments were conducted with 300-400 $\mu$ g adrenal medulla membrane protein and 150-300 $\mu$ g of cerebral cortex membrane protein to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

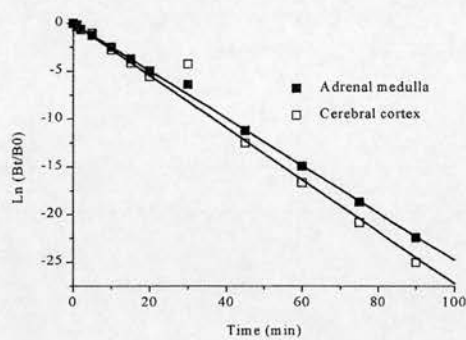
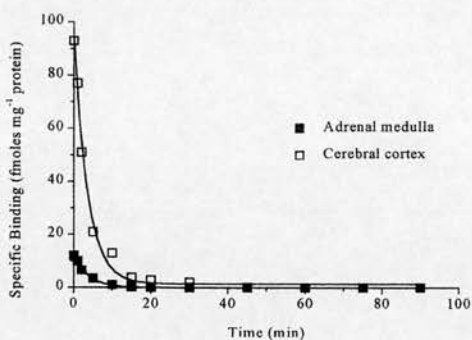
#### **4.2.4.3 Concentration Dependence of [<sup>3</sup>H]Nisoxetine Binding to Rat Adrenal Medulla and Cerebral Cortical Membranes**

Hot saturation analysis of [<sup>3</sup>H]nisoxetine binding to both adrenal medulla and cerebral cortex membranes was carried out using increasing concentrations of [<sup>3</sup>H]nisoxetine (Figure 4.10a). Curve fitting using a logistic model gave an equilibrium dissociation constant ( $K_D$ ) of  $3.84 \pm 0.22$  and  $3.89 \pm 0.13$  M and a binding site density ( $B_{max}$ ) of  $84.67 \pm 4.34$  and  $634.00 \pm 38.57$  fmol mg<sup>-1</sup> protein

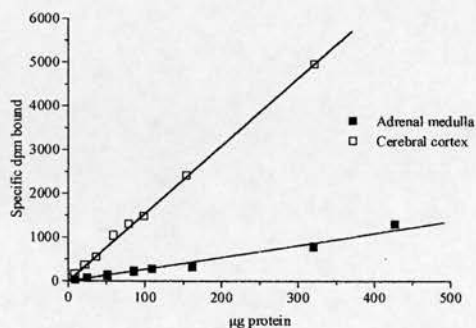
(a)



(b)



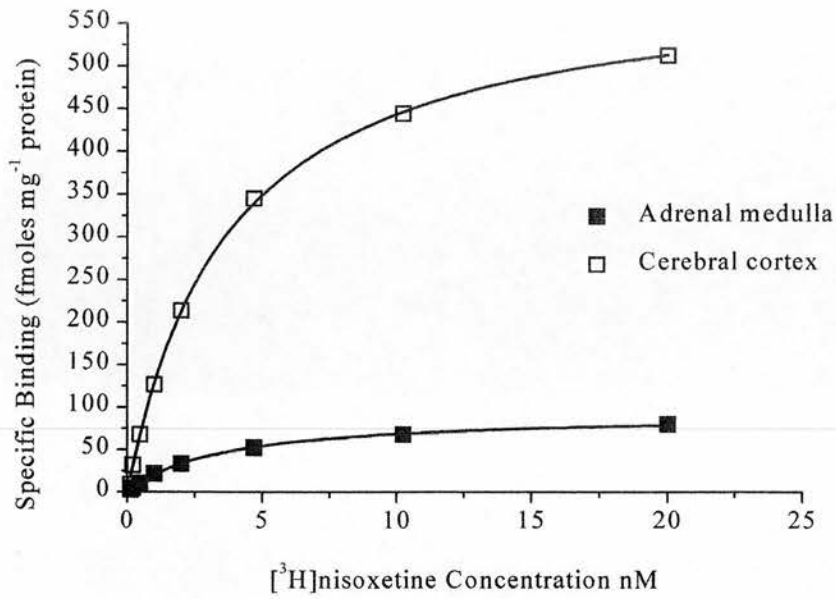
(c)



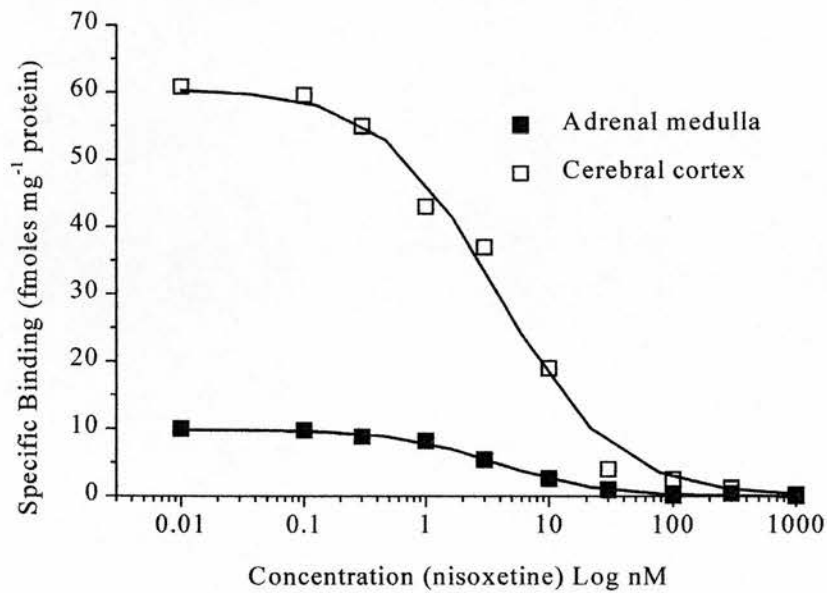
**Figure 4.9: Time Course and Protein Linearity of [<sup>3</sup>H]Nisoxetine Binding.**

The data represent typical (a) time course, and (b) dissociation experiments, and (c) a single protein linearity experiment. Graphs on the right hand side, represent the semilogarithmic transformations of the data. Experiments were performed as described in the text, with mean data obtained from three experiments also in text.

(a)



(b)



**Figure 4.10: Concentration Dependence of Specific  $[^3\text{H}]$ Nisoxetine Binding.**

The results represent (a) hot saturation and (b) cold saturation experiments with each point performed in duplicate. See text for mean values.

(n=3) in adrenal medulla and cerebral cortex membranes respectively. Hill slopes of  $0.93 \pm 0.07$  and  $0.94 \pm 0.06$  (n=3) in adrenal medulla and cerebral cortex membranes respectively were observed.

Competition binding studies using 0.50nM [ $^3\text{H}$ ]nisoxetine and increasing concentrations of unlabelled nisoxetine (0.01nM-1 $\mu\text{M}$ ) (Figure 4.10b), gave a  $K_D$  of  $3.94 \pm 0.14$  and  $3.93 \pm 0.17$  nM, a  $B_{\text{max}}$  of  $98.67 \pm 11.42$  and  $510.21 \pm 53.25$  fmol  $\text{mg}^{-1}$  protein, and a Hill slope of  $0.97 \pm 0.03$  and  $0.90 \pm 0.11$  (n=3) in adrenal medulla and cerebral cortex membranes respectively. This is in very close agreement with data obtained from 'hot' saturation and time course experiments. No significant differences were observed in the  $B_{\text{max}}$  or  $\text{p}K_D$  values between these studies in Wistar Cobb or in Sprague Dawley rats (data not shown).

#### **4.2.4.4 Pharmacology of [ $^3\text{H}$ ]Nisoxetine Binding Sites in Rat Adrenal Medulla and Cerebral Cortical Membranes**

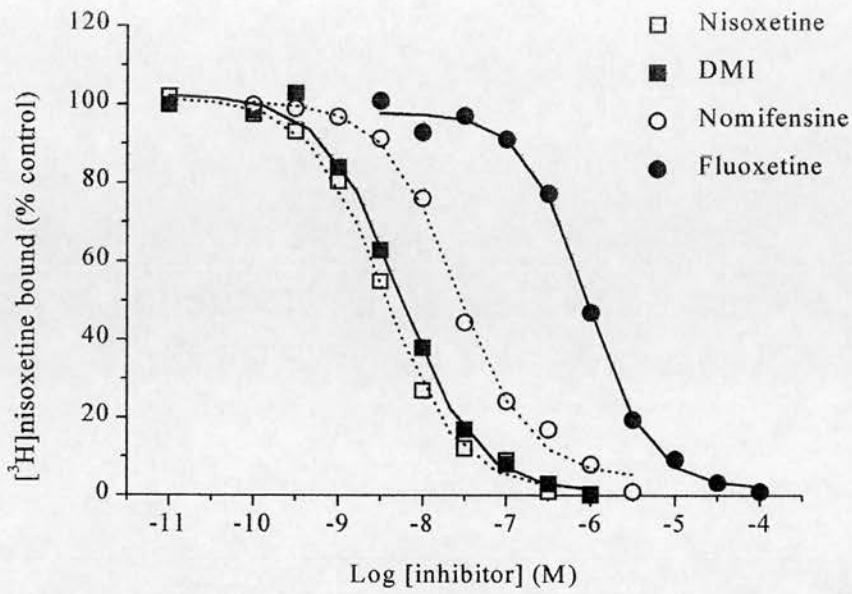
To pharmacologically characterise [ $^3\text{H}$ ]nisoxetine binding sites, the affinity of a number of amine uptake inhibitors (nisoxetine, desmethylimipramine (DMI), mianserin, nomifensine, fluoxetine and citalopram) and amine substrates (5-HT, dopamine, and noradrenaline) was investigated. Experiments with adrenal medulla and neocortical membranes were performed in parallel to allow direct comparison.

Re-uptake inhibitors of noradrenaline transport (nisoxetine and DMI) had the highest affinity in the adrenal medulla and neocortical membranes with  $\text{p}K_i$  values of 8.43 and 8.26, and 8.43 and 8.51 respectively (Table 4.2).

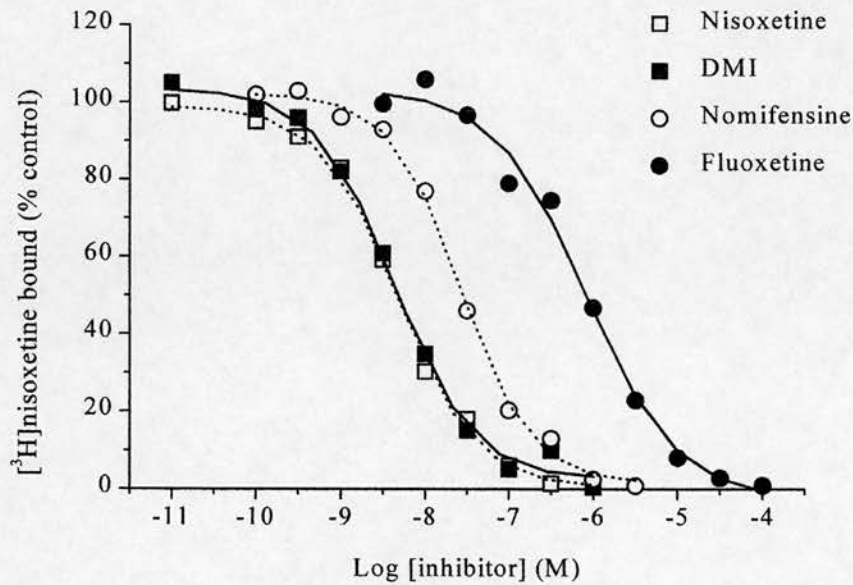
Nomifensine (a selective dopaminergic uptake inhibitor) had lower affinity than the noradrenergic reuptake inhibitors, whilst fluoxetine and citalopram (selective serotonergic ligands) were by far the weakest amine uptake inhibitors tested in both adrenal medulla (Figure 4.11a) and cerebral cortex (Figure 4.11b) membranes. Of the amine substrates tested (5-HT, dopamine and noradrenaline), noradrenaline was the most potent with a  $\text{p}K_i$  value of 5.59 in both adrenal medulla and cerebral cortical membranes (Table 4.2).

Hill coefficients of all amine re-uptake inhibitors and substrates were close to unity (Table 4.2) in all membrane preparations, indicative of a single population of [ $^3\text{H}$ ]nisoxetine binding sites. The rank order of potency of all the drugs tested against

(a)



(b)



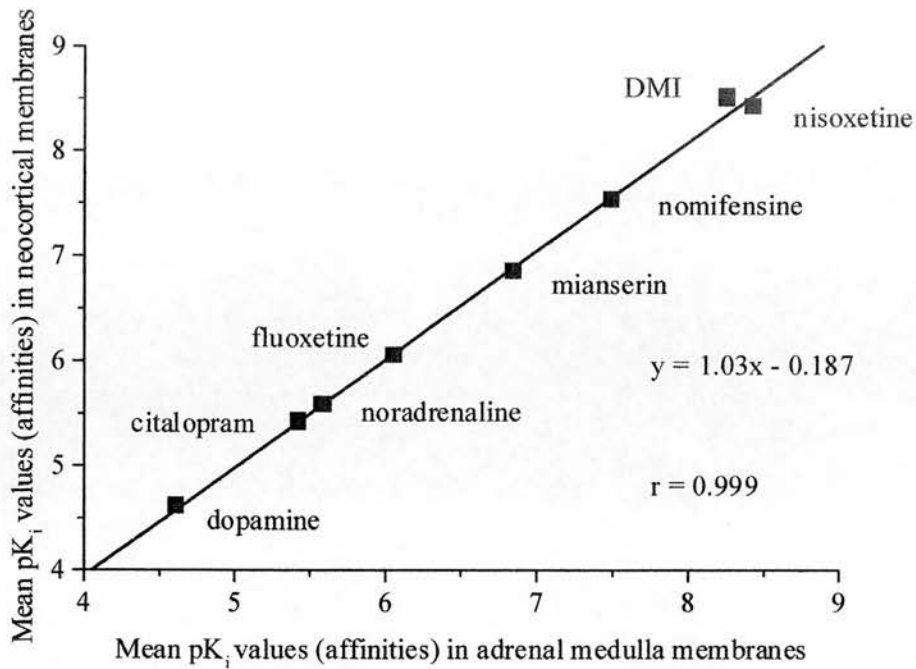
**Figure 4.11: Inhibition of  $[^3\text{H}]$ Nisoxetine Binding by Amine Uptake Inhibitors.**

The data represent a typical experiment in (a) adrenal medulla and (b) cerebral cortical membranes.  $pK_i$  values for competing drugs were determined from at least three independent parallel experiments (Table 4.2).

<u>Inhibitors</u>	<u>Adrenal Medulla Membranes</u>		<u>Cerebral Cortex Membranes</u>	
	<u>pK<sub>i</sub></u>	<u>nH</u>	<u>pK<sub>i</sub></u>	<u>nH</u>
Nisoxetine	8.43 ± 0.01	0.93 ± 0.07	8.43 ± 0.01	0.95 ± 0.06
DMI	8.26 ± 0.04	0.90 ± 0.03	8.51 ± 0.22	0.99 ± 0.07
Nomifensine	7.49 ± 0.08	0.88 ± 0.12	7.53 ± 0.03	0.89 ± 0.08
Mianserin	6.85 ± 0.06	0.95 ± 0.05	6.85 ± 0.05	0.88 ± 0.08
Fluoxetine	6.06 ± 0.02	1.00 ± 0.07	6.05 ± 0.03	1.03 ± 0.06
Citalopram	5.42 ± 0.04	0.96 ± 0.04	5.42 ± 0.04	0.99 ± 0.03
<u>Substrates</u>				
Noradrenaline	5.59 ± 0.09	0.95 ± 0.06	5.59 ± 0.12	0.89 ± 0.12
Dopamine	4.61 ± 0.02	0.91 ± 0.06	4.61 ± 0.03	0.93 ± 0.06
5-HT	< 4	nd	< 4	nd

**Table 4.2: Pharmacology of [<sup>3</sup>H]Nisoxetine Binding.**

The affinity values were determined from the inhibition of 0.50nM [<sup>3</sup>H]nisoxetine binding to rat adrenal medulla and cerebral cortex membranes. The results are expressed as pK<sub>i</sub> values and Hill slope (nH) for a single site logistic fit. Values shown are the mean ± s.e.mean. Data represents the results from at least three independent experiments. nd represents those factors that were not determined.



**Figure 4.12: Correlation of Affinities of [<sup>3</sup>H]nisoxetine Binding in Rat Adrenal Medulla and Cerebral Cortical Membranes.**

Affinities for inhibition of [<sup>3</sup>H]nisoxetine binding were determined from at least three experiments (Table 4.2). The correlation coefficient (*r*) obtained by linear regression analyses of the data was 0.999 (*p*<0.0001, degrees of freedom = 7) with a slope of  $1.03 \pm 0.02$  which is not significantly different from 1 (Student's *t*-test).



[<sup>3</sup>H]nisoxetine binding in adrenal medulla and cerebral cortex membranes was nisoxetine = desmethylimipramine (DMI) > nomifensine > mianserin > fluoxetine > noradrenaline > citalopram > dopamine > 5-HT (Table 4.2). Comparison of drug affinities between adrenal medulla and neocortical membranes, show a very good correlation ( $r = 0.999$ , Figure 4.6), with no one drug being significantly different in affinity between the two membrane preparations ( $P > 0.05$ , Table 4.2).

### 4.3 Discussion

The initial aim of this chapter was to pharmacologically characterise SERT protein sites in the rat adrenal gland and to confirm their distribution using [<sup>3</sup>H]paroxetine autoradiography and Western blot analysis. The pharmacological characterisation used the most selective of the SSRIs, citalopram, in membrane radioligand binding studies. The binding of [<sup>3</sup>H]citalopram to both adrenal medulla and cerebral cortex membranes was reversible, saturable and of high affinity. The  $K_D$  and  $B_{max}$  values were similar in their respective kinetic, saturation and competition studies in both membrane preparations (Figures 4.2 & 4.4). A high density of [<sup>3</sup>H]citalopram binding sites was found in rat adrenal medulla (including inner cortex) membranes corresponding to a  $B_{max}$  value of  $657 \pm 28$  fmoles  $mg^{-1}$  protein (2.5 fold lower than in cerebral cortex membranes) and a  $pK_D$  value of  $8.85 \pm 0.02$  (Figure 4.4). No specific binding could be detected in the adrenal capsular region, even when using 10 times more protein than required for assays with adrenal medulla membranes. A high density of [<sup>3</sup>H]citalopram binding sites were identified in platelet membranes (approximately 10 fold higher than in cerebral cortex) with the same affinity as adrenal medulla and cerebral cortex membranes (Figure 4.3). It was necessary to use two further incubation and centrifugation steps in the platelet membrane preparation for [<sup>3</sup>H]citalopram binding (section 3.2.2.5). The use of this extended membrane preparation in adrenal or brain membranes, compared to standard brain and adrenal membrane preparations, resulted in no change in specific [<sup>3</sup>H]citalopram binding (Figure 4.1). This further confirms the lack of [<sup>3</sup>H]citalopram binding sites in the adrenal capsular region.

In adrenal medulla membranes, the rank order of affinity for amine uptake inhibitors was paroxetine > citalopram > fluoxetine > nisoxetine > DMI > nomifensine (Table 4.1). Of all the drugs tested, each had a Hill coefficient of close to unity suggesting the binding to one population of binding sites (Table 4.1). Thus potent inhibitors of 5-HT uptake, such as the SSRIs; paroxetine, citalopram and fluoxetine had high affinity for [<sup>3</sup>H]citalopram binding sites whereas inhibitors of noradrenaline and dopamine uptake (nisoxetine and nomifensine respectively) had low affinity. DMI, which has affinity for both NET and SERT (a higher affinity for the former), had a lower affinity than the SSRIs, which is comparable to binding at SERT (Figure 4.5; Owens *et al.*, 1997). Of the amine substrates tested, 5-HT had much higher affinity than either dopamine or noradrenaline (Table 4.1).

In parallel comparative experiments, [<sup>3</sup>H]citalopram binding in brain cortex membranes gave a pK<sub>D</sub> value of 8.80 ± 0.05, which was not statistically significantly different to the affinity in adrenal medulla membranes. Moreover, the rank order of all the drugs tested in neocortical membranes was the same as in adrenal medulla membranes (Table 4.1). Comparison of affinities displayed an excellent correlation (Figure 4.6). A similar pharmacological profile was also obtained in platelet membranes (Table 4.1). The pharmacological profile of [<sup>3</sup>H]citalopram binding in adrenal medulla membranes is therefore consistent with SERT binding in brain and platelet membranes (Plenge & Møllerup, 1991; Owens *et al.*, 1997). A recent report has shown a high density of fluoxetine and DMI sensitive [<sup>3</sup>H]citalopram binding sites in the rat adrenal medulla (Pähkla & Rägo, 1997). However a limited range of drugs were tested, including reserpine and β-carbolines, and the authors did not make a clear conclusion as to the nature of these [<sup>3</sup>H]citalopram binding sites. In my study a spectrum of amine transporter uptake inhibitors and a number of substrates were tested in direct comparison to [<sup>3</sup>H]citalopram binding in the brain. This enabled the conclusion that the [<sup>3</sup>H]citalopram binding sites identified in the rat adrenal gland, represented SERT binding sites as in the brain.

The presence and distribution of SERT in the rat adrenal gland has been investigated in some other studies. *In situ* hybridisation mRNA experiments have revealed the adrenal medulla as a potential site for significant SERT expression (Hoffman *et al.*, 1991 and Blakely *et al.*, 1994). Recently [<sup>125</sup>I]RTI-55 has been used

in autoradiographic experiments, and potential SERT expression shown in the rat adrenal medulla (Schroeter *et al.*, 1997). This ligand has very high affinity for SERT, but despite showing low affinity for NET, has a similar high affinity for the dopamine transporter (DAT; Boja *et al.*, 1992; Silverthorn *et al.*, 1995). DAT, however, has immunohistochemically been shown to be present in the rat adrenal medulla (Mitsuma *et al.*, 1998). Therefore it was important to confirm SERT expression and distribution using a more selective ligand. Multiple specific radioligands have been evaluated for the anatomical mapping of SERT expression in rat brain, including [<sup>3</sup>H]citalopram (Lidlow *et al.*, 1989) and [<sup>3</sup>H]paroxetine (Chen *et al.*, 1992). Using the former, SERT expression was confirmed in rat adrenal medulla membranes, and the latter used for autoradiographic distribution experiments. A high density of SERT autoradiographic binding sites were found in the adrenal medulla ( $220 \pm 20$  fmoles  $\text{mg}^{-1}$  tissue), with negligible such sites in the surrounding cortical area ( $7 \pm 6$  fmoles  $\text{mg}^{-1}$  tissue; Figure 4.7). Using both membrane and autoradiographic binding techniques, no evidence was found for SERT expression in the capsular region (including the zona glomerulosa).

To aid the autoradiographic and membrane binding studies to determine if SERT is present in the adrenal capsules, an immunological approach was also adopted. Using the mouse anti-SERT monoclonal antibody (described in chapter 2), SERT immunoreactivity was specifically detected in adrenal medulla and brain cortex with an apparent molecular weight of 76 kDa (Figure 4.8). SERT immunoreactivity was also detected in platelets at 94 kDa (Figure 4.8). This is in agreement with brain and platelet studies in chapter 2 and also by others (e.g. Qian *et al.*, 1995). No immunoreactivity was observed in adrenal capsules using larger quantities of tissue (Figure 4.8), confirming the results obtained by [<sup>3</sup>H]citalopram binding. The specificity of SERT immunodetection was confirmed by the lack of any staining in the cerebellum or liver, two tissues which are known to show negligible SERT expression (Figure 4.8; Steinbusch, 1984; Qian *et al.*, 1995).

The presence of residual platelets from the adrenal medullary blood supply could account for the detection of SERT in the adrenal medulla, especially as the pharmacology of their binding sites are identical (Table 4.1). The difference in size of SERT between the adrenal medulla and platelets as determined by Western blots

is particularly important. Though this difference in size may be explained by the differing degrees of glycosylation (see chapter 2), it indicates that there is no contamination of adrenal SERT with platelet SERT. Furthermore the method for membrane [<sup>3</sup>H]citalopram binding in rat adrenal medulla membranes does not reveal any specific binding in platelet membranes (Figure 4.1). Therefore, despite the identical pharmacology of platelet and adrenal [<sup>3</sup>H]citalopram binding sites (Table 4.1), the detection of SERT in the adrenal medulla by radioligand binding or immunological methods is not due to the presence of platelets. These studies suggest that the pharmacology of the SERT located peripherally in the adrenal medulla is indistinguishable from SERT found in the CNS or SERT found on platelets.

[<sup>3</sup>H]Nisoxetine binding assays were developed to specifically label NET. The binding of [<sup>3</sup>H]nisoxetine to both adrenal medulla and cerebral cortical membranes was reversible, saturable and of high affinity. The  $K_D$  and  $B_{max}$  values were similar in their respective kinetic, saturation and competition studies in both membrane preparations (Figures 4.9 & 4.10). A relatively low density of [<sup>3</sup>H]nisoxetine binding sites was found in the rat adrenal medulla (including inner cortex) membranes with a  $B_{max}$  value of  $84.67 \pm 4.34$  fmoles  $mg^{-1}$  protein (~6 fold lower than in cerebral cortex membranes) and a  $pK_D$  value of  $8.43 \pm 0.01$  (Figure 4.10).

In adrenal medulla membranes, the rank order of affinity for amine uptake inhibitors was nisoxetine = DMI > nomifensine > mianserin > fluoxetine > citalopram (Table 4.2). Of all the drugs tested, each had a Hill coefficient of close to unity suggesting the binding to one population of binding sites (Table 4.2). Thus potent inhibitors of noradrenaline uptake, such as nisoxetine and DMI, had high affinity for [<sup>3</sup>H]nisoxetine binding sites whereas inhibitors of serotonergic and dopamine uptake (citalopram and fluoxetine, and nomifensine respectively) had lower affinity (Figure 4.11; Table 4.2). Of the amine substrates tested, noradrenaline had much higher affinity than either dopamine or 5-HT (Table 4.2). This would suggest that [<sup>3</sup>H]nisoxetine binding had labelled and detected the NET protein in the rat adrenal medulla membranes, albeit at a low density. In parallel comparative experiments, [<sup>3</sup>H]nisoxetine binding in brain cortical membranes gave a  $pK_D$  value of  $8.43 \pm 0.01$ , which was not statistically significantly different to the affinity in adrenal medulla membranes. Moreover, the rank order of all the drugs tested in

neocortical membranes was identical to in the adrenal medulla membranes (Table 4.2). The comparison of affinities displayed a very good correlation with no one drug being statistically different between adrenal medulla and brain membranes (Figure 4.12; Table 4.2). This shows that the pharmacology of the NET located peripherally in the adrenal medulla is indistinguishable from NET found in the CNS. Indeed a similar pharmacology has been shown in previous studies in the CNS (Tejani-Butt *et al.*, 1990; Tejani-Butt, 1992; Cheetham *et al.*, 1996). These studies show that 5-HT re-uptake sites are pharmacologically distinguishable from noradrenergic re-uptake sites in both the brain and adrenal medulla.

The confirmation of high levels of SERT protein in the rat adrenal medulla raises the question as to role of the transporter in this peripheral organ. Adrenal physiology shows blood moving in one direction to a central single vein within the medulla of the rat adrenal gland (Nussdorfer, 1980; Breslow, 1992). The specific location of SERT in the medullary region of the adrenal gland may serve as the uptake machinery for 5-HT in the blood. SERT is therefore positioned where 5-HT is potentially most concentrated. Furthermore the evidence supporting chromaffin cell 5-HT synthesis is indirect. After *in vitro* precursor loading, 5-HT has been detected in the adrenal gland (Holzwarth *et al.*, 1984). Although this suggests local biosynthesis, others have suggested that mast cell 5-HT biosynthesis could account for these findings (Hinson *et al.*, 1989). This may explain why very low levels of tryptophan hydroxylase (TPH) mRNA are detected by PCR techniques in the adrenal gland (Vandenbergh *et al.*, 1991). However, the authors of this above study could not rule out the possibility of a post-translational modified form of TPH (Kim *et al.*, 1991). In a more recent study the lack of any TPH protein has been confirmed immunologically (Schroeter *et al.*, 1997). The lack of TPH suggests *de novo* synthesis of 5-HT in the rat adrenal gland does not occur.

Adrenal 5-HT accumulation could possibly involve a mechanism involving NET, since these transporters are expressed by chromaffin cells, and will transport 5-HT (Thoa *et al.*, 1969). However, recent studies indicate only a low level of plasma membrane catecholamine transport activity in the intact adrenal gland resulting from corticosteroid suppression of NET gene expression (Wakade *et al.*, 1996). Furthermore the low affinity of 5-HT for NET (Pacholczyk *et al.*, 1991) suggests an

alternative mechanism for adrenal 5-HT accumulation. The confirmation of a high density of SERT protein in the adrenal medulla, compared to the lower density of the pharmacologically distinct NET, as shown in this chapter, supports the hypothesis that SERT mediated 5-HT plasma membrane transport, may be involved in loading chromaffin granules with 5-HT for later release (Blakely *et al.*, 1994). Chromaffin secretory granules, within chromaffin cells, possess large quantities of catecholamines, and are packaged by vesicular monoamine transporters (VMATs; Edwards, 1992). These transporters are less selective than plasma membrane transporters and can transport 5-HT with high affinity. It is known that chromaffin granule fusion can significantly elevate the free plasma concentration of 5-HT above nanomolar concentrations (Verhofstad & Jonsson, 1983; Holzwarth & Brownfield, 1985), about the  $K_D$  for a number of 5-HT receptors (Boess & Martin, 1994). These previous studies thus suggest that 5-HT may be transported across the plasma membrane from blood and further concentrated by VMAT in chromaffin secretory granules before release. This mechanism is analogous to those involved in the uptake, storage and secretion of 5-HT by platelets where SERT is also known to be expressed (Rudnick, 1977; Gillis & Pitt, 1982; Stoltz, 1985).

Within the adrenal gland it is known that 5-HT stimulation of the cells of the adrenal cortex and zona glomerulosa causes the secretion of steroids (Racz *et al.*, 1979; Rocco *et al.*, 1990; Rocco *et al.*, 1992). Is it also known that this stimulation of steroid secretion is mediated by a variety of 5-HT receptors in these adrenal cell types (Williams *et al.*, 1984; Matsouka *et al.*, 1985; Lefebvre *et al.*, 1992; Welch & Saphier, 1994). The discovery of SERT protein in the rat adrenal medulla suggests a potential regulatory mechanism for steroid secretion. It was perhaps surprising that the adrenal capsular region, which includes the zona glomerulosa layer where many of these receptors are located, did not therefore contain SERT. However in a recent study, the most intense SERT immunolabelling was found in medullary chromaffin cells in close proximity to steroid synthesising cells of the cortex, suggesting a paracrine interaction between the two cell types mediated by 5-HT (Schroeter *et al.*, 1997).

#### 4.4 Summary

A [<sup>3</sup>H]citalopram binding assay was developed to label SERT. A high density of binding sites specifically located in the rat adrenal medulla was revealed, with a pharmacological profile identical to brain and platelet SERT. The specific location of SERT in the adrenal medulla was confirmed using autoradiographic and immunological techniques.

A [<sup>3</sup>H]nisoxetine binding assay was developed to specifically label SERT. Studies. A low density of binding sites in the rat adrenal medulla with a pharmacological profile identical to brain NET was identified.

## **CHAPTER 5**

### **CHARACTERISATION OF 5-HT RECEPTOR RADIOLIGAND BINDING ASSAYS**



In the previous chapter radioligand binding assays were characterised for the 5-HT and noradrenaline transporters. In this chapter, binding assays were established for the 5-HT<sub>1A</sub> receptor and developed for 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> receptors. The purpose of these experiments was to use such assays to measure possible changes in receptor density and affinity after chronic antidepressant treatments and repeated MDMA administration (chapters 6 & 7).

The 5-HT<sub>1A</sub> receptor was one of the original 5-HT receptors characterised. Previous studies have used the 5-HT<sub>1A</sub> receptor agonist, 8-OH DPAT to radiolabel this 5-HT receptor sub-type (Gozlan *et al.*, 1983). [<sup>3</sup>H]8-OH DPAT binding to rat brain membranes was therefore pharmacologically characterised prior to drug treatments to ensure specific labelling of 5-HT<sub>1A</sub> receptors.

The most recent addition to the 5-HT receptor family is the 5-HT<sub>7</sub> receptor. The cDNA encoding this 5-HT receptor sub-type has been sequenced from a number of species including; mouse (Plassat *et al.*, 1993), guinea-pig (Tsou *et al.*, 1994), human (Bard *et al.*, 1993; Heidmann *et al.*, 1997; Jasper *et al.*, 1997), and rat (Lovenberg *et al.*, 1993a; Meyerhof *et al.*, 1993; Ruat *et al.*, 1993b; Shen *et al.*, 1993). At present, however, there are no commercially available selective 5-HT<sub>7</sub> receptor ligands. The identification of 5-HT<sub>7</sub> binding sites has been established using the protocol of rank order of agonist and antagonist potency at recombinant receptors (Sleight *et al.*, 1995b; Eglen *et al.*, 1997). The expressed recombinant 5-HT<sub>7</sub> receptor has; high affinity (pK<sub>i</sub> 8.0-10.00) for the agonists 5-CT, 5-HT, and 5-MeOT, and the 5-HT<sub>2A/2B/2C</sub> antagonist mesulergine, moderate affinity (pK<sub>i</sub> 6-7.9) for the 5-HT<sub>2A/2B/2C</sub> antagonist ritanserin, the 'selective' 5-HT<sub>1A</sub> agonist 8-OH DPAT, the non-selective antagonists methysergide and ergotamine, and low affinity (pK<sub>i</sub> <6.0) for the 5HT<sub>1A/1B</sub> antagonists pindolol and cyanopindolol (Table 5.1). Several antipsychotic compounds, including pimozide and clozapine also display high to moderate affinity for the cloned 5-HT<sub>7</sub> receptor (Roth *et al.*, 1994; Table 5.1). These attributes comprise a unique pharmacological profile for the 5-HT<sub>7</sub> receptor, which distinguishes it from closely related receptors such as the 5-HT<sub>1A</sub> receptor (Hoyer *et al.* 1994; To *et al.*, 1995; Table 5.2).

Though the pharmacology of 5-HT<sub>7</sub> receptors has been characterised using recombinant receptors, the development of a radioligand binding assay to selectively

**Table 5.1: Summary of Drug Affinity Values (pK<sub>i</sub>) at Rat and Guinea-pig 5-HT<sub>7</sub> Receptors.**

Compound	Rat 5-HT <sub>7(a)</sub> [ <sup>3</sup> H]5-HT Shen <i>et al.</i> , 1993	Rat 5-HT <sub>7(a)</sub> [ <sup>3</sup> H]LSD Shen <i>et al.</i> , 1993	Rat 5-HT <sub>7(e)</sub> [ <sup>3</sup> H]5-HT Meyerhof <i>et al.</i> , 1993	Rat 5-HT <sub>7</sub> [ <sup>3</sup> H]LSD Roth <i>et al.</i> , 1994	Rat 5-HT <sub>7(a)</sub> [ <sup>3</sup> H]5-HT Ruat <i>et al.</i> , 1993b	Rat 5-HT <sub>7</sub> [ <sup>3</sup> H]5-HT Stowe & Barnes, 1998b	Guinea-pig 5-HT <sub>7</sub> [ <sup>3</sup> H]5-HT Tsou <i>et al.</i> , 1994	Guinea-pig 5-HT <sub>7</sub> [ <sup>3</sup> H]5-HT To <i>et al.</i> , 1995	Guinea-pig 5-HT <sub>7</sub> ** [ <sup>3</sup> H]5-HT To <i>et al.</i> , 1995
<b>Agonists</b>									
5-CT	9.80	9.48	8.9		9.92	9.28	9.4	9.65	9.01
5-MeOT	9.24	8.75			9.02		9.0	9.28	8.80
5-HT	8.82	8.74	7.6		9.22	8.70	9.0	9.60	8.87
8-OH DPAT	7.46	7.45	5.7		7.28	6.81	7.3	7.39	6.87
Sumatriptan	6.60	6.24			6.30	6.16			
DP-5-CT								8.27	7.87
<b>Antagonists</b>									
Mesulergine	7.71	8.15						7.81	7.18
Metergoline	8.21	8.69			7.2			8.16	7.31
Mianserin	7.43	6.95	4.4		7.17			6.97	6.18
Clozapine	7.40	6.87		8.2	7.21	6.10		7.32	6.83
Ketanserin	6.58	6.69			<5.12	<5		6.20	5.54
Methiothepin	8.82	8.99				8.03	8.00	8.43	7.34
Methysergide	7.87	7.9	6.4			7.08	7.40	7.65	7.03
Ritanserin	7.82	7.66						7.34	6.72
Pindolol					<5.6			<4	<5
Pimozide				9.3		8.00			
Cyanopindolol									
Ergotamine								7.31	6.86

All Values are for recombinant receptors expressed in cell lines except \* values from rat whole brain (minus striatum and cerebellum) membranes and \*\* values from guinea-pig cortical homogenates.

**Table 5.2: Summary of Affinity Values at 5-HT Receptor Subtypes.**

Compound	5-HT <sub>1A</sub>	5-HT <sub>1B</sub>	5-HT <sub>1D</sub>	5-HT <sub>1E</sub>	5-HT <sub>1F</sub>	5-HT <sub>2A</sub>	5-HT <sub>2C</sub>	5-HT <sub>3</sub>	5-HT <sub>4</sub>	5-HT <sub>5A</sub>	5-HT <sub>5B</sub>	5-HT <sub>6</sub>	5-HT <sub>7</sub>
<b>Agonists</b>													
5-CT	9.53 <sup>a</sup>	8.29 <sup>a</sup>	8.6 <sup>c</sup>	5.15 <sup>e</sup>	5.6 <sup>h</sup>	4.66 <sup>i</sup>	6.23 <sup>a</sup>		5.2 <sup>p</sup>	9.5 <sup>q</sup>	7.4 <sup>i</sup>	6.6 <sup>q</sup>	9.48 <sup>q</sup>
5-MeOT	8.04 <sup>s</sup>	7.34 <sup>r</sup>	8.4 <sup>s</sup>	<6 <sup>g</sup>	<6 <sup>h</sup>	6.61 <sup>i</sup>	8.04 <sup>k</sup>	5.72 <sup>n</sup>	6.5 <sup>p</sup>			7.41 <sup>q</sup>	8.75 <sup>q</sup>
5-HT	8.38 <sup>s</sup>	8.61 <sup>a</sup>	8.4 <sup>c</sup>	8.14 <sup>e</sup>	7.17 <sup>h</sup>	7.1 <sup>i</sup>	7.95 <sup>k</sup>	6.80 <sup>m</sup>	7.3 <sup>p</sup>	8.1 <sup>q</sup>	6.6 <sup>i</sup>	7.25 <sup>q</sup>	8.74 <sup>q</sup>
8-OH DPAT	8.61 <sup>a</sup>	5.75 <sup>a</sup>	6.09 <sup>f</sup>	<6 <sup>g</sup>	<6 <sup>h</sup>	<6 <sup>i</sup>	5.14 <sup>b</sup>	<5 <sup>m</sup>	<5 <sup>p</sup>	7.0 <sup>q</sup>	6.4 <sup>i</sup>	<5 <sup>q</sup>	7.45 <sup>q</sup>
Sumatriptan		6.33 <sup>r</sup>	8.02 <sup>f</sup>		7.19 <sup>h</sup>					6.78 <sup>q</sup>	5.1 <sup>i</sup>		6.24 <sup>q</sup>
DP-5-CT	9.54 <sup>s</sup>	<5 <sup>r</sup>	7.24 <sup>s</sup>										
<b>Antagonists</b>													
Mesulergine	6.75 <sup>a</sup>	5.94 <sup>a</sup>	5.48 <sup>f</sup>			8.33 <sup>i</sup>	7.45 <sup>k</sup>	8.79 <sup>a</sup>		<6.0 <sup>q</sup>	<6.0 <sup>q</sup>	5.76 <sup>q</sup>	8.15 <sup>q</sup>
Metergoline	7.89 <sup>b</sup>	7.4 <sup>c</sup>	9.1 <sup>e</sup>	5.95 <sup>e</sup>	6.29 <sup>h</sup>	9.0 <sup>i</sup>	9.2 <sup>c</sup>	5.13 <sup>m</sup>		<6.0 <sup>q</sup>	<6.0 <sup>q</sup>	7.52 <sup>q</sup>	8.69 <sup>q</sup>
Mianserin	6.03 <sup>s</sup>	5.21 <sup>s</sup>	7.52 <sup>f</sup>				6.70 <sup>r</sup>					7.34 <sup>q</sup>	6.95 <sup>q</sup>
Clozapine	5.75 <sup>c</sup>					8.4 <sup>i</sup>	7.5 <sup>k</sup>	7.31 <sup>n</sup>				7.89 <sup>q</sup>	7.87 <sup>q</sup>
Ketanserin	5.86 <sup>s</sup>	5.72 <sup>s</sup>	6.00 <sup>s</sup>			8.30 <sup>r</sup>	7.55 <sup>r</sup>			4.8 <sup>q</sup>	5.8 <sup>i</sup>	<5 <sup>q</sup>	6.69 <sup>q</sup>
Methiothepin	7.1 <sup>e</sup>	7.89 <sup>r</sup>	9.23 <sup>f</sup>	6.68 <sup>e</sup>	<6 <sup>h</sup>	8.79 <sup>i</sup>	8.76 <sup>i</sup>			7.0 <sup>q</sup>	7.8 <sup>i</sup>	8.74 <sup>q</sup>	8.99 <sup>q</sup>
Methysergide	7.63 <sup>s</sup>	5.74 <sup>r</sup>	8.42 <sup>s</sup>		7.87 <sup>h</sup>					7.2 <sup>q</sup>	6.9 <sup>i</sup>	6.43 <sup>q</sup>	7.9 <sup>q</sup>
Ritanserin	6.08 <sup>a</sup>	5.76 <sup>a</sup>	6.39 <sup>a</sup>			8.51 <sup>i</sup>	8.29 <sup>k</sup>	5.23 <sup>m</sup>				7.36 <sup>q</sup>	7.66 <sup>q</sup>
Pindolol	7.71 <sup>s</sup>	6.82 <sup>r</sup>	<5.0 <sup>f</sup>	<6 <sup>g</sup>	<6 <sup>h</sup>				<5 <sup>p</sup>	4.7 <sup>q</sup>		<5.0 <sup>q</sup>	<6 <sup>q</sup>
Pimozide						8.10 <sup>j</sup>	<5 <sup>j</sup>					7.2 <sup>j</sup>	9.3 <sup>j</sup>
Ergotamine	8.37 <sup>s</sup>	8.69 <sup>s</sup>	10.11 <sup>r</sup>		7.28 <sup>h</sup>					8.4 <sup>q</sup>	8.5 <sup>q</sup>		7.49 <sup>q</sup>
DHE	8.91 <sup>s</sup>	8.38 <sup>r</sup>	10.19 <sup>r</sup>				6.84 <sup>f</sup>					7.88 <sup>q</sup>	6.82 <sup>q</sup>
WAY100635	8.42 <sup>d</sup>												

References: <sup>a</sup>Van Wijngaarden *et al.*, 1990; <sup>b</sup>Peroutka, 1986; <sup>c</sup>Wander *et al.*, 1987; <sup>d</sup>Khawaja *et al.*, 1995; <sup>e</sup>Hoyer & Schoeffer, 1991; <sup>f</sup>Herrick-Davies & Teitler, 1988; <sup>g</sup>McCallister *et al.*, 1992; <sup>h</sup>Lovenberg *et al.*, 1993b; <sup>i</sup>Hoyer *et al.*, 1986b; <sup>j</sup>Roth *et al.*, 1994; <sup>k</sup>Wainscott *et al.*, 1996; <sup>l</sup>Hoyer *et al.*, 1985a; <sup>m</sup>Nelson & Thomas, 1989; <sup>n</sup>Schmidt & Peroutka, 1990; <sup>p</sup>Grossman *et al.*, 1993; <sup>q</sup>Hoyer *et al.*, 1994; <sup>r</sup>Boess & Martin, 1994; <sup>s</sup>Hoyer *et al.*, 1988. All data is represented as pK<sub>i</sub> values except reference <sup>d</sup> (pIC<sub>50</sub>). Values are reported for rat receptors, except references; <sup>a</sup>pig (5-HT<sub>2C</sub>) only; <sup>f</sup>human recombinant receptor; <sup>n</sup>guinea pig. DHE = dihydroergotamine.

label a population of native 5-HT<sub>7</sub> receptors has proved difficult. Studies have been performed using the endogenous ligand, 5-HT, as a radiolabel. 5-HT has similar affinity for a variety of 5-HT receptors (Table 5.2) and therefore selective binding to 5-HT<sub>7</sub> receptors requires the use of masking agents to block binding to other 5-HT receptor sub-types. Previous studies have used this ligand (in the presence of the 5-HT<sub>1A/1B</sub> and  $\beta$ -adrenoreceptor ligand ( $\pm$ )-pindolol to reduce non-5-HT<sub>7</sub> receptor ligand binding) in an attempt to label native 5-HT<sub>7</sub> receptors in rat hypothalamus. Shallow competition curves for a number of compounds were indicative of labelling a heterogeneous population of binding sites in these studies (Sleight *et al.*, 1995a; Gobbi *et al.*, 1996). Indeed a more recent study using [<sup>3</sup>H]5-HT in the presence of 3 $\mu$ M pindolol, a saturating concentration to block all 5-HT<sub>1A/1B</sub> binding, again revealed very low Hill coefficients for some compounds confirming the non-specific labelling of 5-HT<sub>7</sub> receptors (Clemett *et al.*, 1999).

The higher potency of 5-CT in functional models (Leung *et al.*, 1996; Lovenberg *et al.*, 1993a; Martin & Wilson, 1995; Terrón, 1996, 1997a & 1997b; Terrón & Falcón-Neri, 1999), in conjunction with its higher affinity at 5-HT<sub>7</sub> receptors, high affinity at 5-HT<sub>1A/1B/1D</sub> and 5-HT<sub>5A</sub> receptors and low affinity at other 5-HT receptors (5-HT<sub>1E/1F/2A/2C/4/5B</sub> and 5-HT<sub>6</sub> receptors; Table 5.2) suggest that the radioligand binding of this ligand in comparison to [<sup>3</sup>H]5-HT might be less complex (Hoyer *et al.*, 1994). The use of this relatively more selective radioligand in the presence of (-)-cyanopindolol (1 $\mu$ M) and sumatriptan (1 $\mu$ M), to block binding to 5-HT<sub>1A</sub> and 5-HT<sub>1B/1D</sub> receptors, labelled a single population of receptors in guinea-pig cerebral cortex membranes, which displayed a pharmacological profile broadly comparable to recombinant 5-HT<sub>7</sub> receptors (To *et al.*, 1995; Boyland *et al.*, 1995; Table 5.3). [<sup>3</sup>H]5-CT binding in rat cerebral cortex homogenates, using the same masking conditions, was to a heterogeneous receptor population, as revealed by shallow competition curves for some compounds (eg. methiothepin; Boyland *et al.*, 1995; Table 5.3). In a study using rat whole brain membranes [<sup>3</sup>H]5-CT in the presence of 10 $\mu$ M pindolol and 100nM WAY100635 was used to label a homogenous population of receptors which correlated well to binding at the 5-HT<sub>7</sub> receptor (Stowe & Barnes, 1998b; Tables 5.1, 5.2 & 5.3). Using such masking agents, [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors would be blocked. 5-CT

**Table 5.3: Summary of Assay Conditions Used to Label 5-HT<sub>7</sub> Receptors in Mammalian Brain.**

Radioligand and Masking Compounds	NSB-defining compound	Receptors visualised	Anatomical Localisation	Technique	Reference
[ <sup>3</sup> H]5-HT	100nM Pindolol*	5-HT <sub>7</sub>	rat hypothalamus	radioligand binding	Sleight <i>et al.</i> , 1995a
[ <sup>3</sup> H]5-HT	3μM Pindolol	heterogeneous	rat hypothalamus	radioligand binding	Gobbi <i>et al.</i> , 1996
[ <sup>3</sup> H]5-HT	3μM Pindolol	heterogeneous	rat hypothalamus	radioligand binding	Clemett <i>et al.</i> , 1999
[ <sup>3</sup> H]5-HT	10μM 5-HT	heterogeneous	rat hypothalamus	radioligand binding	Stowe & Barnes, 1996, 1998a
10μM pindolol, 1 μM sumatriptan	1μM 5-HT	heterogeneous	rat cortex	radioligand binding	Boylard <i>et al.</i> , 1995
[ <sup>3</sup> H]5-HT	1μM 5-HT	5-HT <sub>7</sub>	guinea-pig cortex	radioligand binding	
1μM cyanopindolol, 1μM sumatriptan	1μM 5-HT	5-HT <sub>7</sub>	guinea-pig cortex	radioligand binding	To <i>et al.</i> , 1995
1μM cyanopindolol, 1μM sumatriptan	1μM 5-HT	5-HT <sub>7</sub>	guinea-pig cortex	radioligand binding autoradiography	
1μM cyanopindolol, 1μM sumatriptan	10μM 5-HT	heterogeneous	human cortex	radioligand binding	Barnes <i>et al.</i> , 1997
10μM pindolol, 1μM sumatriptan	10μM 5-HT	5-HT <sub>7</sub> >> 5-HT <sub>5A, 5B</sub> **	rat brain	autoradiography	Gustafson <i>et al.</i> , 1996
[ <sup>3</sup> H]5-HT	10μM 5-HT	5-HT <sub>7</sub>	guinea-pig brain	autoradiography	Waeber & Moskowitz, 1995a
30nM PAPP, 160nM pindolol ***	10μM 5-HT	Heterogeneous (mainly 5-HT <sub>7</sub> )	rat and guinea-pig brain	autoradiography	Mengod <i>et al.</i> , 1996
[ <sup>3</sup> H]5-HT	10μM 5-HT	5-HT <sub>7</sub>	rat whole brain	radioligand binding	Stowe & Barnes, 1998b
100μM WAY100135, 250μM GR127935	10μM 5-HT	5-HT <sub>7</sub>	rat whole brain	radioligand binding	
[ <sup>3</sup> H]5-HT	10μM 5-HT	5-HT <sub>7</sub>	rat whole brain, guinea-pig ileum rat jejunum	radioligand binding	Hemedah <i>et al.</i> , 1999
10μM pindolol, 100nM WAY100635 ****	1μM risperidone	5-HT <sub>7</sub>			
[ <sup>3</sup> H]Mesulergine*****					
30nM cianserin, 3μM RS 102221, 1μM raclopride, 0.1μM prazosin + 0.1μM yohimbine					

\* The insufficient block of 5-HT<sub>1A/1B</sub> binding by 100nM Pindolol, questions the effects reported after chronic fluoxetine treatment (Sleight *et al.*, 1995a). \*\* The affinity of 5-HT for 5-HT<sub>5A/5B</sub> receptors is three-fold lower than for 5-HT<sub>7</sub>; as [<sup>3</sup>H]5-HT was used at the K<sub>D</sub> for 5-HT<sub>7</sub> receptors, residual binding under these conditions is believed to represent binding mainly to 5-HT<sub>7</sub> receptors (Gustafson *et al.*, 1996). \*\*\* A binding profile similar to that of the 5-HT<sub>7</sub> receptor was determined by displacement of [<sup>3</sup>H]5-HT binding by spiperone, clozapine and methiothepin, but not by sumatriptan, CP122228, GR127935 or dihydroergotamine (Waeber & Moskowitz, 1995a). NSB – non-specific binding. \*\*\*\* First report of labelling of homogeneous population of 5-HT<sub>7</sub> receptors in rat brain. \*\*\*\*\* First use of an antagonist ligand.

also binds to 5-HT<sub>1D</sub> receptors with high affinity, although this receptor is in low abundance in the rat whole brain (Bruinvels *et al.*, 1993a & b). However in certain brain regions, such as the striatum, 5-HT<sub>1D</sub> receptors are in relatively greater abundance (Bruinvels *et al.*, 1993a & b). In such brain regions it is therefore unlikely that a homogeneous population of receptors would be identified using the masking agents employed by Stowe & Barnes (1998b). In this chapter, conditions were established for [<sup>3</sup>H]5-CT selective binding to the 5-HT<sub>7</sub> receptor in the individual brain areas of the cortex, hippocampus and striatum.

Despite the shortfalls of the blocks used in earlier experiments, [<sup>3</sup>H]5-CT has been used to autoradiographically visualise the 5-HT<sub>7</sub> receptor in rat and/or guinea pig brain (Waeber and Moskowitz, 1995a; Gustafson *et al.*, 1996). 5-HT<sub>7</sub> mRNA expression appears to correlate well with 5-HT<sub>7</sub> receptor binding densities (Lovenberg *et al.*, 1993a; Ruat *et al.*, 1993b; Shen *et al.*, 1993). Highest concentrations of 5-HT<sub>7</sub> mRNA exist in the hypothalamus, cortex, hippocampus, striatum, brainstem and thalamus of the rat brain, as demonstrated by Northern blot analysis and *in situ* hybridisation experiments (Lovenberg *et al.*, 1993a; Ruat *et al.*, 1993b; Shen *et al.*, 1993; Table 5.3). In this chapter, the 5-HT<sub>7</sub> binding assay conditions established and evaluated in membrane binding studies were also adapted for use in [<sup>3</sup>H]5-CT autoradiography experiments to assess the distribution of the 5-HT<sub>7</sub> receptor in the rat brain.

The 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors have become of particular interest recently, because of their apparent role in migraine and hypothesised roles in depression and diseases involving the basal ganglia (Saxena and Ferrari, 1989; Peroutka, 1990b; Waeber *et al.*, 1990; Moskowitz, 1992; Rebeck *et al.*, 1994;). 5-HT<sub>1B</sub> receptors are present in rat brain at much higher densities than 5-HT<sub>1D</sub> receptors (Bruinvels *et al.*, 1993), with 5-HT<sub>1D</sub> receptors reaching a maximum density in brain structures such as the substantia nigra, globus pallidus and caudate-putamen (Bruinvels *et al.*, 1993a & b). At present there are very few ligands that can selectively differentiate between 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors or these receptors and other 5-HT receptor sub-types. GR125,743 is one of the most recent 5-HT<sub>1B/1D</sub> receptor antagonists with a high affinity and marked selectivity over other 5-HT receptor subtypes (Scopes *et al.*, 1994). The radiolabelled form of GR125,743 has been successfully used to

characterise human and guinea pig 5-HT<sub>1B/1D</sub> receptors (Audinot *et al.*, 1997; Doménech *et al.*, 1997). Finally, the binding of [<sup>3</sup>H]GR125,743 in rat cortex, hippocampus and striatum membranes was also characterised in this chapter.

## 5.1 Methods

[<sup>3</sup>H]8-OH DPAT binding assays, to label 5-HT<sub>1A</sub> receptors, were conducted as described in chapter 3, adapting a previously reported method (Gozlan *et al.*, 1983). [<sup>3</sup>H]5-CT binding assays, to label 5-HT<sub>7</sub> receptors, were conducted as described in chapter 3. As [<sup>3</sup>H]5-CT is a non-selective ligand, the use of masking agents to block binding to non-5-HT<sub>7</sub> receptors was investigated. The rationale for the use of such blocks is described throughout the appropriate results sections. In order to visualise the distribution of 5-HT<sub>7</sub> receptors, [<sup>3</sup>H]5-CT autoradiography experiments were performed using the masking agents that would block non-5-HT<sub>7</sub> receptor membrane binding. [<sup>3</sup>H]5-CT autoradiography experiments were performed using adult male Wistar Cobb rats (200-300g; bred in-house; Department of Neuroscience, University of Edinburgh), as described in chapter 3. [<sup>3</sup>H]GR125,743 binding to label 5-HT<sub>1B/1D</sub> receptors was performed as described in chapter 3.

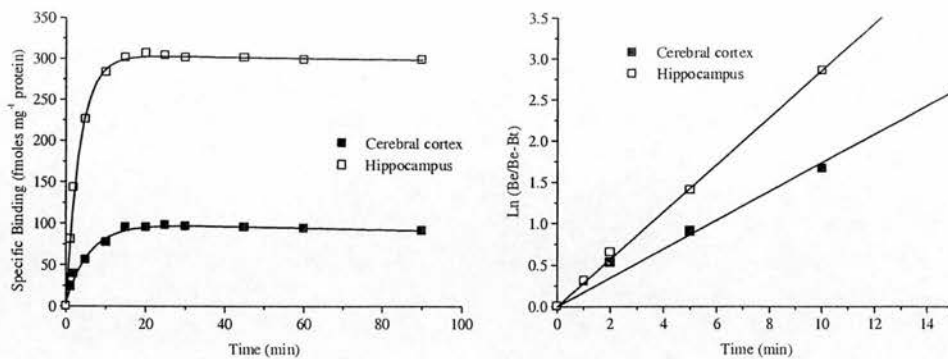
## 5.2 Results

### 5.2.1 [<sup>3</sup>H]8-OH DPAT Binding Assays

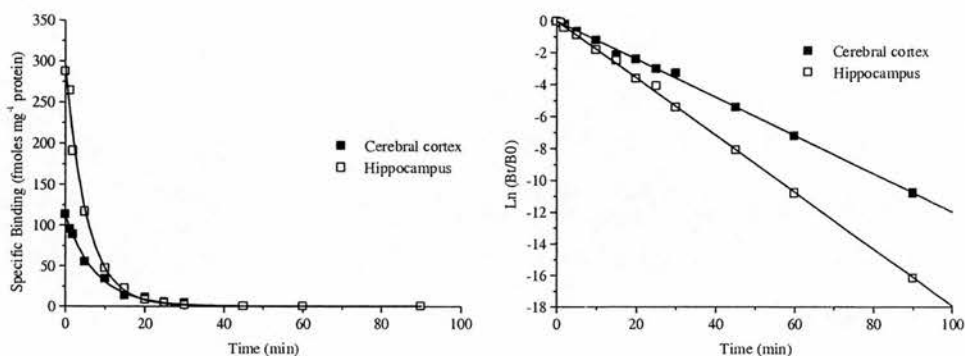
#### 5.2.1.1 Time Course of [<sup>3</sup>H]8-OH DPAT Binding to Rat Cerebral Cortex and Hippocampus Membranes

A time course (0-90 min) of [<sup>3</sup>H]8-OH DPAT (0.25nM) binding to rat cerebral cortex and hippocampus membranes was carried out at 37°C. Equilibrium was attained by 15 min being stable for at least another 75 min (Figure 5.1a). Curve fitting using non-linear regression gave an observed constant ( $k_{\text{obs}}$ ) of  $0.189 \pm 0.0118 \text{ min}^{-1}$  with a  $t_{1/2}$  of  $6.46 \pm 1.31 \text{ min}$  ( $n=3$ ) in cerebral cortex membranes, and an observed constant ( $k_{\text{obs}}$ ) of  $0.2977 \pm 0.046 \text{ min}^{-1}$  ( $n=3$ ) with a  $t_{1/2}$  of  $3.75 \pm 0.29 \text{ min}$  ( $n=3$ ) in hippocampus membranes. Dissociation was initiated after addition of 10 $\mu$ M 5-HT following incubation for 30 min at 37°C, and followed first order kinetics with a  $t_{1/2}$  of  $6.94 \pm 0.37$  and  $4.54 \pm 0.60 \text{ min}$  (Figure 5.1b) and a dissociation rate constant

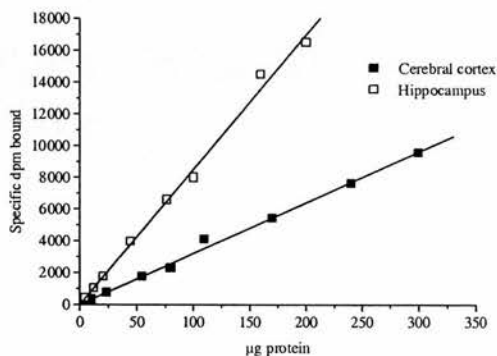
(a)



(b)



(c)



**Figure 5.1: Time Course & Protein Linearity of [<sup>3</sup>H]8-OH DPAT Binding.**

The data represent typical (a) time course, and (b) dissociation experiments, and (c) a single protein linearity experiment. Graphs on the right hand side, represent the semilogarithmic transformations of the data. Experiments were performed as described in the text, with mean data obtained from three experiments also in text.



( $k_{-1}$ ) of  $0.139 \pm 0.002$  and  $0.210 \pm 0.021 \text{ min}^{-1}$  ( $n=3$ ) in cerebral cortex and hippocampus membranes respectively. Subsequent calculation gave an association rate constant ( $k_{+1}$ ) of  $0.211 \pm 0.033$  and  $0.3832 \pm 0.167 \text{ min}^{-1}\text{M}^{-1}$  in cerebral cortex and hippocampus membranes respectively. The data from these time course experiments gave an equilibrium constant ( $K_D$ ) of  $0.692 \pm 0.127$  and  $0.995 \pm 0.586 \text{ nM}$  in cerebral cortex and hippocampus membranes respectively. In the following competition experiments [ $^3\text{H}$ ]8-OH DPAT ( $0.25\text{nM}$ ) binding to rat cortex and hippocampus membranes was carried out at equilibrium at  $37^\circ\text{C}$  for 30 min.

### **5.2.1.2 Effect of Membrane Protein Concentration on [ $^3\text{H}$ ]8-OH DPAT Binding to Rat Cerebral Cortex and Hippocampus Membranes**

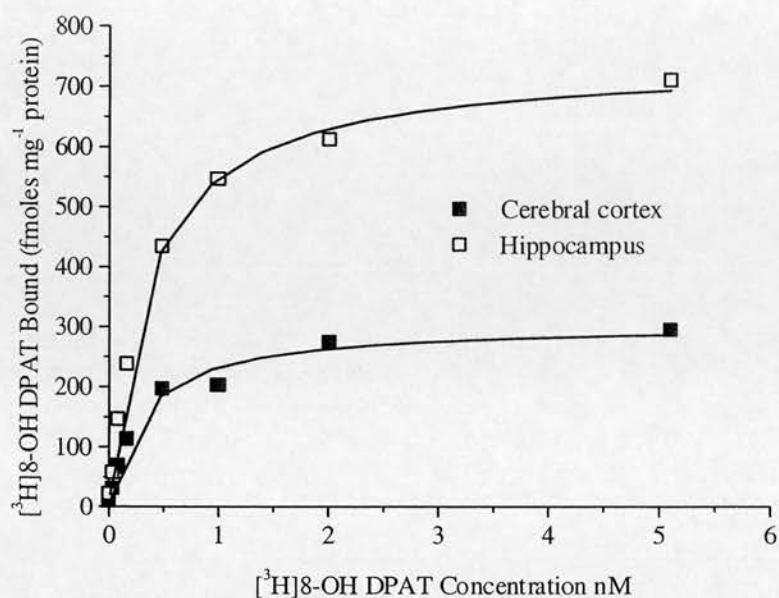
Specific [ $^3\text{H}$ ]8-OH DPAT binding was dependent on membrane protein (Figure 5.1c) and was linear up to the concentrations measured ( $\sim 300\mu\text{g}$  protein/assay). Subsequent experiments were conducted with  $40\text{-}160\mu\text{g}$  cerebral cortex or  $10\text{-}60\mu\text{g}$  hippocampus membrane protein, to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

### **5.2.1.3 Concentration Dependence of [ $^3\text{H}$ ]8-OH DPAT Binding to Rat Cerebral Cortex and Hippocampus Membranes**

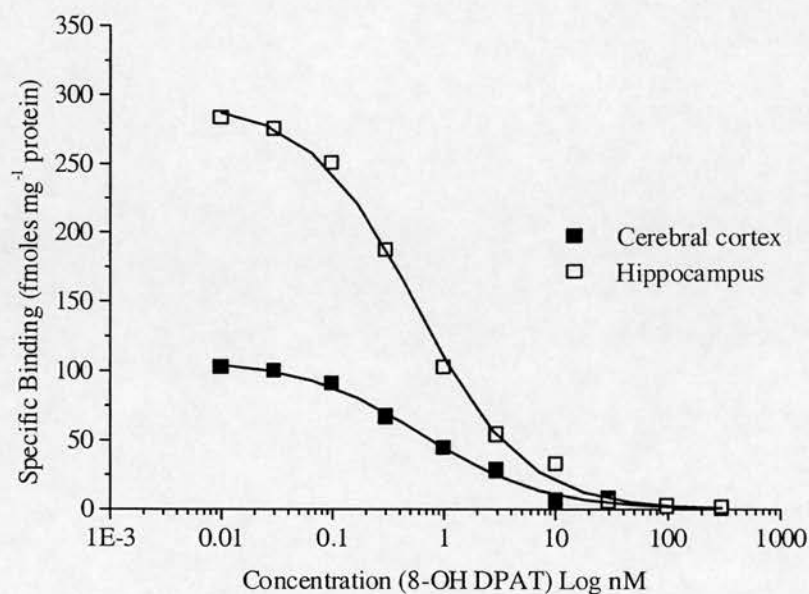
Hot saturation analysis of [ $^3\text{H}$ ]8-OH DPAT binding to rat cerebral cortex and hippocampus membranes was carried out using increasing concentrations of [ $^3\text{H}$ ]8-OH DPAT (Figure 5.2a). Curve fitting using a logistic model gave an equilibrium dissociation constant ( $K_D$ ) of  $0.41 \pm 0.13 \text{ nM}$  and a binding site density ( $B_{\text{max}}$ ) of  $0.29 \pm 0.02 \text{ pmol mg}^{-1} \text{ protein}$  ( $n=3$ ) in cerebral cortex membranes. In hippocampus membranes hot saturation analysis gave a similar dissociation constant ( $K_D$ ) of  $0.45 \pm 0.17 \text{ nM}$  and a higher binding site density ( $B_{\text{max}}$ ) of  $0.75 \pm 0.08 \text{ pmol mg}^{-1} \text{ protein}$  ( $n=3$ ). Hill Slopes of  $0.91 \pm 0.08$  and  $1.06 \pm 0.03$  in hippocampus and cortex membranes ( $n=3$ ), were consistent with binding to one population of binding sites.

Competition binding studies using  $0.25\text{nM}$  [ $^3\text{H}$ ]8-OH DPAT and increasing concentrations of unlabelled 8-OH DPAT ( $0.003\text{-}100\text{nM}$ ) (Figure 5.2b), gave  $K_D$  values of  $0.69 \pm 0.34$  and  $0.37 \pm 0.13 \text{ nM}$  and  $B_{\text{max}}$  values of  $0.41 \pm 0.11$  and  $0.74 \pm$

(a)



(b)



**Figure 5.2: Concentration Dependence of Specific [<sup>3</sup>H]8-OH DPAT Binding.**

The results represent (a) hot saturation and (b) cold saturation experiments with each point performed in duplicate. See text for mean values.

0.13 pmol mg<sup>-1</sup> protein with Hill coefficients of  $0.87 \pm 0.10$  and  $1.00 \pm 0.03$  (n=3) in cerebral cortex and hippocampus membranes respectively. This was in reasonable agreement with data obtained from both 'hot' saturation and time course studies.

#### **5.2.1.4 Pharmacology of [<sup>3</sup>H]8-OH DPAT Binding Sites in Rat Cerebral Cortex and Hippocampus Membranes**

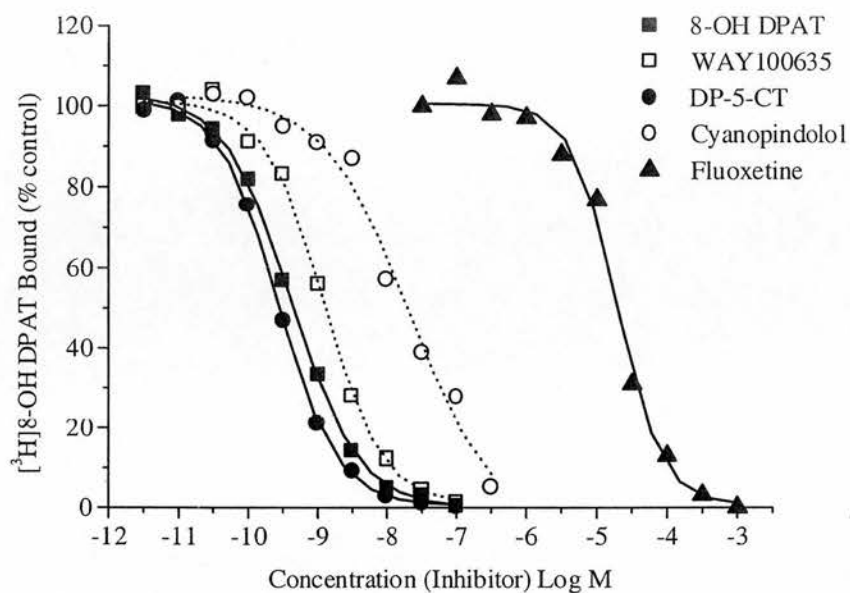
The pharmacology of [<sup>3</sup>H]8-OH DPAT binding was assessed in both cerebral cortex and hippocampus membranes using 8-OH DPAT (5-HT<sub>1A</sub> agonist), WAY100635 (5-HT<sub>1A</sub> antagonist), DP-5-CT (5-HT<sub>1A/1B</sub> agonist) and cyanopindolol (5-HT<sub>1A/1B</sub> antagonist). The rank order of affinity was the same in both membrane preparations: DP-5-CT > 8-OH DPAT > WAY100635 > cyanopindolol > any of the antidepressants and MDMA tested (Table 5.4; Figure 5.3). The affinity values were not significantly different between the two brain areas (P>0.05) and agreed with published binding at 5-HT<sub>1A</sub> receptors. Furthermore the Hill slopes of each compound were not significantly different from unity (P>0.05; Table 5.4), suggesting binding to a single population of binding sites.

### **5.2.2 [<sup>3</sup>H]5-CT Binding Assays**

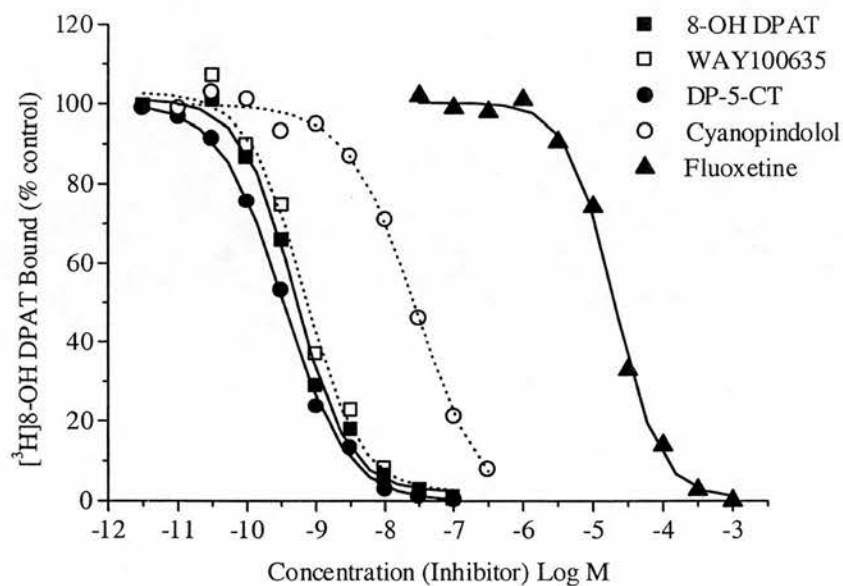
#### **5.2.2.1 Time Course of [<sup>3</sup>H]5-CT Binding to Rat Cerebral Cortex Membranes**

A time course (0-90 min) of [<sup>3</sup>H]5-CT (0.25nM) binding to rat cerebral cortex membranes was carried out at 25°C, with equilibrium attained by 30 min being stable for at least another 60 min (Figure 5.4a). Curve fitting using non-linear regression gave an observed constant ( $k_{obs}$ ) of  $0.130 \pm 0.008$  min<sup>-1</sup> (n=3) with a  $t_{1/2}$  of  $5.044 \pm 0.021$  min (n=3). Dissociation was initiated after addition of 10µM 5-HT following incubation for 60 min at 25°C, and followed first order kinetics with a  $t_{1/2}$  of  $7.575 \pm 0.202$  min (Figure 5.4b) and a dissociation rate constant ( $k_{-1}$ ) of  $0.094 \pm 0.002$  min<sup>-1</sup> (n=3). Subsequent calculation gave an association rate constant ( $k_{+1}$ ) of  $0.148 \pm 0.032$  min<sup>-1</sup>M<sup>-1</sup>. The data from these time course experiments gave an equilibrium constant ( $K_D$ ) of  $0.684 \pm 0.124$  nM. In the following competition experiments

(a)



(b)



**Figure 5.3: Inhibition of  $[^3\text{H}]8\text{-OH DPAT}$  Binding.**

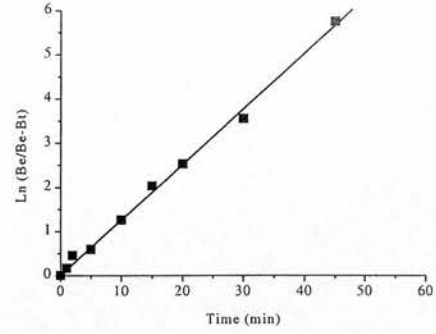
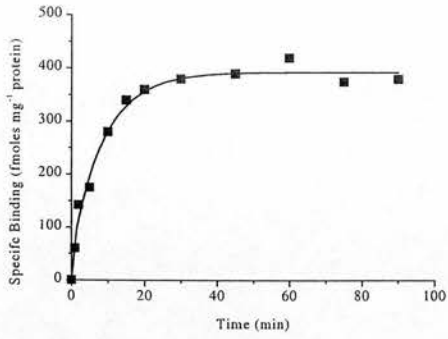
The data represent a typical experiment in (a) cerebral cortex & (b) hippocampus membranes. Mean data from at least three different experiments (Table 5.4).

	Cerebral Cortex Membranes			Hippocampus Membranes		
	n	pK <sub>i</sub>	nH	n	pK <sub>i</sub>	nH
<u>5-HT<sub>1A</sub></u>						
<u>Receptor Drugs</u>						
8-OH DPAT	3	9.28 ± 0.22	0.87 ± 0.10	3	9.50 ± 0.19	1.00 ± 0.03
WAY100635	3	9.14 ± 0.03	1.02 ± 0.03	3	9.14 ± 0.20	1.06 ± 0.05
DP-5-CT	3	9.73 ± 0.05	1.06 ± 0.06	3	9.79 ± 0.03	1.10 ± 0.07
Cyanopindolol	3	8.05 ± 0.04	0.99 ± 0.09	3	8.02 ± 0.14	0.98 ± 0.02
<u>Antidepressants</u>						
Citalopram	3	4.93 ± 0.02	0.96 ± 0.02	3	5.00 ± 0.06	1.00 ± 0.07
Fluoxetine	3	4.62 ± 0.01	1.07 ± 0.05	3	4.59 ± 0.06	1.06 ± 0.02
Tianeptine*	3	No affinity		3	No affinity	
<u>Neurotoxin</u>						
MDMA	3	5.18 ± 0.02	0.93 ± 0.01	3	5.16 ± 0.02	0.99 ± 0.04

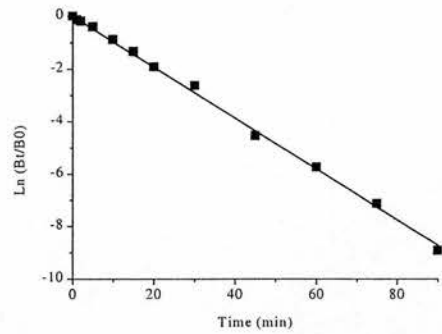
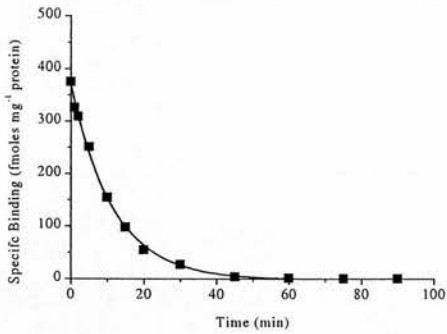
**Table 5.4: Pharmacology of [<sup>3</sup>H]8-OH DPAT Binding in Rat Cerebral Cortex and Hippocampus Membranes.**

The affinity values were determined from the inhibition of 0.25nM [<sup>3</sup>H]8-OH DPAT binding to rat cerebral cortex and hippocampus membranes. The results are expressed as pK<sub>i</sub> values and Hill slopes (nH) for a logistic fit. Values shown are the mean ± s.e.mean. \* = tianeptine tested up to 1mM.

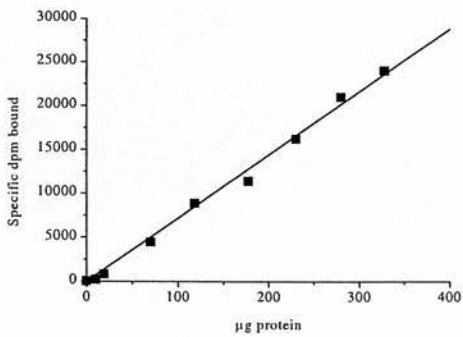
(a)



(b)



(c)



**Figure 5.4: Time Course and Protein Linearity of [<sup>3</sup>H]5-CT Binding.**

The data represent typical (a) time course, and (b) dissociation experiments, and (c) a single protein linearity experiment. Graphs on the right hand side, represent the semilogarithmic transformations of the data. Experiments were performed as described in the text, with mean data obtained from three experiments also in text.

[<sup>3</sup>H]5-CT (0.25nM) binding to rat cortex membranes was carried out at equilibrium at 25°C for 60 min.

### **5.2.2.2 Effect of Membrane Protein Concentration on [<sup>3</sup>H]5-CT Binding to Rat Cerebral Cortex Membranes**

Specific [<sup>3</sup>H]5-CT binding was dependent on membrane protein (Figure 5.4c) and was linear up to the concentrations measured (~300µg protein/assay). Subsequent experiments were conducted with 25-50µg membrane protein to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

### **5.2.2.3 Concentration Dependence of [<sup>3</sup>H]5-CT Binding to Rat Cerebral Cortex Membranes**

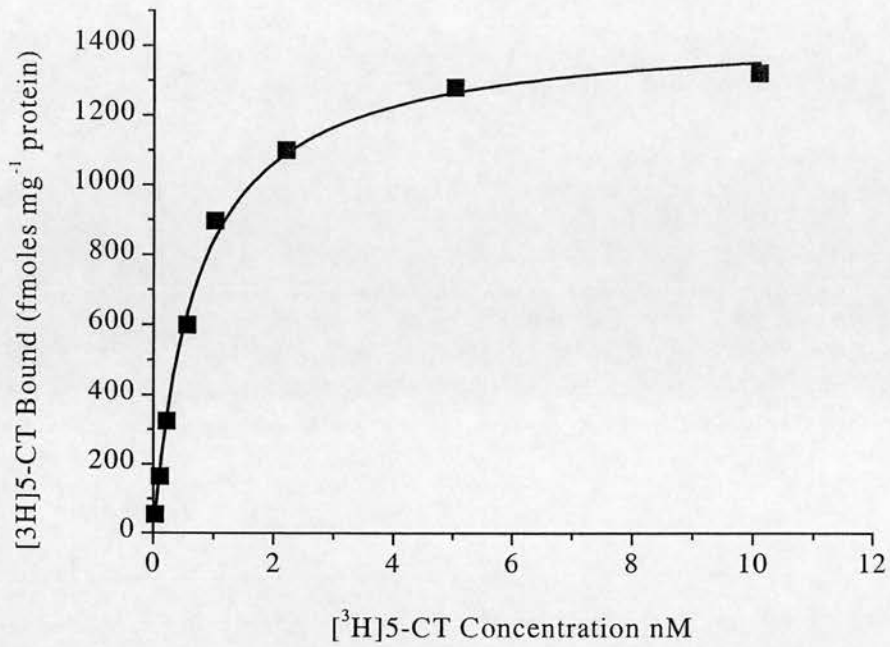
Hot saturation analysis of [<sup>3</sup>H]5-CT binding to rat cerebral cortex was carried out using increasing concentrations of [<sup>3</sup>H]5-CT (Figure 5.5a). Curve fitting using a logistic model gave an equilibrium dissociation constant ( $K_D$ ) of  $0.57 \pm 0.09$  nM, binding site density ( $B_{max}$ ) of  $1.48 \pm 0.22$  pmol mg<sup>-1</sup> protein and a Hill slope of  $0.92 \pm 0.11$  (n=3). This Hill slope was not significantly different from unity ( $P>0.05$ ), and is consistent to binding to one population of binding sites or a number of receptors with similar affinity.

Competition binding studies using 0.25nM [<sup>3</sup>H]5-CT and increasing concentrations of unlabelled 5-CT (0.01-300nM) (Figure 5.5b), gave a  $K_D$  of  $0.58 \pm 0.02$ nM and a  $B_{max}$  of  $1.33 \pm 0.08$  pmol mg<sup>-1</sup> protein with a Hill coefficient of  $1.01 \pm 0.05$  (n=12). This is in reasonable agreement with data obtained from both 'hot' saturation and time course studies.

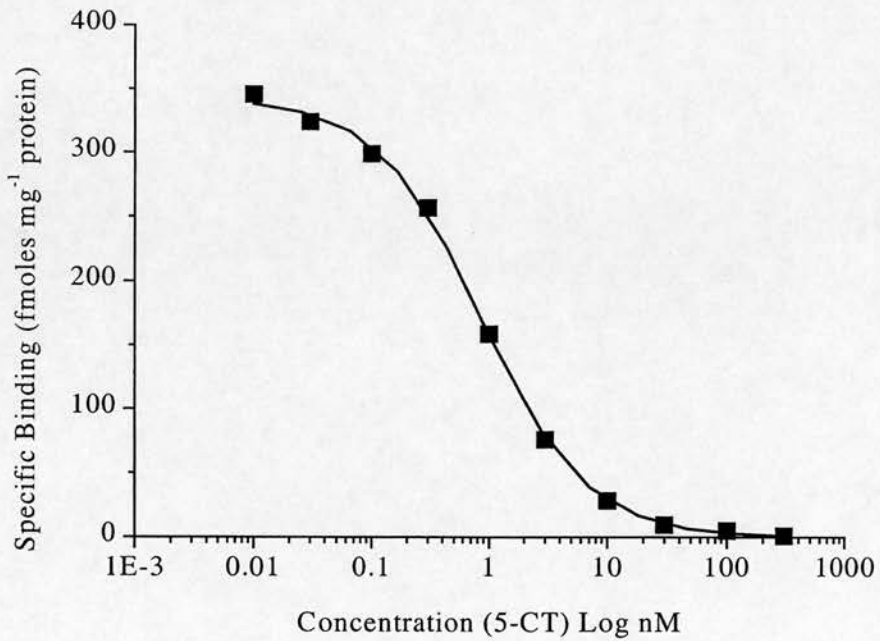
### **5.2.2.4 Pharmacological Profile of [<sup>3</sup>H]5-CT Binding Sites in Rat Cortex Membranes: Blockade of the 5-HT<sub>1A</sub> Component of Binding**

Competition studies using [<sup>3</sup>H]5-CT were used in conjunction with drugs that have high affinity at 5-HT<sub>1A</sub> receptors, to block the 5-HT<sub>1A</sub> component of [<sup>3</sup>H]5-CT binding. Unlabelled 5-CT was included in each experiment to enable calculations of

(a)



(b)



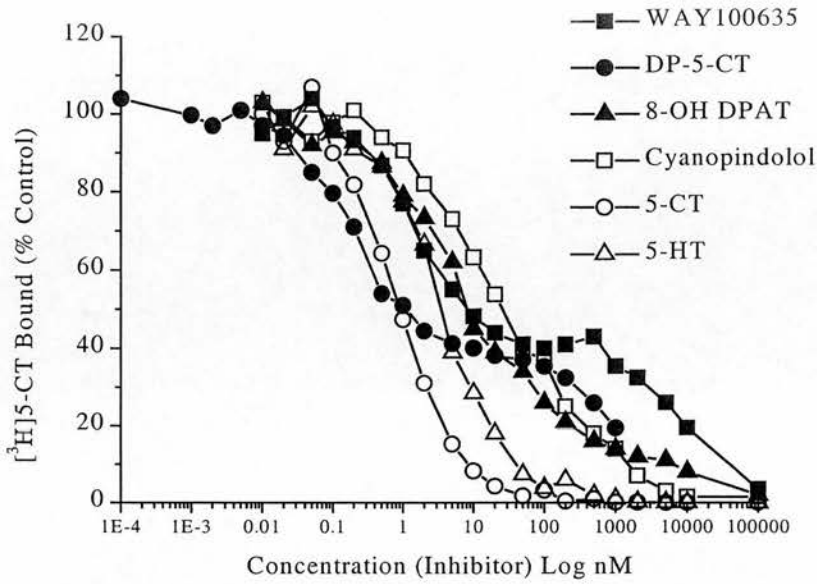
**Figure 5.5: Concentration Dependence of Specific [<sup>3</sup>H]5-CT Binding.**

The results represent (a) hot saturation and (b) cold saturation experiments with each point performed in duplicate. See text for mean values.

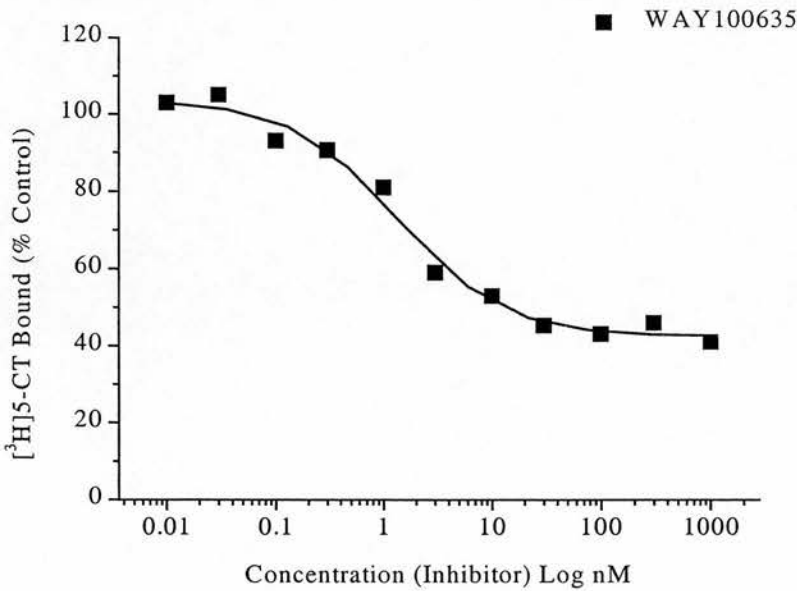


$pK_i$  values. Both 5-CT and 5-HT inhibited binding fully with Hill slopes not different from unity and best fitted a one-site model of binding (Figure 5.6a; Table 5.5). Cyanopindolol (5-HT<sub>1A/1B</sub> antagonist), DP-5-CT (5-HT<sub>1A/1B</sub> agonist), 8-OH DPAT (5-HT<sub>1A</sub> agonist), and WAY100635 (5-HT<sub>1A</sub> antagonist) all inhibited binding fully when used at concentrations up to 100 $\mu$ M (Figure 5.6a). However curves gave Hill slopes significantly less than 1, and best fit a two site model of binding (Table 5.5). The proportion of high affinity sites was approximately 60% for DP-5-CT, Cyanopindolol, WAY100635 and 8-OH DPAT, with approximately 40% constituting the low affinity site (Table 5.5). The affinities of the high affinity site for each of the compounds tested is indicative of binding at 5-HT<sub>1A</sub> receptors (Table 5.5). The small difference (<200 fold) in IC<sub>50</sub> values between the high and low affinity sites for cyanopindolol and 8-OH DPAT, as indicated by moderately low Hill slopes (0.60-0.80), made it difficult to graphically determine a concentration that could be used to only block the high affinity component of binding, i.e. binding to 5-HT<sub>1A</sub> receptors. On the otherhand for DP-5-CT and WAY100635, the larger separation of IC<sub>50</sub>s (>1000 fold) between the high and low affinity sites, as indicated by low Hill slopes (<0.60), made it much easier to graphically determine a concentration that could be used to block the high affinity component of binding. Whereas DP-5-CT started to plateau between 5 and 50nM, WAY100635 reached a more obvious plateau between 100nM and 1 $\mu$ M due to the greater IC<sub>50</sub> separation of high and low affinity binding sites (Figure 5.6a). Inhibition binding curves were subsequently generated for WAY100635 between 0.01nM and 1 $\mu$ M. Analysis of this WAY100635 sensitive component of [<sup>3</sup>H]5-CT binding revealed one population of binding sites, with a  $pK_i$  value of  $8.91 \pm 0.02$ , indicative of binding to 5-HT<sub>1A</sub> receptors (Table 5.5). The inhibition binding profile of WAY100635 reached a plateau between 100nM and 1 $\mu$ M (Figure 5.6b). At the level of the plateau, [<sup>3</sup>H]5-CT binding was reduced by  $61.65 \pm 0.99\%$  (n=12). In subsequent experiments 200nM WAY100635 was used to block binding to 5-HT<sub>1A</sub> receptors and to monitor the level of the 5-HT<sub>1A</sub> receptor block within and between experiments.

(a)



(b)



**Figure 5.6: The Effect of 5-HT<sub>1A</sub> Receptor Ligands on Inhibition of [3H]5-CT Binding in Rat Cortex Membranes.**

The data represent a typical experiment (a) single points and (b) duplicate points with mean data from at least three different experiments (Table 5.5)

**Table 5.5: Inhibition of [<sup>3</sup>H]5-CT Binding to Rat Cortex Membranes by 5-HT<sub>1A</sub> Receptor Ligands.**

Competing ligand	n	Single site fit		Two site fit	
		pK <sub>i</sub>	nH	pIC <sub>50</sub>	%
5-CT	12	9.23 ± 0.02	0.98 ± 0.02		
			High	9.07 ± 0.02	99.20 ± 0.08
			Low	5.81 ± 0.25	1.03 ± 0.54
5-HT	8	8.53 ± 0.03	0.99 ± 0.03		
			High	8.46 ± 0.11	97.45 ± 2.51
			Low	6.14 ± 0.20	3.21 ± 2.12
DP-5-CT*	8	8.53 ± 0.04	0.30 ± 0.02		
			High	9.69 ± 0.15	61.25 ± 2.51
			Low	5.93 ± 0.21	40.75 ± 1.53
WAY100635*	12	8.43 ± 0.02	0.69 ± 0.11		
			High	8.81 ± 0.16	61.30 ± 2.51
			Low	4.05 ± 0.25	40.27 ± 2.58
8-OH DPAT*	8	8.07 ± 0.04	0.67 ± 0.03		
			High	8.76 ± 0.25	65.23 ± 3.28
			Low	6.49 ± 0.31	37.85 ± 4.21
Cyanopindolol*	8	7.69 ± 0.17	0.76 ± 0.02		
			High	8.20 ± 0.21	58.25 ± 3.52
			Low	6.46 ± 0.28	39.71 ± 5.65

Analysis of data up to 1μM				%Inhibition by 200nM	
WAY100635	12	8.91 ± 0.02	1.00 ± 0.03	61.65 ± 0.99	

The affinity values were determined from the inhibition of 0.25nM [<sup>3</sup>H]5-CT to rat cerebral cortex membranes. The results are expressed as pK<sub>i</sub> values and Hill slope for a one site fit, and for a two site fit the pIC<sub>50</sub> and % contribution of each component is given. Values shown are the mean ± s.e.mean. \* Statistically better at fitting a two site model (F test, p <0.0001)

### 5.2.2.5 Pharmacological Profile of [<sup>3</sup>H]5-CT Binding Site in Rat Cortex Membranes in the Presence of 200nM WAY100635

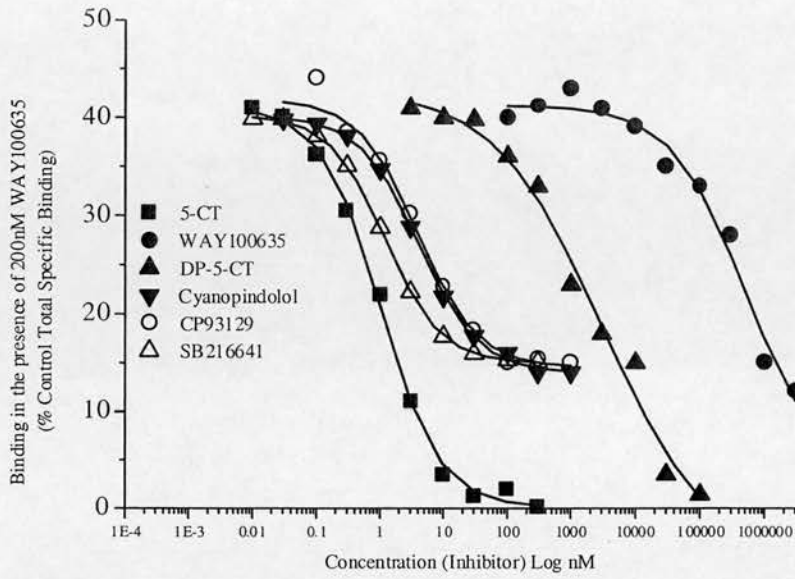
Using conditions which allowed blockade of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> receptors (above), the proportion of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors in rat cortex membranes was determined. Unlabelled 5-CT was included in each experiment to enable calculations of pK<sub>i</sub> values.

A number of drugs with varying degrees of selectivity for these and other 5-HT receptor subtypes were tested up to concentrations of 100μM to 1mM. The non-selective agonists, 5-CT and 5-HT, fully inhibited this remaining binding with Hill slopes of 1 and with similar affinities as in the absence of 200nM WAY100635 (Table 5.6). The non-selective antagonist metergoline, also completely inhibited WAY100635 insensitive [<sup>3</sup>H]5-CT binding with a Hill slope of 1 (Table 5.6). The other non-selective drugs tested, all completely inhibited WAY100635 insensitive [<sup>3</sup>H]5-CT binding but with Hill slopes of <1 and with a rank order of potency of: Metergoline > Methysergide > Mianserin > Ketanserin > Clozapine > 8-OH DPAT > ritanserin (Table 5.6).

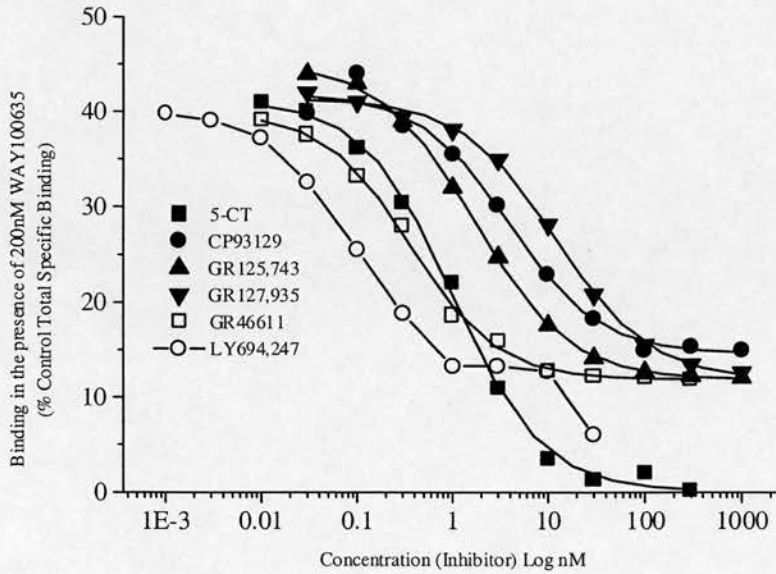
For those drugs which have a reported selectivity for 5-HT<sub>1B</sub> (DP-5-CT, SB216642, CGS12066B & CP93129) or 5-HT<sub>1D</sub> receptors (sumatriptan, PAPP, BRL15572, LY694,247 & GR46611) or a combination of these receptors (GR125,743, GR127,935 & GR85548), the WAY100635 insensitive component of [<sup>3</sup>H]5-CT binding was almost completely inhibited with Hill slopes of <1 (data not shown). However when some of these inhibition curves were fitted to a one site logistic model up to concentrations of 1μM, varying amounts of the inhibition of the WAY100635 insensitive component of [<sup>3</sup>H]5-CT binding was observed.

Drugs with reported selective affinities at 5-HT<sub>1B</sub> receptors (SB216641, CGS12066B & CP93129) all had Hill slopes of 1 and inhibited approximately 60% of the WAY100635 insensitive component of [<sup>3</sup>H]5-CT binding, which corresponds to approximately 85% of the total specific [<sup>3</sup>H]5-CT binding (Table 5.6). The 5-HT<sub>1A/1B</sub> antagonist, cyanopindolol also blocked approximately 60% of the remaining binding with a Hill slope of one. Figure 5.7a shows the binding of "selective" 5-HT<sub>1B</sub> drugs and the non-5-HT<sub>1A</sub> binding profile of WAY100635 and DP-5-CT in the

(a)



(b)



**Figure 5.7: Inhibition of  $[^3\text{H}]5\text{-CT}$  Binding in the Presence of 200nM WAY100635 (which blocks approximately 60% of control binding)**

The data represent a typical experiment (a) 5-HT<sub>1B</sub> ligands & WAY100635 and (b) 5-HT<sub>1B/1D</sub> ligands and the selective 5-HT<sub>1B</sub> agonist, CP93129 with mean data from at least three different experiments (Table 5.6).

**Table 5.6: Inhibition of [<sup>3</sup>H]5-CT Binding in the presence of 200nM WAY100635 to Rat Cortex Membranes by 5-HT Receptor Ligands.**

Competing ligand	n	pK <sub>i</sub>	nH	% Maximum	% Maximum
				Inhibition of WAY100635 Insensitive [ <sup>3</sup> H]5-CT Binding	Inhibition of Total Specific [ <sup>3</sup> H]5-CT Binding
LY694,247	5	10.84 ± 0.28	0.78 ± 0.09	100	100
GR46611*	3	9.63 ± 0.01	0.86 ± 0.13	73.28 ± 3.12	89.23 ± 2.51
5-CT	12	9.15 ± 0.03	1.01 ± 0.05	100	100
SB216642*	5	9.04 ± 0.06	1.11 ± 0.17	62.21 ± 2.28	84.79 ± 1.25
5-HT	3	8.76 ± 0.02	0.93 ± 0.08	100	100
GR125743*	8	8.73 ± 0.06	1.05 ± 0.03	72.19 ± 2.02	88.24 ± 0.55
Cyanopindolol*	8	8.56 ± 0.11	0.98 ± 0.04	58.24 ± 3.12	83.79 ± 1.25
CP93129*	7	8.47 ± 0.08	0.94 ± 0.03	61.23 ± 2.15	84.75 ± 0.75
Metergoline	3	8.08 ± 0.05	1.00 ± 0.08	100	100
GR127935*	8	8.05 ± 0.10	1.08 ± 0.06	73.41 ± 2.28	89.24 ± 0.98
GR85548*	4	7.90 ± 0.15	1.01 ± 0.06	74.56 ± 2.46	89.90 ± 0.94
Sumatriptan	3	7.50 ± 0.17	0.76 ± 0.03	100	100
CGS12066B*	3	7.22 ± 0.07	1.07 ± 0.03	57.28 ± 3.16	84.12 ± 1.28
Methysergide	3	6.95 ± 0.07	0.37 ± 0.05	100	100
PAPP	4	6.60 ± 0.09	0.76 ± 0.06	100	100
BRL15572	4	6.01 ± 0.19	1.17 ± 0.17	100	100
Mianserin	3	5.73 ± 0.10	0.69 ± 0.12	100	100
DP-5-CT	4	5.44 ± 0.28	0.84 ± 0.12	100	100
Ketanserin	3	5.32 ± 0.49	0.86 ± 0.03	100	100
Clozapine	3	5.29 ± 0.30	0.55 ± 0.07	100	100
8-OH DPAT	3	4.69 ± 0.30	0.48 ± 0.03	100	100
Ritanserin	3	3.32 ± 0.17	0.66 ± 0.11	100	100

The affinity values were determined from the inhibition of 0.25nM [<sup>3</sup>H]5-CT to rat cerebral cortex membranes in the presence of 200nM WAY100635. The results are expressed as pK<sub>i</sub> values and Hill slope for a one site logistic fit. Inhibition curves were fit up to concentrations of 100µM for all compounds except those indicated by \* which were fit up to concentrations of 1µM. The % maximum inhibition of total specific and WAY100635 insensitive [<sup>3</sup>H]5-CT binding are given. Values shown are the mean ± s.e.mean, of at least three experiments as shown by the n value.

presence of 200nM WAY100635. WAY100635 only inhibited at concentrations greater than 1 $\mu$ M, with an affinity close to that seen for the low affinity non-5-HT<sub>1A</sub> receptors previously. DP-5-CT inhibited binding fully, with a pK<sub>i</sub> value of 5.44, reflecting the low affinity site seen in the absence of WAY100635. CP93129 reached an inhibition of specific binding maximum at between 100 and 1000nM. 300nM CP93129 in the presence of 200nM WAY100635 was consequently used to determine the proportion of 5-HT<sub>1B</sub> and 5-HT<sub>1A</sub> receptors labelled and calculated to be 84.75  $\pm$  0.75% of total specific [<sup>3</sup>H]5-CT binding (n=7; Table 5.6). Taking into account the inhibition of total specific binding [<sup>3</sup>H]5-CT by WAY100635 (61.65  $\pm$  0.99%) the proportion of 5-HT<sub>1B</sub> receptors labelled by [<sup>3</sup>H]5-CT corresponded to 23.10%.

The majority of drugs tested with reported higher affinities at 5-HT<sub>1D</sub> receptors compared to 5-HT<sub>1B</sub> receptors inhibited WAY insensitive [<sup>3</sup>H]5-CT binding fully with Hill slopes of < 1 (Table 5.6). However, the inhibition curve for LY694,247 was clearly biphasic inhibiting 70 % of the binding up to a plateau of between 1 and 10nM, with a Hill slope of <1 when fit up to a concentration of 1 $\mu$ M (Figure 5.7b). GR46611, on the otherhand, only inhibited binding by approximately 70% with a Hill slope of < 1.

In contrast to the 5-HT<sub>1B</sub> selective drugs, the selective 5-HT<sub>1B/1D</sub> compounds with similar affinities at 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors (GR125,743, GR127,935 & GR85548) all inhibited approximately 70% of the WAY100635 insensitive component of [<sup>3</sup>H]5-CT binding with Hill slopes of 1, which corresponds to approximately 90% of the total specific [<sup>3</sup>H]5-CT binding (Table 5.6). Therefore drugs with a relatively high potency at 5-HT<sub>1B/1D</sub> receptors (and LY694,247 & GR46611), inhibited greater amounts of WAY100635 insensitive [<sup>3</sup>H]5-CT binding (approximately 10%), compared to those that only inhibited 5-HT<sub>1B</sub> receptor binding. Figure 5.7b shows the binding of "selective" 5-HT<sub>1B/1D</sub> drugs in the presence of 200nM WAY100635. A curve to CP93129 is included to emphasise the increased inhibition by 5-HT<sub>1B/1D</sub> drugs. The most potent of these drugs, GR125,743, reached an inhibition of binding maximum at between 100 and 1000nM. 200nM GR125,743 in the presence of 200nM WAY100635 was consequently used to determine the proportion of 5-HT<sub>1B/1D</sub> and 5-HT<sub>1A</sub> receptors labelled and calculated to be 88.24  $\pm$

0.55% of total specific [ $^3\text{H}$ ]5-CT binding ( $n=8$ ; Table 5.6). Taking into account the inhibition of total specific [ $^3\text{H}$ ]5-CT binding by WAY100635 ( $61.65 \pm 0.99\%$ ) the proportion of 5-HT<sub>1B/1D</sub> receptors labelled by [ $^3\text{H}$ ]5-CT corresponded to  $26.59 \pm 3.58\%$ . Consequently the proportion of 5-HT<sub>1D</sub> receptors labelled by [ $^3\text{H}$ ]5-CT corresponded to  $3.49 \pm 2.41\%$ .

Therefore the WAY100635 insensitive component of [ $^3\text{H}$ ]5-CT binding includes 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors and also another unknown component(s). This unknown component(s) represented  $11.76 \pm 0.55\%$  of total specific [ $^3\text{H}$ ]5-CT binding.

### **5.2.2.6 [ $^3\text{H}$ ]5-CT Binding in the Presence of 200nM WAY100635 & 200nM GR125,743**

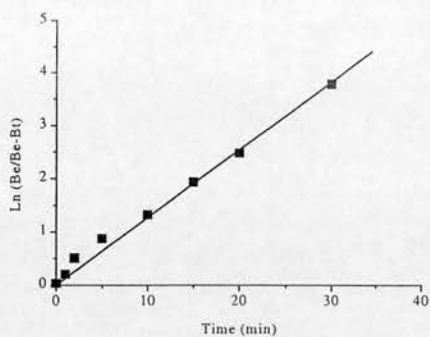
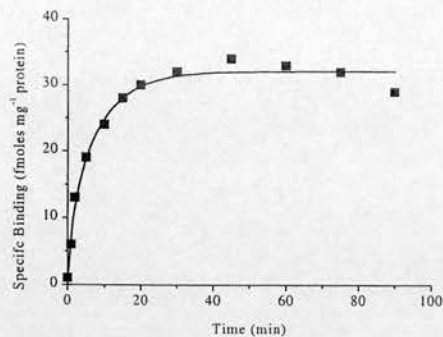
200nM GR125,743 in the presence of 200nM WAY100635 was sufficient to block binding to 5-HT<sub>1B/1D</sub> receptors. These conditions were used to investigate the kinetics and pharmacology of the remaining component(s) of WAY100635 & GR125,743 insensitive [ $^3\text{H}$ ]5-CT binding which could not include the 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> or 5-HT<sub>1D</sub> receptor population. In subsequent experiments the inhibition of [ $^3\text{H}$ ]5-CT binding by 200nM WAY100635, 200nM WAY100635 & 300nM CP93129 and 200nM WAY100635 & 200nM GR125,743 was also used to monitor the level of 5-HT<sub>1A</sub>, 5-HT<sub>1A/1B</sub> and 5-HT<sub>1A/1B/1D</sub> receptors respectively blocked within and between experiments.

#### **5.2.2.6.1 Time Course of [ $^3\text{H}$ ]5-CT Binding to Rat Cerebral Cortex Membranes in the Presence of 200nM WAY100635 & 200nM GR125,743**

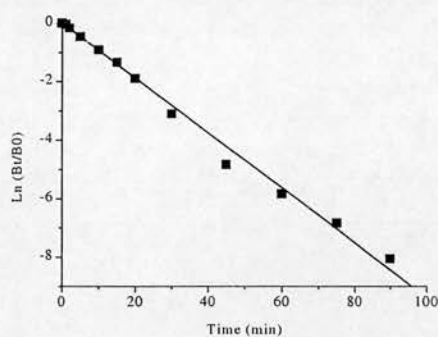
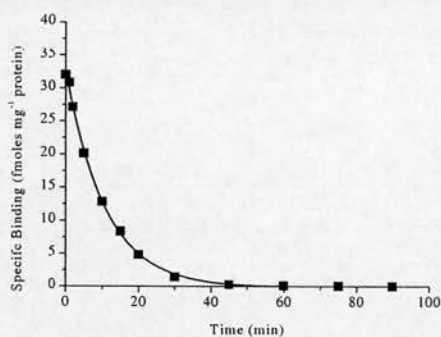
A time course (0-90 min) of [ $^3\text{H}$ ]5-CT (0.25nM) binding to rat cerebral cortex membranes was carried out at 25°C, with equilibrium attained by 20 min being stable for at least another 70 min (Figure 5.8a). Curve fitting using non-linear regression gave an observed constant ( $k_{\text{obs}}$ ) of  $0.142 \pm 0.007 \text{ min}^{-1}$  ( $n=3$ ) with a  $t_{1/2}$  of  $3.789 \pm 0.076 \text{ min}$  ( $n=3$ ). Dissociation was initiated after addition of 10 $\mu\text{M}$  5-HT following incubation for 60 min at 25°C, and followed first order kinetics with a  $t_{1/2}$  of  $6.716 \pm 0.476 \text{ min}$  (Figure 5.8b) and a dissociation rate constant ( $k_{-1}$ ) of  $0.095 \pm 0.001 \text{ min}^{-1}$



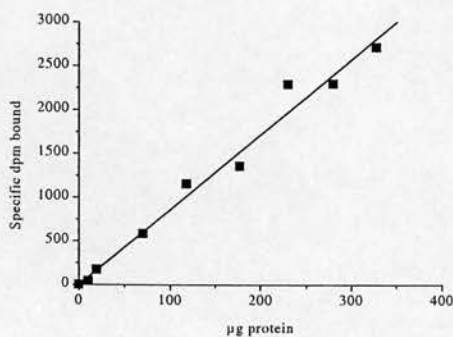
(a)



(b)



(c)



**Figure 5.8: Time Course and Protein Linearity of [<sup>3</sup>H]5-CT Binding in the Presence of 200nM WAY100635 & 200nM GR125,743.**

The data represent typical (a) time course, and (b) dissociation experiments, and (c) a single protein linearity experiment. Graphs on the right hand side represent the semilogarithmic transformations of the data. Experiments were performed as described in the text, with mean data obtained from three experiments also in text.

(n=3). Subsequent calculation gave an association rate constant ( $k_{+1}$ ) of  $0.196 \pm 0.153 \text{ min}^{-1}\text{M}^{-1}$ . The data from these time course experiments gave an equilibrium constant ( $K_D$ ) of  $0.488 \pm 0.039 \text{ nM}$ . In the following competition experiments [ $^3\text{H}$ ]5-CT (0.25nM) binding to rat cortex membranes was carried out at equilibrium at  $25^\circ\text{C}$  for 60 min.

#### **5.2.2.6.2 Effect of Membrane Protein Concentration on [ $^3\text{H}$ ]5-CT Binding to Rat Cerebral Cortex Membranes in the Presence of 200nM WAY100635 & 200nM GR125,743**

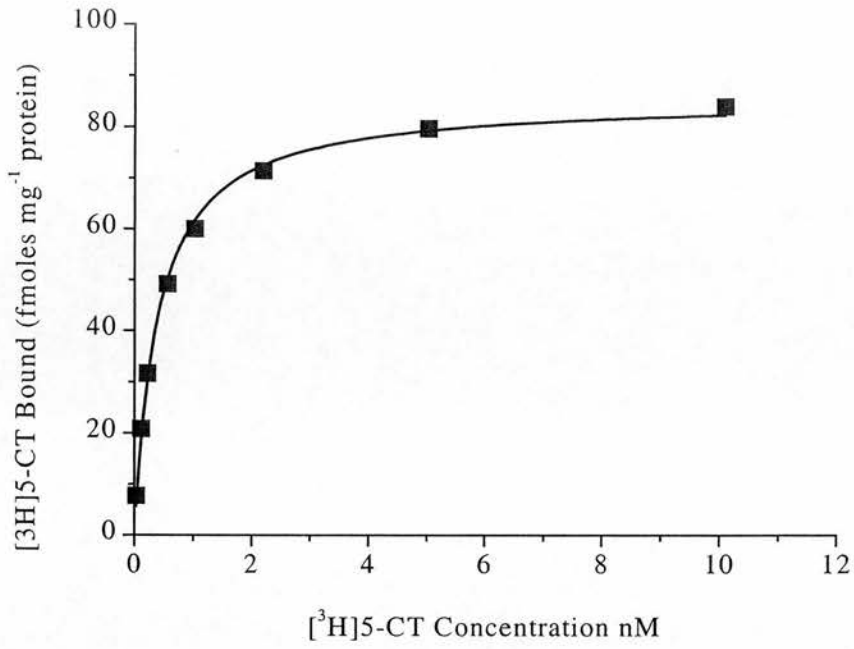
Specific [ $^3\text{H}$ ]5-CT binding was dependent on membrane protein (Figure 5.8c) and was linear up to the concentrations measured ( $\sim 300\mu\text{g}$  protein/assay). Subsequent experiments were conducted with 100-300 $\mu\text{g}$  membrane protein to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

#### **5.2.2.6.3 Concentration Dependence of [ $^3\text{H}$ ]5-CT Binding to Rat Cerebral Cortex Membranes in the presence of 200nM WAY100635 & 200nM GR125,743**

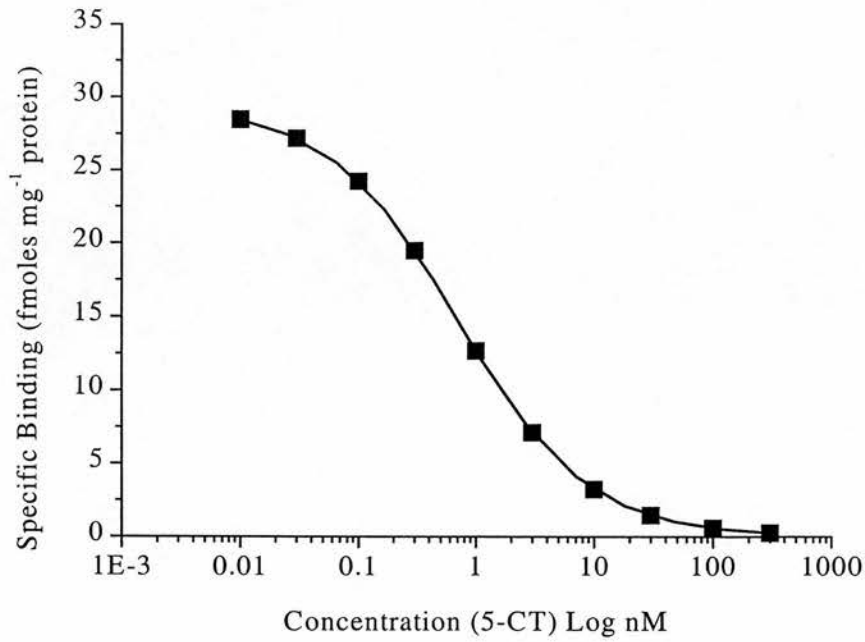
Hot saturation analysis of [ $^3\text{H}$ ]5-CT binding, in the presence of 200nM WAY100635 & 200nM GR125743, to rat cerebral cortex membranes was carried out using increasing concentrations of [ $^3\text{H}$ ]5-CT (Figure 5.9a). Curve fitting using a logistic model gave an equilibrium dissociation constant ( $K_D$ ) of  $0.36 \pm 0.04 \text{ nM}$ , a binding site density ( $B_{\text{max}}$ ) of  $85.13 \pm 1.09 \text{ fmol mg}^{-1} \text{ protein}$  (n=3) and a Hill slope of  $0.79 \pm 0.03$  (n=3).

Competition binding studies using 0.25nM [ $^3\text{H}$ ]5-CT and increasing concentrations of unlabelled 5-CT (0.01-300nM) (Figure 5.9b), gave a  $K_D$  of  $0.45 \pm 0.08\text{nM}$  and a  $B_{\text{max}}$  of  $80.44 \pm 5.70 \text{ fmol mg}^{-1} \text{ protein}$  with a Hill coefficient of  $0.84 \pm 0.03$  (n=9). This was in reasonable agreement with data obtained from both saturation and time course studies. The Hill slopes for both hot and cold saturations were significantly less than 1 ( $P < 0.05$ ).

(a)



(b)



**Figure 5.9: Concentration Dependence of Specific [<sup>3</sup>H]5-CT Binding in the Presence of 200nM WAY100635 & 200nM GR125,743.**

The results represent (a) hot saturation and (b) cold saturation experiments with each point performed in duplicate. See text for mean values.

#### 5.2.2.6.4 Pharmacology of [<sup>3</sup>H]5-CT Binding in the Presence of 200nM WAY100635 & 200nM GR125,743

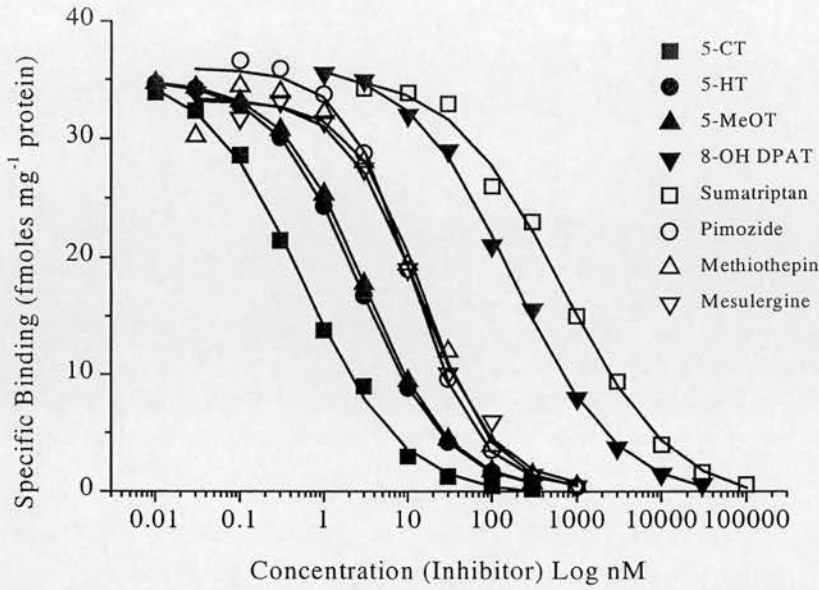
The WAY100635 & GR125,743 insensitive component of [<sup>3</sup>H]5-CT binding, representing the non-5-HT<sub>1A/1B/1D</sub> receptor population, was pharmacologically characterised using a number of structurally diverse compounds. These were tested up to 100µM and all inhibited the remaining binding fully (Figure 5.10). Of the antagonists tested, mesulergine was the most potent, with a mean pK<sub>i</sub> value of 8.15 ± 0.02 and Ketanserin the least potent, with a mean pK<sub>i</sub> value of 6.56 ± 0.11 (Table 5.7). The rank order of potency of these antagonists was: mesulergine > methiothepin > metergoline > pimozone > methysergide > ergotamine > ritanserin > dihydroergotamine > clozapine > mianserin > ketanserin (Table 5.7). All antagonists displayed binding to a single population of binding sites as indicated by their Hill slopes not differing from 1 (Table 5.7; Figure 5.10). Of the agonists tested, 5-CT was the most potent with a mean pK<sub>i</sub> value of 9.35 ± 0.08 and DP-5-CT the least potent with a pK<sub>i</sub> value of <5 (Table 5.7). The rank order of potency of the agonists tested was: 5-CT > 5-HT > 5-MeOT > 8-OH DPAT > Sumatriptan > DP-5-CT. In contrast to the antagonists, agonists generally had a Hill slope of between 0.76 and 1. Other compounds tested up to a concentration of 100µM, some of which had been used to characterise the blocking of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A/1B/1D</sub> receptors, had pK<sub>i</sub>s of <6. These included pindolol, cyanopindolol and WAY100635 (Table 5.7).

Comparison of drug affinities obtained for [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 and 200nM GR125,743 with the known affinities of these drugs at 5-HT<sub>7</sub> rat recombinant receptors gave a good positive correlation (r = 0.90; Figure 5.11). [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743 in rat cerebral cortex membranes is therefore likely to specifically label the native 5-HT<sub>7</sub> receptor population in rat cortex membranes.

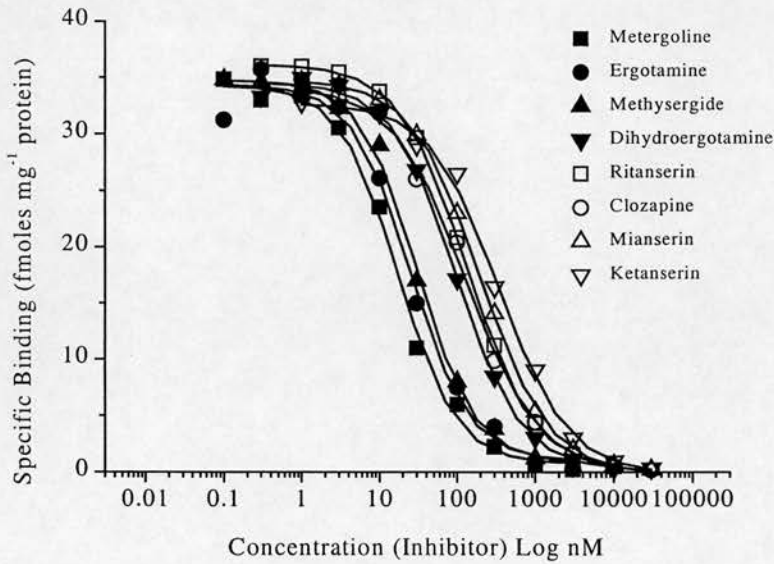
#### 5.2.2.7 Characterisation of [<sup>3</sup>H]5-CT Binding in Guinea Pig Cortex Membranes

The low Hill slopes of agonists in rat 5-HT<sub>7</sub> binding above, may be indicative of the reported complex nature of [<sup>3</sup>H]5-CT binding to rat native 5-HT<sub>7</sub> receptors

(a)



(b)



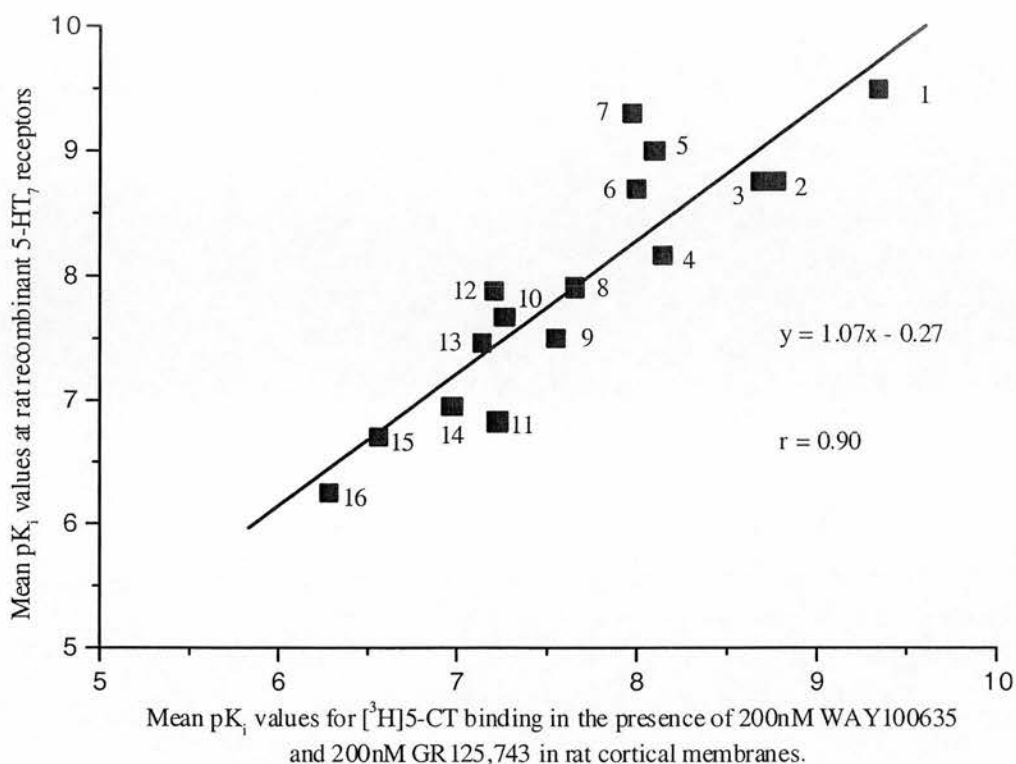
**Figure 5.10: Inhibition of [<sup>3</sup>H]5-CT Binding in the Presence of 200nM WAY100635 & 200nM GR125743.**

The data represent a typical experiment using 0.25nM [<sup>3</sup>H]5-CT in the presence of 200nM WAY100635 & 200nM GR125,743.(a) & (b) represent two graphs from the same experiment with mean data from at least three different experiments (Table 5.7).

**Table 5.7: Inhibition of [<sup>3</sup>H]5-CT Binding in the Presence of 200nM WAY100635 and 200nM GR125743 to Rat Cortex Membranes by 5-HT Receptor Ligands.**

	n	pK <sub>i</sub>	nH
<u>Agonists</u>			
5-CT	9	9.35 ± 0.08	0.84 ± 0.03
5-HT	4	8.78 ± 0.05	0.82 ± 0.01
5-MeOT	4	8.69 ± 0.03	0.84 ± 0.01
8-OH DPAT	4	7.15 ± 0.15	0.82 ± 0.04
Sumatriptan	4	6.29 ± 0.10	0.83 ± 0.03
DP-5-CT	3	<5	n.d.
<u>Antagonists</u>			
Mesulergine	4	8.15 ± 0.02	1.03 ± 0.04
Methiothepin	4	8.10 ± 0.11	0.96 ± 0.05
Metergoline	4	8.00 ± 0.06	1.03 ± 0.03
Pimozide	4	7.97 ± 0.19	0.95 ± 0.01
Methysergide	4	7.66 ± 0.16	0.94 ± 0.11
Ergotamine	4	7.55 ± 0.09	0.94 ± 0.06
Ritanserin	4	7.27 ± 0.08	0.98 ± 0.02
Dihydroergotamine	4	7.23 ± 0.05	1.00 ± 0.05
Clozapine	4	7.21 ± 0.16	0.95 ± 0.08
Mianserin	4	6.98 ± 0.08	0.95 ± 0.03
Ketanserin	4	6.56 ± 0.11	1.00 ± 0.03
Cyanopindolol	3	<6	n.d.
Pindolol	3	<5	n.d.
WAY100635	3	<5	n.d.

The affinity values were determined from the inhibition of 0.25nM [<sup>3</sup>H]5-CT to rat cerebral cortex membranes in the presence of 200nM WAY100635 & 200nM GR125,743. Values are the mean ± s.e.mean pK<sub>i</sub> values and Hill slope (nH). n.d. = not determined.



**Figure 5.11: Correlation Between [<sup>3</sup>H]5-CT Binding Site Affinities in the Presence of 200nM WAY100635 & 200nM GR125,743 and Affinities at Rat Recombinant 5-HT<sub>7</sub> Receptors.**

Affinities at rat cortical [<sup>3</sup>H]5-CT binding sites in the presence of 200nM WAY100635 & 200nM GR125743 determined from atleast three experiments (Table 5.6). (1) 5-CT; (2) 5-HT; (3) 5-MeOT; (4) mesulergine; (5) methiothepin; (6) metergoline; (7) pimozide; (8) methysergide; (9) ergotamine; (10) ritanserin; (11) dihydroergotamine; (12) clozapine; (13) 8-OH DPAT; (14) Mianserin; (15) ketanserin; (16) sumatriptan. Affinities at the recombinant 5-HT<sub>7</sub> receptor taken from Hoyer *et al.*, (1994).  $r$ =linear correlation coefficient.

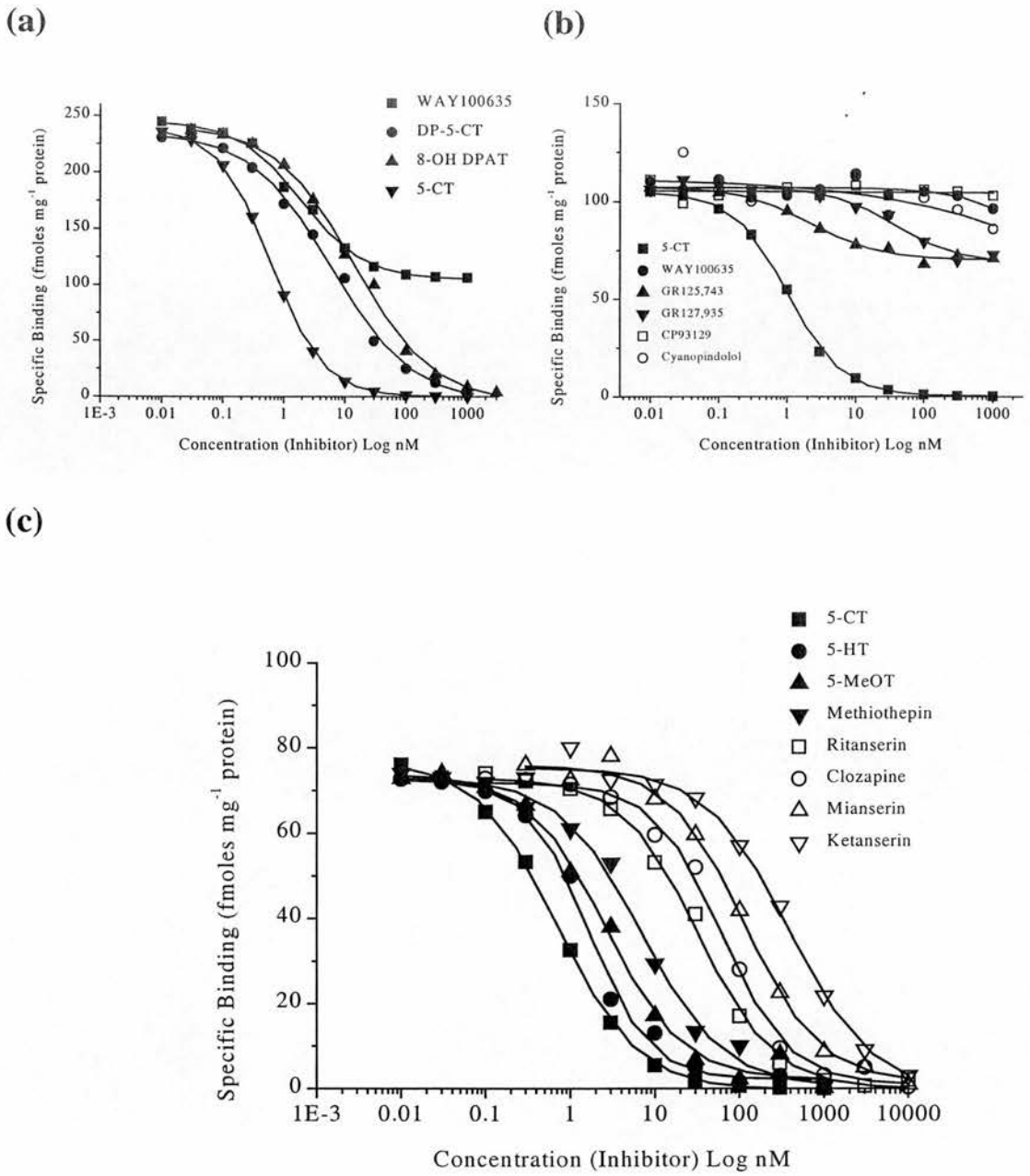
compared to the apparently less complex binding to guinea pig brain membranes (Boyland *et al.*, 1995). [<sup>3</sup>H]5-CT binding in guinea pig cortex membranes using the same conditions as in the rat 5-HT<sub>7</sub> receptor binding assay was investigated. However the pharmacology of the guinea pig 5-HT<sub>1B</sub> receptor is different to the rodent 5-HT<sub>1B</sub> receptor, but the same as in man (see Hoyer *et al.*, 1994). It was therefore important to firstly determine the affinities of the drugs used in the rat cortex membranes, in order to find the concentration of drugs required to achieve full block of non-5-HT<sub>7</sub> receptors in guinea pig cortex membranes.

In the absence of masking drugs, cold saturation experiments revealed a single population of binding sites, as indicated by a Hill slope of unity, with a K<sub>D</sub> value of 0.44 ± 0.08 nM and a B<sub>max</sub> of 0.893 ± 0.101 pmoles mg<sup>-1</sup> protein (n=4). DP-5-CT and 8-OH DPAT completely inhibited [<sup>3</sup>H]5-CT binding at concentrations greater than 1 μM (Figure 5.12a). Despite Hill slopes <1, there was no obvious graphical separation of binding site affinities (Figure 5.12a). Inhibition of [<sup>3</sup>H]5-CT binding by WAY100635 on the other hand, reached a plateau between 100 and 1000nM with a mean pK<sub>i</sub> value of 8.73 ± 0.07 and a Hill slope of 1 (Figure 5.12a; Table 5.8), which is close to the value obtained in rat cortex membranes. To define the proportion of non-5-HT<sub>7</sub> receptors labelled by [<sup>3</sup>H]5-CT, subsequent experiments were performed in the presence of 200nM WAY100635, which caused a 59.76 ± 3.09% reduction in [<sup>3</sup>H]5-CT binding (n=3, Table 5.8; Figure 5.12a).

Binding inhibition curves were generated for CP93129, cyanopindolol, GR125743 and GR127935. The latter two 5-HT<sub>1B/1D</sub> antagonists inhibited [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 by approximately a further 9%, with Hill slopes of 1 and respective mean pK<sub>i</sub> values of 8.69 ± 0.14 and 7.78 ± 0.13 (Table 5.8; Figure 5.12b). GR125,743 in the presence of 200nM WAY100635 reached a plateau between 100 and 1000nM (Figure 5.12b), as in the rat, blocking 68.20 ± 5.40% of total specific [<sup>3</sup>H]5-CT binding. CP93129 and cyanopindolol inhibition curves, up to 1 μM, inhibited WAY100635 insensitive [<sup>3</sup>H]5-CT binding with much lower affinity (pK<sub>i</sub> <6) than in rat cortex membranes. Due to this low affinity, the proportion of 5-HT<sub>1B</sub> receptors labelled by [<sup>3</sup>H]5-CT could not be determined.

In subsequent experiments 200nM GR125743 was used in addition to 200nM WAY100635 to block binding to 5-HT<sub>1A/1B/1D</sub> receptors. The affinity (K<sub>D</sub>) of this





**Figure 5.12: Inhibition of [<sup>3</sup>H]5-CT Binding to Guinea Pig Cortex Membranes by 5-HT Receptor Ligands.**

The data represent typical 0.25nM [<sup>3</sup>H]5-CT binding experiments in the presence of (a) no block (b) 200nM WAY100635 and (c) 200nM WAY100635 and 200nM GR125,743 with mean data from at least three different experiments (Table 5.8).

**Table 5.8: Inhibition of [<sup>3</sup>H]5-CT Binding to Guinea Pig Cortex Membranes by 5-HT Receptor Ligands.**

Competing Ligand	n	pK <sub>i</sub>	nH
<u>In the absence of any blocking agents</u>			
5-CT	3	9.37 ± 0.07	0.98 ± 0.02
DP-5-CT	3	8.66 ± 0.06	0.65 ± 0.07
8-OH DPAT	3	8.11 ± 0.04	0.81 ± 0.05
WAY100635	3	8.73 ± 0.07	0.98 ± 0.01
<u>In the presence of 200nM WAY100635</u>			
5-CT	3	9.11 ± 0.03	0.94 ± 0.05
GR125,743	3	8.69 ± 0.14	0.96 ± 0.04
GR127,935	3	7.78 ± 0.13	0.99 ± 0.02
Cyanopindolol	3	<6	n.d.
CP93129	3	<6	n.d.
<u>In the presence of 200nM WAY100635 and 200nM GR125,743</u>			
<u>Agonists</u>			
5-CT	4	9.25 ± 0.12	0.96 ± 0.13
5-HT	4	8.78 ± 0.05	0.99 ± 0.02
5-MeOT	4	8.63 ± 0.07	1.00 ± 0.03
DP-5-CT	4	7.95 ± 0.09	0.98 ± 0.02
8-OH DPAT	4	6.91 ± 0.09	0.90 ± 0.09
<u>Antagonists</u>			
Methiothepin	4	8.29 ± 0.10	0.95 ± 0.04
Metergoline	4	8.03 ± 0.07	0.93 ± 0.07
Ritanserin	4	7.69 ± 0.06	0.87 ± 0.11
Clozapine	4	7.47 ± 0.05	0.95 ± 0.03
Mianserin	4	6.99 ± 0.04	0.94 ± 0.05
Ketanserin	4	6.49 ± 0.06	1.08 ± 0.09

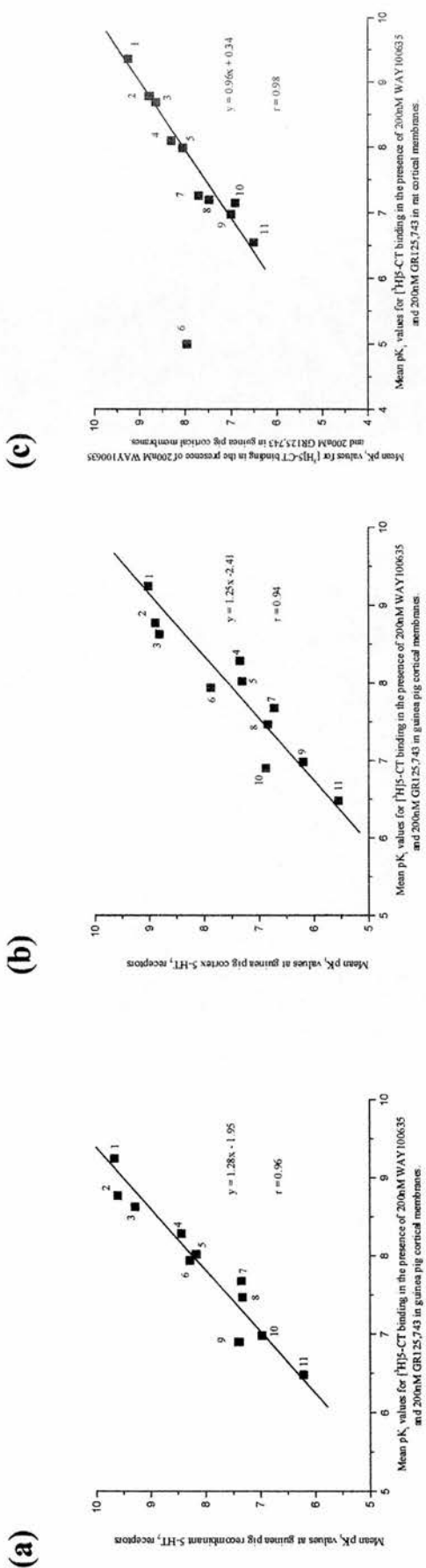
The affinity values were determined from the inhibition of 0.25nM [<sup>3</sup>H]5-CT to guinea pig cerebral cortex membranes using the conditions stated. Values are mean ± s.e.mean pK<sub>i</sub> values and Hill slope (nH). n.d. = not determined.

remaining WAY100635 & GR125,743 binding was  $0.56 \pm 0.12$  nM, with a binding site density ( $B_{\max}$ ) of  $0.355 \pm 0.056$  pmoles  $\text{mg}^{-1}$  protein and a Hill slope of 1 ( $n = 3$ ; Table 5.8).

The pharmacology of WAY100635 & GR125,743 insensitive [ $^3\text{H}$ ]5-CT binding, which represented  $31.80 \pm 5.40\%$  of total specific [ $^3\text{H}$ ]5-CT binding, was essentially the same as in rat cortex membranes. Unlike in the rat, all drugs tested, whether agonists or antagonists, inhibited binding fully with a Hill slope close to unity (Figure 5.12c; Table 5.8). Of the antagonists tested, methiothepin was the most potent with a mean  $\text{pK}_i$  value of  $8.29 \pm 0.10$  and ketanserin the weakest with a mean  $\text{pK}_i$  value of  $6.49 \pm 0.06$  (Table 5.8). The rank order of potency of the antagonists was the same as in the rat, whereby: Methiothepin > Metergoline > Ritanserin > Clozapine > Mianserin > Ketanserin (Table 5.8). Of the agonists tested, 5-CT was the most potent with a mean  $\text{pK}_i$  value of  $9.25 \pm 0.12$  and 8-OH DPAT the least potent with a mean  $\text{pK}_i$  value of  $6.91 \pm 0.09$  (Table 5.8). The rank order of potency of the agonists tested was slightly different than in rat: 5-CT > 5-HT > 5-MeOT > DP-5-CT > 8-OH DPAT. (Table 5.8). DP-5-CT had 2000 fold higher affinity in guinea pig cortex membranes compared to corresponding rat membranes. As in the rat, pindolol, cyanopindolol & WAY100635 all had  $\text{pIC}_{50\text{s}} < 6$  (Table 5.8)

Comparison of drug affinities obtained for [ $^3\text{H}$ ]5-CT binding in the presence of 200nM WAY100635 and 200nM GR125,743 in guinea pig cortex with the known affinities of these drugs at 5-HT<sub>7</sub> guinea pig recombinant receptors gave a very good positive correlation ( $r = 0.96$ ; Fig 5.13a). Comparison of drug affinities obtained for [ $^3\text{H}$ ]5-CT binding in the presence of 200nM WAY100635 and 200nM GR125,743 in guinea pig cortex membranes with the affinities of these drugs in a previous study in the same membranes using different blocking drugs (To *et al.*, 1995) also gave a good positive correlation ( $r = 0.94$ ; Figure 5.13b). Comparison of experimental affinities for WAY100635 & GR125,743 insensitive [ $^3\text{H}$ ]5-CT binding in native rat and guinea pig cortex membranes also displayed a good positive correlation ( $r = 0.98$ , Figure 5.13c), when excluding the obvious outlier, DP-5-CT.

Radioligand binding using 0.25nM [ $^3\text{H}$ ]5-CT in the presence of 200nM WAY100635 & 200nM GR125,743 can therefore label the 5-HT<sub>7</sub> receptor in both native rat and guinea pig cortex membranes.



**Figure 5.13: Correlation Between [<sup>3</sup>H]5-CT Binding Site Affinities in the Presence of 200nM WAY100635 & 200nM GR125,743 in Guinea Pig Cortex Membranes and (a) Recombinant and (b) Native Guinea Pig 5-HT<sub>7</sub> Receptors and (c) Experimental Affinities Using Same Conditions in Rat Cortex Membranes.**

Affinities at guinea pig cortical [<sup>3</sup>H]5-CT binding sites in the presence of 200nM WAY100635 & 200nM GR125743 determined from at least three experiments (Table 5.8). (1) 5-CT; (2) 5-HT; (3) 5-MeOT; (4) methiothepin; (5) metergoline; (6) DP-5-CT; (7) ritanserlin; (8) clozapine; (9) Mianserin; (10) 8-OH DPAT; (11) ketanserin. Affinities for comparison to experimentally determined affinities obtained from To *et al.*, (1995), whose binding conditions are outlined in Table 5.3. Rat data for (c) taken from Table 5.7.  $r$  = linear correlation coefficient. Linear regression analysis for (c) does not include the obvious outlier DP-5-CT.

### 5.2.2.8 Characterisation of [<sup>3</sup>H]5-CT Binding in Rat Striatum and Hippocampal Brain Membranes

For the purpose of investigating the effects of chronic antidepressant treatments on 5-HT<sub>7</sub> receptor density in other brain areas, as in chapter 6, the conditions of the blocks in the rat striatum and hippocampus to reveal the 5-HT<sub>7</sub> binding site were determined.

To block binding to 5-HT<sub>1A</sub> receptors, WAY100635 was again used. Inhibition binding curves to WAY100635 yielded a plateau between 100 and 1000nM in both brain areas with a Hill slope of 1, with a mean pK<sub>i</sub> value of 8.45 ± 0.02 and 8.44 ± 0.02 in striatum and hippocampus membranes respectively (n = 3; data not shown). This was in good agreement with data obtained in the rat cortex and hence 200nM WAY100635 was used to mask binding to 5-HT<sub>1A</sub> receptors. However the proportion of [<sup>3</sup>H]5-CT binding which is attributable to 5-HT<sub>1A</sub> receptors as defined in the presence of 200nM WAY100635, was dramatically different to that in rat cortex membranes. In the striatum 200nM WAY100635 blocked only 21.96 ± 0.38% of total specific [<sup>3</sup>H]5-CT binding, whereas 200nM WAY100635 blocked 77.41 ± 0.96% of total specific [<sup>3</sup>H]5-CT binding in the hippocampus (Table 5.9).

The pharmacology of the WAY100635 insensitive [<sup>3</sup>H]5-CT binding sites was identical comparing the striatum, hippocampus and cortex (data not shown). CP93129 reached a plateau between 100 and 1000nM and yielded similar pK<sub>i</sub> values of 8.43 ± 0.11 and 8.35 ± 0.04 (n = 3, data not shown) with Hill slopes of 1, in striatum and hippocampus membranes respectively. 300nM CP93129 in the presence of 200nM WAY100635 blocked 79.63 ± 2.40% and 91.67 ± 1.45% of total specific [<sup>3</sup>H]5-CT binding in the striatum and hippocampus respectively (Table 5.9). GR125,743 in the presence of 200nM WAY100635 reached a plateau between 100 and 1000nM and yielded pK<sub>i</sub> values of 8.75 ± 0.03 and 8.85 ± 0.06 with Hill slopes of 1, in striatum and hippocampus membranes respectively (n=3, data not shown). 200nM GR125743 in the presence of 200nM WAY100635 blocked 90.68 ± 0.66% and 93.33 ± 0.88% of total specific [<sup>3</sup>H]5-CT binding in the striatum, whereas [<sup>3</sup>H]5-CT binding was blocked in the hippocampus (Table 5.9).

**Table 5.9: Summary of Inhibition of [<sup>3</sup>H]5-CT Binding in Membranes from Rat and Guinea Pig Tissue and Relative Proportions of Experimentally Determined 5-HT<sub>1A/1B/1D</sub> and 5-HT<sub>7</sub> Receptors.**

Brain area	n	% of total specific [ <sup>3</sup> H]5-CT control binding blocked in the presence of				B <sub>max</sub> (fmoles mg <sup>-1</sup> protein)
		200nM WAY100635	200nM WAY100635 & 300nM CP93129	200nM WAY100635 & 200nM GR125,743	200nM WAY100635	
Rat cortex	12	61.65 ± 0.99	84.75 ± 0.75	88.24 ± 0.55	80.44 ± 5.70 (89.17 ± 5.23)	
Rat hippocampus	3	77.41 ± 0.96	91.67 ± 1.45	94.53 ± 0.88	100.29 ± 8.03 (99.91 ± 7.26)	
Rat striatum	3	21.96 ± 0.38	79.63 ± 2.40	93.33 ± 1.88	75.22 ± 7.21 (83.17 ± 6.23)	
Guinea pig cortex	4	59.76 ± 3.09	*	68.20 ± 5.40	355.25 ± 56.22	

Brain area	n	Relative percent proportions of receptors occupied by 0.25nM [ <sup>3</sup> H]5-CT			
		5-HT <sub>1A</sub>	5-HT <sub>1B</sub>	5-HT <sub>1D</sub>	5-HT <sub>7</sub>
Rat cortex	12	61.65 ± 0.99	23.10 ± 1.25	3.49 ± 2.41	26.59 ± 3.58
Rat hippocampus	3	77.41 ± 0.96	14.26 ± 2.12	2.86 ± 1.21	17.12 ± 2.12
Rat striatum	3	21.96 ± 0.38	57.67 ± 3.45	12.65 ± 2.35	71.37 ± 6.50
Guinea pig cortex	4	59.76 ± 3.09	*	*	8.44 ± 2.45

The results are expressed as the % of total specific [<sup>3</sup>H]5-CT control binding in the presence of various blocking conditions or as the relative percent proportions of receptors occupied by 0.25nM [<sup>3</sup>H]5-CT. Values shown are the mean ± s.e.mean. B<sub>max</sub> values relate to the binding that remains after block with 200nM WAY100635 & 200nM GR125,742 (5-HT<sub>7</sub> binding). Data in brackets relates to studies in Sprague Dawley rats. In guinea pig cortex the proportion of 5-HT<sub>1B</sub> or 5-HT<sub>1D</sub> receptors could not be determined due to low affinity of CP93129 and cyanopindolol as compared to in rat membranes due to the known difference in pharmacology of rodent and non-rodent 5-HT<sub>1B</sub> receptors (\*).

The  $K_D$  of this remaining WAY100635 & GR125,743 insensitive [ $^3$ H]5-CT binding was  $0.53 \pm 0.12$  and  $0.43 \pm 0.11$  nM, with  $B_{max}$  values of  $75.22 \pm 7.21$  and  $100.29 \pm 8.03$  fmoles  $mg^{-1}$  protein in striatum and hippocampus membranes respectively ( $n = 3$ , Table 5.9). The pharmacology of this remaining binding was assessed using a range of drugs used in rat cortex experiments. In both tissues the rank order of potency was 5-CT > 5-HT > methiothepin > clozapine > 8-OH DPAT > ketanserin, with the antagonists having Hill slopes of 1, and the agonists Hill slopes of <1 as seen in cortex membranes (data not shown). Once again the affinities of these drugs correlated well to binding at rat recombinant 5-HT<sub>7</sub> receptors (data not shown). Therefore the same conditions employed in the rat cortex can be used in the rat striatum and hippocampus to label native 5-HT<sub>7</sub> receptors.

#### **5.2.2.9 Characterisation of [ $^3$ H]5-CT Binding in a Different Strain of Rat-Sprague Dawley**

The binding experiments described thus far were carried out using Wistar Cobb rats. For the drug treatment studies described in chapter 6 adult male Sprague Dawley rats were used. Therefore the characterisation of [ $^3$ H]5-CT binding was repeated in adult male Sprague Dawley rats (200-300g; Charles River) in cortex, hippocampus, and striatum membranes. There were no differences observed in either the % of [ $^3$ H]5-CT binding blocked by the different blocking agents, or in the pharmacology of the binding sites after these blocks (data not shown). Importantly there was a strong correlation in the [ $^3$ H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743 with results obtained in Wistar Cobb rat membranes and with binding at 5-HT<sub>7</sub> recombinant receptors (data not shown). The  $B_{max}$  values of this remaining WAY100635 & GR125,743 binding site was no different to Wistar Cobb rat membranes, with  $89.17 \pm 5.23$ ,  $83.17 \pm 6.23$ , and  $99.81 \pm 7.26$  fmoles  $mg^{-1}$  protein in cortex, striatum and hippocampus Sprague Dawley rat membranes respectively (Table 5.9).

### 5.2.2.10 Relative Proportions of 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub>

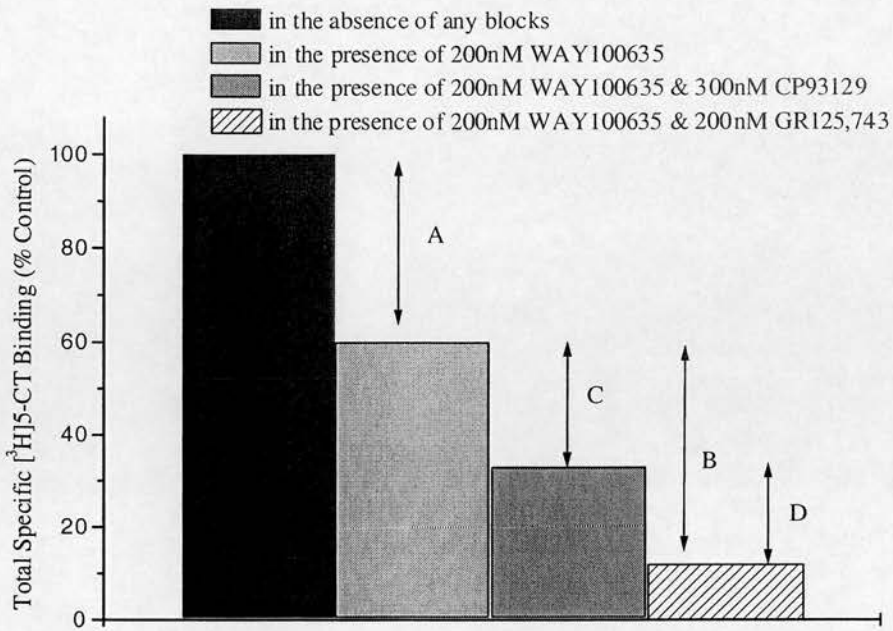
#### Receptors Labelled by [<sup>3</sup>H]5-CT

Irrespective of the source of tissue or the brain area investigated, the studies described throughout this chapter utilised 200nM WAY100635 to block [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> receptors, 200nM WAY100635 & 200nM CP93129 to block [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors and 200nM WAY100635 & 200nM GR125743 to block [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> and 5-HT<sub>1B/1D</sub> receptors. From these studies it was important to calculate the relative proportions of each receptor subtype labelled by [<sup>3</sup>H]5-CT, to act as control reference points between experiments and for the antidepressant or MDMA treatments (chapters 6 & 7).

Figure 5.14 summarises the calculation of the proportions of receptors labelled by 0.25nM [<sup>3</sup>H]5-CT. Relative proportions were calculated as the percentage of specific unmasked [<sup>3</sup>H]5-CT binding for each of the three blocks. The % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> receptors was calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635. The % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1B</sub> & 5-HT<sub>1D</sub> receptors was calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 200nM GR125743 minus the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635. The % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1B</sub> receptors was calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 300nM CP93129 minus the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635. The % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1D</sub> receptors was calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 200nM GR125,743 minus the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 300nM CP93129.

The relative proportions of [<sup>3</sup>H]5-CT binding to 5-HT receptors varies through the rat brain (Table 5.9). The highest proportion of [<sup>3</sup>H]5-CT binding corresponding to binding at 5-HT<sub>1A</sub> receptors was in the hippocampus, an area known to have a high density of 5-HT<sub>1A</sub> receptors (Deshmukh *et al.*, 1983). The highest proportion of any combination of 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors was in the striatum, an area rich in these receptors (Bruinvels *et al.*, 1993a & b).





**Figure 5.14: Calculation of Proportions of 5-HT Receptors Labelled By [<sup>3</sup>H]5-CT.**

This Figure represents how the proportion of 5-HT receptors labelled by 0.25nM [<sup>3</sup>H]5-CT were calculated irrespective of tissue source. This figure is purely a summary of how the calculations are performed and is not representative of experimental data.

A= % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> receptors calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635.

B= % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1B</sub> & 5-HT<sub>1D</sub> receptors calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 200nM GR125743 minus the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635.

C= % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1B</sub> receptors calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 300nM CP93129 minus the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635.

D= % of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1D</sub> receptors calculated as the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 200nM GR125,743 minus the % of [<sup>3</sup>H]5-CT binding blocked in the presence of 200nM WAY100635 & 300nM CP93129.

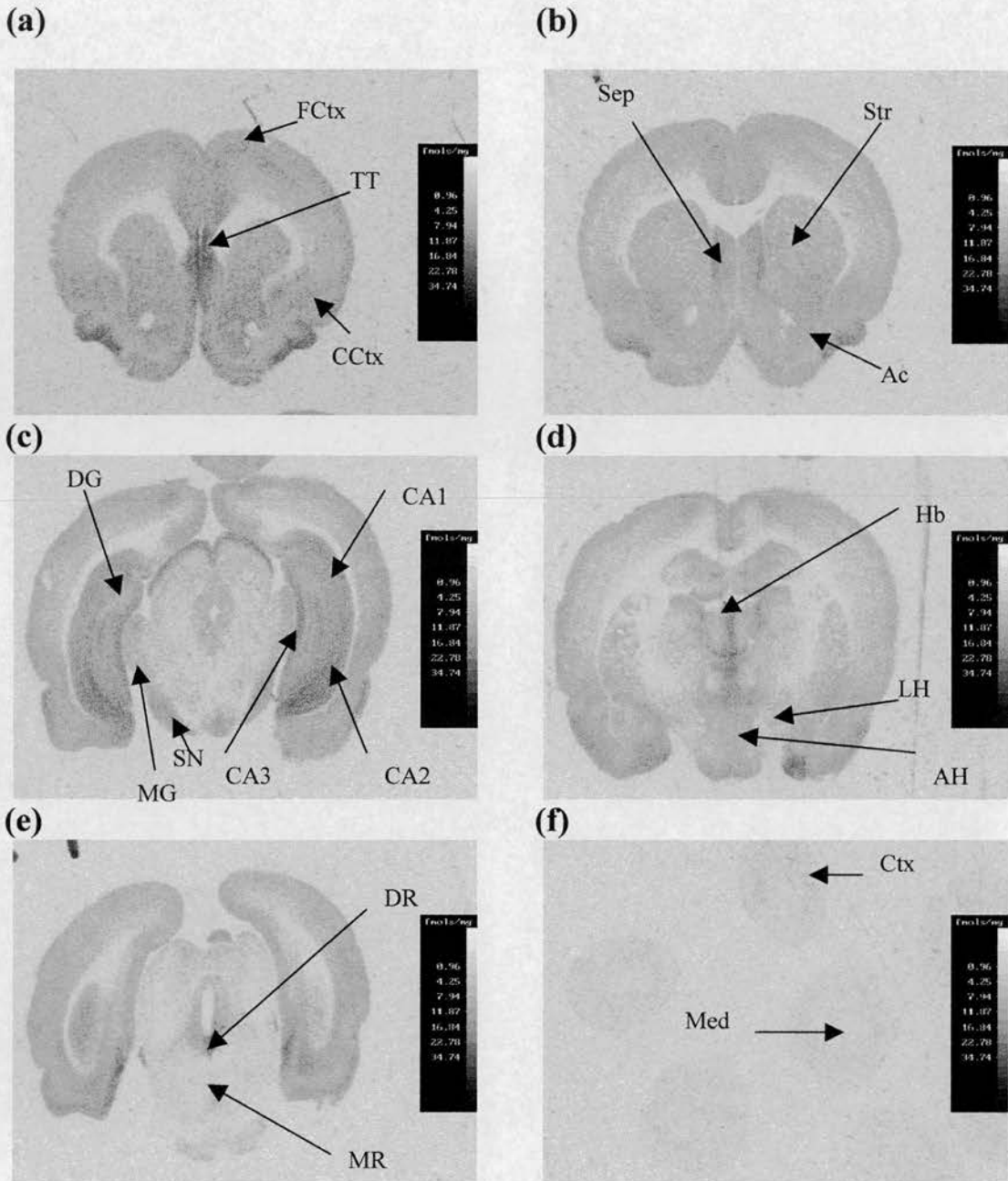
### 5.2.3 [<sup>3</sup>H]5-CT Autoradiography

The distribution of 5-HT<sub>7</sub> binding sites labelled by 0.5nM [<sup>3</sup>H]5-CT was studied in rat brain and adrenal glands by *in vitro* autoradiography, using the same blocking conditions employed in the corresponding radioligand binding assays, i.e. in the presence of 200nM WAY100635 (to prevent binding to 5-HT<sub>1A</sub> receptors) and 200nM GR125,743 (to prevent binding to 5-HT<sub>1B/1D</sub> receptors). Initial experiments were conducted after a 6 week exposure to Hyperfilm™, however the resultant images in the presence of 200nM WAY100635 & 200nM GR125,743 to define the 5-HT<sub>7</sub> population were faint, and revealed low optical densities (data not shown). The experiments were therefore repeated at 12 weeks exposure to obtain images at a reasonable resolution.

Following a 12 week exposure, non-specific binding in consecutive sections was not significantly greater than background, but specific binding was detected in various brain regions. Table 5.10 and Figure 5.15, shows the density of 5-HT<sub>7</sub> binding sites in 14 areas of rat brain and also 2 areas of the rat adrenal gland. The rank order of [<sup>3</sup>H]5-CT binding site densities in the presence of 200nM WAY100635 and 200nM GR125,743, was: dorsal raphé > septum > hippocampus structures > substantia nigra > cortex regions = striatum regions > hypothalamic regions = median raphé. Specific [<sup>3</sup>H]5-CT binding sites under these conditions were negligible in the rat adrenal gland indicating no 5-HT<sub>7</sub> receptors in this peripheral tissue.

Figures 5.16a, 5.17a & 5.18a represent total [<sup>3</sup>H]5-CT binding and Figures 5.16b, 5.17b & 5.18b represent total [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635. Within these images many of the brain areas gave optical densities that were not on the linear portion of the [<sup>3</sup>H]microscales generated standard curve, as shown by the saturating signal. Therefore no specific binding (fmols mg<sup>-1</sup> protein) was determined for these images and hence the proportion of receptors labelled by [<sup>3</sup>H]5-CT could not be determined.

The autoradiograms in the adrenal glands (Figure 5.18), however reveal differences between the total binding (Figure 5.18a) and in the presence of 200nM WAY100635 (Figure 5.18b) in the centrally located rat adrenal medulla, suggesting



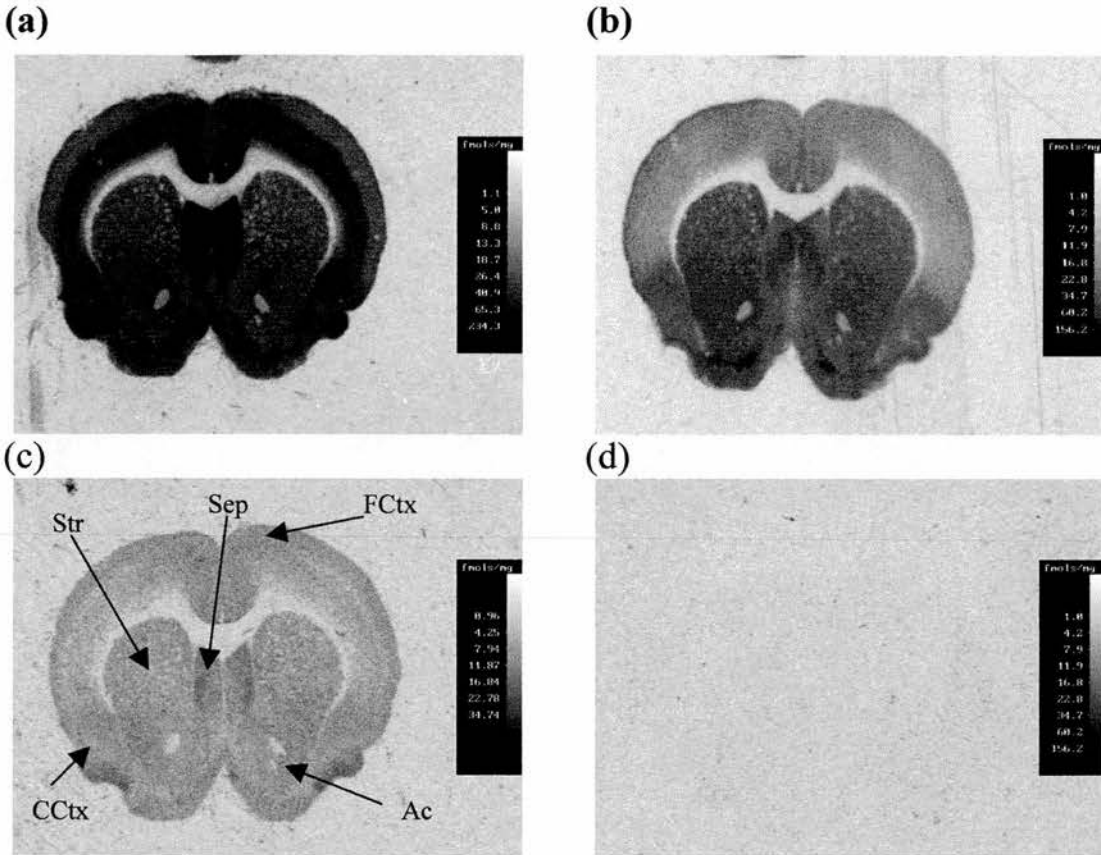
**Figure 5.15: 5-HT<sub>7</sub> Distribution in Rat Brain and Adrenal Glands**

Representative autoradiograms in the presence of 0.5nM [<sup>3</sup>H]5-CT and 200nM WAY100635 and 200nM GR125,743 after 12 weeks exposure, at the levels of the; (a)- tenia tecta (TT), (b)- septum (Sep), (c)- medial geniculate (MG), (d)- habenula (Hb), (e)- raphé nuclei and (f)- adrenal gland. CA1, CA2 & CA3 represent corresponding areas of the hippocampus, DG = dentate gyrus, SN = substantia nigra, FCtx = frontal cortex, CCtx = caudal cortex, LH & AH = lateral and anterior hypothalamus respectively, Str = striatum, Ac = accumbens, DR = dorsal raphé, MR = median raphé, Ctx = adrenal cortex and Med = adrenal medulla.

<u>Brain Area</u>	<u>fmols mg<sup>-1</sup> tissue</u>
<b>At the level of the tenia tecta</b>	
Frontal cortex	8.06 ± 1.13
Caudal cortex	7.24 ± 0.61
<b>At the level of the septum</b>	
Accumbens	9.25 ± 1.15
Septum	15.75 ± 1.50
Striatum	7.12 ± 1.00
<b>At the level of the medial geniculate</b>	
CA1	13.25 ± 0.61
CA2	14.22 ± 1.21
CA3	16.75 ± 1.23
Dentate gyrus	12.25 ± 1.25
Substantia nigra	12.76 ± 0.57
<b>At the level of the habenula</b>	
Anterior hypothalamus	7.05 ± 0.54
Lateral hypothalamus	5.12 ± 0.38
<b>At the level of the raphé nuclei</b>	
Dorsal raphé	23.27 ± 1.24
Median raphé	5.19 ± 0.26
<b>Adrenal gland</b>	
Medulla	None
Cortex	None

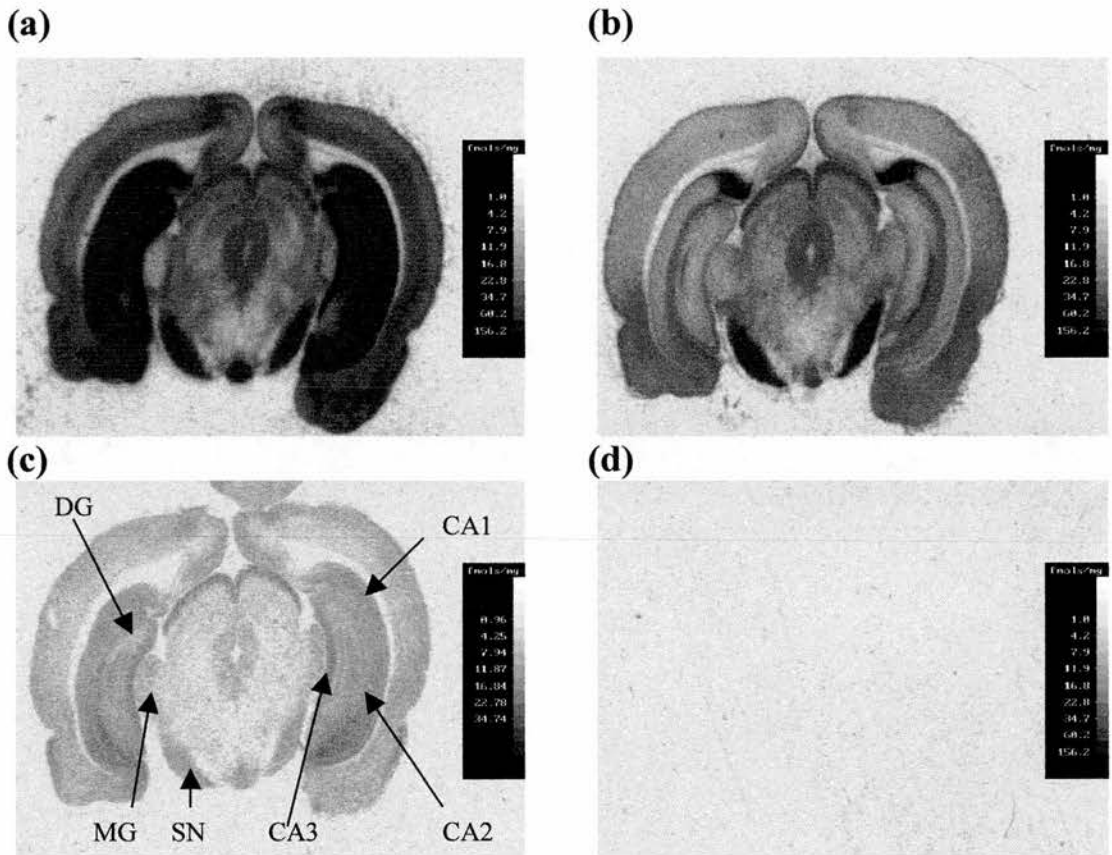
**Table 5.10: Autoradiographic Distribution of Rat 5-HT<sub>7</sub> Receptors.**

Results are expressed as the mean ± s.e. mean from three individual animals after a 12 week exposure to 0.5nM [<sup>3</sup>H]5-CT in the presence of 200nM WAY100635 and 200nM GR125,743. Adjacent sections in the presence of 0.5nM [<sup>3</sup>H]5-CT and 10μM 5-HT determined the level of non-specific binding. The amount of specific binding for the 5-HT<sub>7</sub> receptor is expressed as fmols mg<sup>-1</sup> tissue, after optical density values were corrected using appropriate microscale standards.



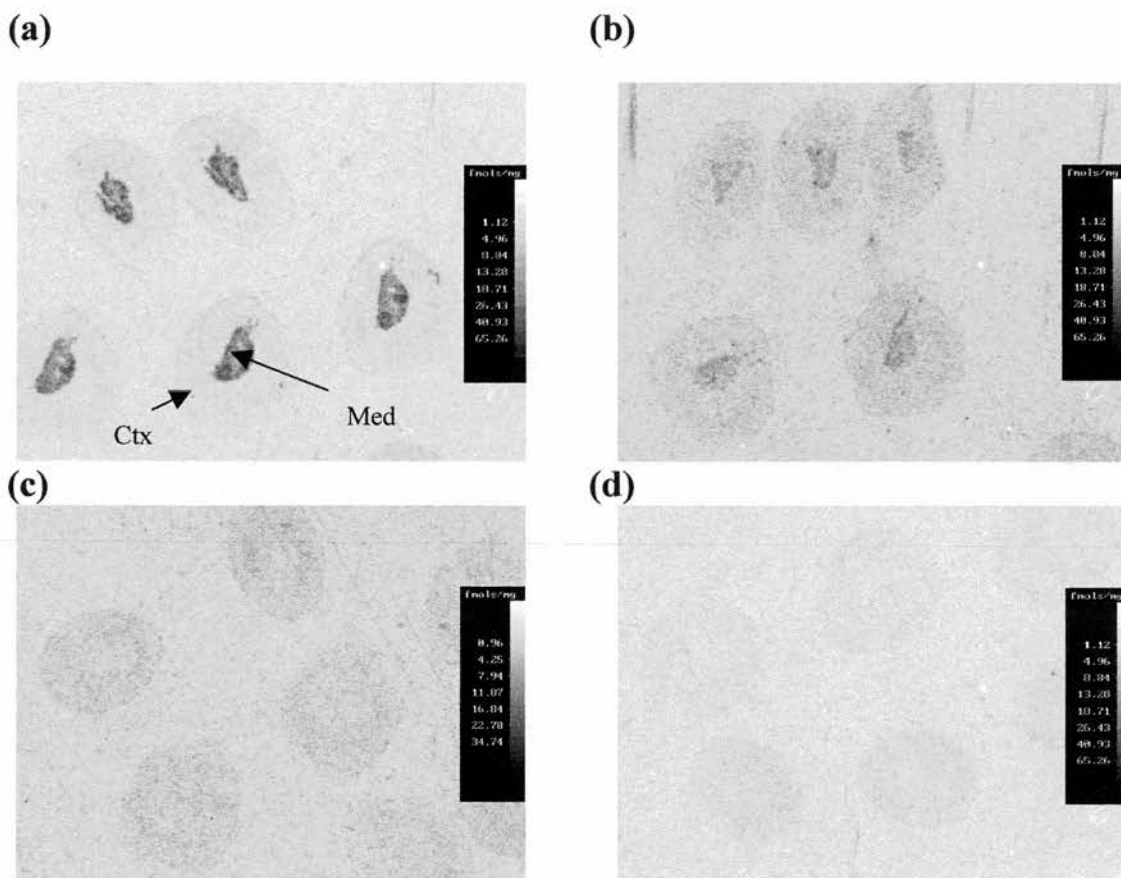
**Figure 5.16:  $[^3\text{H}]5\text{-CT}$  Autoradiography at the Level of the Septum.**

Representative autoradiograms after 12 weeks exposure in the presence of; (a)- 0.5nM  $[^3\text{H}]5\text{-CT}$ , (b)- 0.5nM  $[^3\text{H}]5\text{-CT}$  & 200nM WAY100635, (c)- 0.5nM  $[^3\text{H}]5\text{-CT}$  & 200nM WAY100635 & 200nM GR125,743 and (d)- 0.5nM  $[^3\text{H}]5\text{-CT}$  in the presence of 10 $\mu\text{M}$  5-HT to define non-specific binding. FCtx = frontal cortex, CCtx = caudal cortex, Ac = accumbens, Str = striatum and Sep = septum.



**Figure 5.17: [<sup>3</sup>H]5-CT Autoradiography at the Level of the Medial Geniculate.**

Representative autoradiograms after 12 weeks exposure in the presence of; (a)- 0.5nM [<sup>3</sup>H]5-CT, (b)- 0.5nM [<sup>3</sup>H]5-CT & 200nM WAY100635, (c)- 0.5nM [<sup>3</sup>H]5-CT & 200nM WAY100635 & 200nM GR125,743 and (d)- 0.5nM [<sup>3</sup>H]5-CT in the presence of 10µM 5-HT to define non-specific binding. MG = medial geniculate, SN = substantia nigra, DG = dentate gyrus and CA1, CA2 & CA3 = corresponding layers of the hippocampus.



**Figure 5.18: [<sup>3</sup>H]5-CT Autoradiography in Rat Adrenal Glands.**

Representative autoradiograms after 12 weeks exposure in the presence of; (a)- 0.5nM [<sup>3</sup>H]5-CT, (b)- 0.5nM [<sup>3</sup>H]5-CT & 200nM WAY100635, (c)- 0.5nM [<sup>3</sup>H]5-CT & 200nM WAY100635 & 200nM GR125,743 and (d)- 0.5nM [<sup>3</sup>H]5-CT in the presence of 10 $\mu$ M 5-HT to define non-specific binding. Ctx = adrenal cortex and Med = adrenal medulla.

the presence of 5-HT<sub>1A</sub> receptors. Radioligand binding studies using [<sup>3</sup>H]8-OH DPAT however, revealed no specific binding in either rat adrenal medulla or adrenal capsule membranes (data not shown; up to 300µg of protein). Visual comparison of [<sup>3</sup>H]5-CT autoradiograms representing total [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 (Figure 5.18b) and those in the presence of 200nM WAY100635 & 200nM GR125,743 (Figure 5.18c) clearly show a difference. This may suggest the presence of 5-HT<sub>1B/1D</sub> receptors in the rat adrenal medulla. However radioligand membrane binding studies in rat adrenal medulla or capsule membranes gave no specific [<sup>3</sup>H]5-CT binding (data not shown; up to 300µg of protein). In membrane binding studies, the complete dissection of the adrenal medulla proved too difficult, and so essentially decapsulated adrenals were used. The diffuse localisation and low density of 5-HT<sub>1A/1B/1D</sub> (but not 5-HT<sub>7</sub>) receptors seen autoradiographically in the adrenal medulla (Figure 5.18a), may have therefore not been detected in membrane binding studies.

#### **5.2.4 [<sup>3</sup>H]GR125,743 Binding Assays**

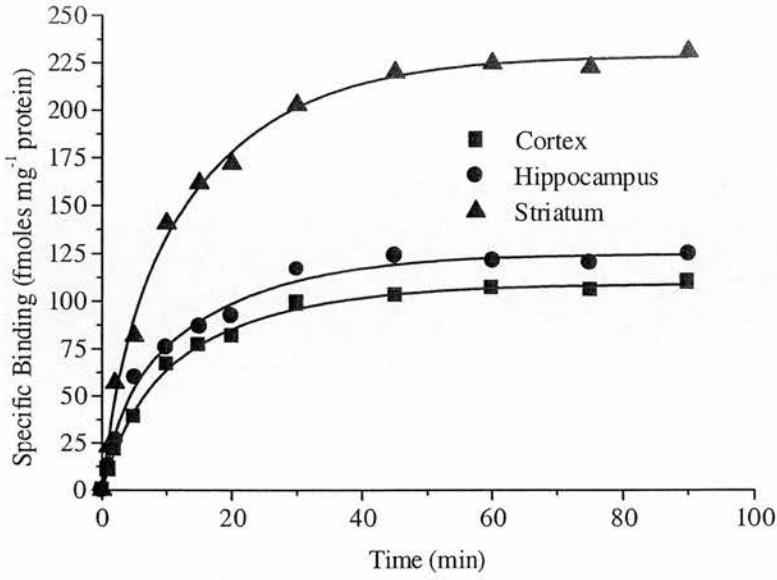
The 5-HT<sub>1B/1D</sub> receptor population could not be isolated using [<sup>3</sup>H]5-CT membrane binding as none of the drugs tested were suitable to block the 5-HT<sub>7</sub> component without inhibiting binding to the 5-HT<sub>1B/1D</sub> receptors. In addition there was no selective 5-HT<sub>7</sub> ligands commercially available. [<sup>3</sup>H]GR125,743 was therefore used to label the 5-HT<sub>1B/1D</sub> receptor population in the following experiments. Characterisation was limited to single time course and protein linearity experiments performed.

##### **5.2.4.1 Time Course of [<sup>3</sup>H]GR125,743 Binding to Rat Brain Membranes**

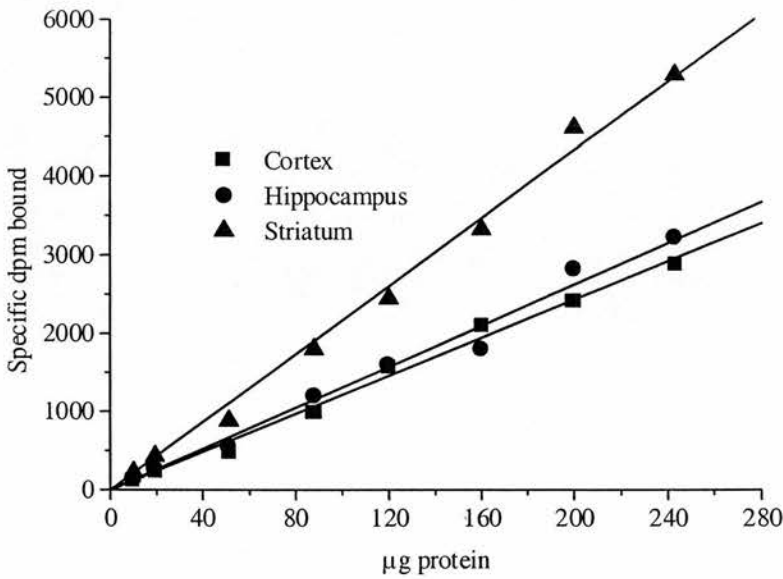
A time course (0-90 min) of [<sup>3</sup>H]GR125,743 (0.25nM) binding to rat cortex, hippocampus and striatum membranes was carried out at 25°C. Equilibrium was attained by 45 min being stable for at least another 45 min (Figure 5.19a). In the following competition experiments [<sup>3</sup>H]GR125,743 (0.25nM) binding to rat membranes was carried out at equilibrium at 25°C for 60 min as described in chapter 3.



(a)



(b)



**Figure 5.19: Time Course and Protein Linearity of [<sup>3</sup>H]GR125,743 Binding.**

The data represent a single (a) time course, and (b) protein linearity experiments. Experiments were performed in cortex, hippocampus and striatum brain areas as described in the text.

#### **5.2.4.2 Effect of Membrane Protein Concentration on [<sup>3</sup>H]GR125,743 Binding to Rat Brain Membranes**

Specific [<sup>3</sup>H]GR125,743 binding was dependent on membrane protein (Figure 5.19b) and was linear up to the concentrations measured (~250µg protein/assay) in cortex, hippocampus and striatum. Subsequent experiments were conducted with 80-200µg of cortex or hippocampus membrane protein, or 60-120µg of striatum membrane protein to ensure that less than 10% of added ligand was bound whilst maintaining a workable amount of specific binding.

#### **5.2.4.3 Concentration Dependence of [<sup>3</sup>H]GR125,743 Binding in Rat Brain Membranes.**

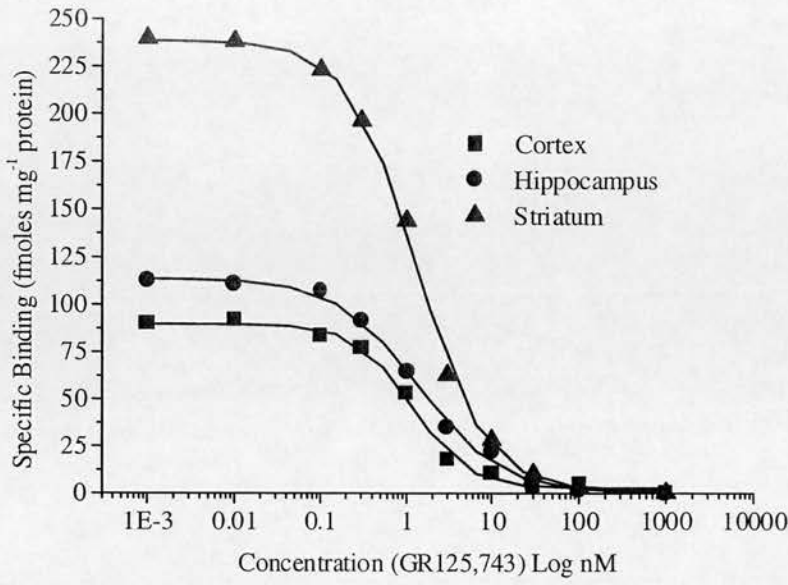
Competition ('cold saturation') binding studies using 0.25nM [<sup>3</sup>H]GR125,743 and increasing concentrations of unlabelled GR125,743 (0.003-1000nM) (Figure 5.20a) inhibited binding fully with Hill slopes of 1 and gave  $K_D$  values of  $1.29 \pm 0.06$ ,  $1.20 \pm 0.03$ , and  $1.20 \pm 0.04$  nM in cortex, hippocampus and striatum membranes respectively (n=4; Table 5.11). The corresponding  $B_{max}$  values were of  $0.50 \pm 0.12$ ,  $0.57 \pm 0.10$  and  $1.25 \pm 0.08$  pmol mg<sup>-1</sup> protein (Table 5.11). This confirms that the striatum, as in the [<sup>3</sup>H]5-CT binding studies, is rich in these receptors whereas both the cortex and hippocampus are relatively less abundant in 5-HT<sub>1B/1D</sub> receptors.

#### **5.2.4.4 Pharmacology of [<sup>3</sup>H]GR125,743 Binding in Rat Brain Membranes**

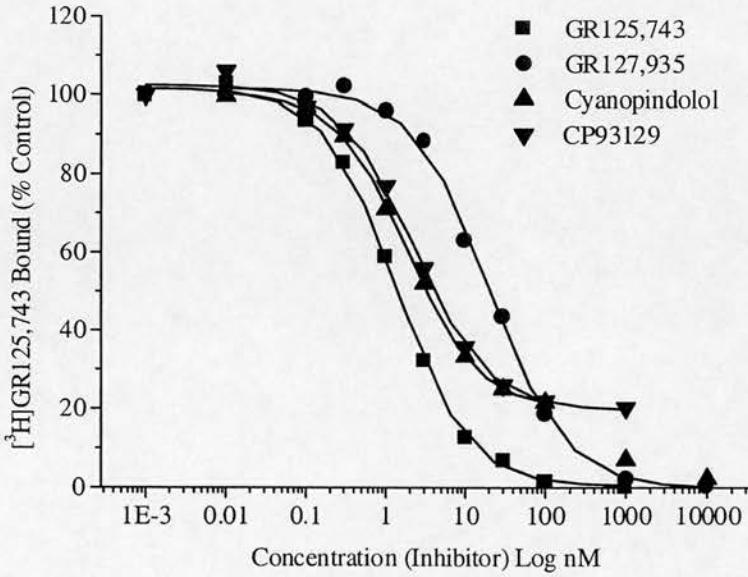
Six classes of drugs were tested which included: selective 5-HT<sub>1B/1D</sub> drugs (GR125,743 & GR127,935), selective 5-HT<sub>1B</sub> drugs (SB216641 & CP93129), selective 5-HT<sub>1D</sub> drugs (BRL15572), drugs with high affinity for both 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors (cyanopindolol), non-selective drugs (ritanserin & ketanserin) and drugs with high affinity at 5-HT<sub>1A</sub> receptors (WAY100635 & DP-5-CT).

GR125,743 and GR127,935 inhibited binding fully with Hill slopes of 1 and with similar affinities for the WAY100635 insensitive component of [<sup>3</sup>H]5-CT binding in section 5.2.2.5. (Table 5.11; Figure 5.20b). SB216641, cyanopindolol (fit

(a)



(b)



**Figure 5.20: Inhibition of [<sup>3</sup>H]GR125,743 Binding.**

The data represent typical experiments (a) cold saturation in cortex, hippocampus and striatum, and (b) inhibition of [<sup>3</sup>H]GR125,743 binding in the striatum. Mean data from at least three independent experiments (Table 5.12).

**Table 5.11: Pharmacology of [<sup>3</sup>H]GR125,743 Binding in Rat Brain Membranes.**

Competing ligand	Cortex			Hippocampus			Striatum		
	pK <sub>i</sub>	Hill slope	% Maximal inhibition	pK <sub>i</sub>	Hill slope	% Maximal inhibition	pK <sub>i</sub>	Hill slope	% Maximal inhibition
GR125,743	8.89 ± 0.06	0.91 ± 0.06	100	8.92 ± 0.03	1.11 ± 0.12	100	8.92 ± 0.04	0.97 ± 0.01	100
GR127,935	7.72 ± 0.12	0.94 ± 0.02	100	7.76 ± 0.13	1.12 ± 0.02	100	7.78 ± 0.10	1.04 ± 0.04	100
Cynaopindolol	9.00 ± 0.06	1.29 ± 0.02	88.25 ± 1.25	8.77 ± 0.06	0.98 ± 0.07	93.13 ± 1.29	8.78 ± 0.07	1.03 ± 0.07	80.26 ± 1.50
CP93129	8.45 ± 0.10	1.12 ± 0.15	86.33 ± 1.33	8.58 ± 0.16	1.17 ± 0.08	94.12 ± 1.10	8.50 ± 0.12	1.13 ± 0.10	81.12 ± 1.24
5-CT	9.06 ± 0.09	0.99 ± 0.10	100		not tested			not tested	
5-HT	8.73 ± 0.15	1.07 ± 0.09	100		not tested			not tested	
Ritanserin	5.79 ± 0.08	1.41 ± 0.30	100		not tested			not tested	
SB216641	8.71 ± 0.08	0.96 ± 0.02	87.25 ± 1.05		not tested			not tested	
Ketanserin	<6 (n=1)	n.d.	n.d.		not tested			not tested	
WAY100635	<6 (n=1)	n.d.	n.d.		not tested			not tested	
BRL15572	<6 (n=1)	n.d.	n.d.		not tested			not tested	
DP-5-CT	<6 (n=1)	n.d.	n.d.		not tested			not tested	
B <sub>max</sub>		503.10 ± 116.34			570.25 ± 101.20			1253.60 ± 178.80	
% binding remaining after CP93129 block		13.43 ± 1.10			8.43 ± 1.30			20.39 ± 1.05	
B <sub>max</sub> 5-HT <sub>1B</sub> site		435.55 ± 85.26			522.16 ± 83.78			997.99 ± 130.20	
B <sub>max</sub> 5-HT <sub>1D</sub> site		67.55 ± 14.66			48.09 ± 15.32			255.61 ± 23.27	

The affinity values (pK<sub>i</sub>) were determined from the inhibition of 0.25 nM [<sup>3</sup>H]GR125,743 to rat cerebral cortex, hippocampus and striatum membranes from at least three independent experiments (unless otherwise stated). Data is expressed as the mean ± s.e.mean. The B<sub>max</sub> values are expressed as fmoles mg<sup>-1</sup> protein. Maximal inhibition is quoted as the percentage of binding blocked by 100 nM cyanopindolol/SB216641 or 1 μM CP93129. The B<sub>max</sub> for the 5-HT<sub>1D</sub> component of binding was calculated according to the degree of block by 300 nM CP93129, assuming that GR125,743 has the same affinity for the 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> binding site (see text). n.d. = not determined.

up to 100nM) and CP93129 (fit up to 1 $\mu$ M) inhibited approximately 87, 92, and 80% of specific [ $^3$ H]GR125,743 binding in cortex, hippocampus and striatum membranes respectively, with affinities indicative of binding to 5-HT<sub>1B</sub> receptors and Hill Slopes of 1 (Table 5.11; Figure 5.20b). The affinity values were also similar to those obtained for the WAY100635 insensitive component of [ $^3$ H]5-CT binding in section 5.2.2.5. (Table 5.11; Figure 5.20b). Cyanopindolol also has high affinity at 5-HT<sub>1A</sub> receptors, but binding to this subtype was excluded by the low affinity of the selective 5-HT<sub>1A</sub> antagonist WAY100635 (Table 5.11). DP-5-CT also had low affinity indicative of 5-HT<sub>1B</sub> binding and non-5-HT<sub>1A</sub> binding. In rat brain membranes, the rank order of potency of all the drugs tested was: 5-CT > cyanopindolol > GR125,743 > 5-HT > SB216641 > CP93129 > GR127,935 > ritanserin, ketanserin, WAY100635, BRL15572, and DP-5-CT (Table 5.11).

The majority of [ $^3$ H]GR125,743 binding to 5-HT<sub>1B/1D</sub> receptors in these tissues is therefore to the 5-HT<sub>1B</sub> receptor. Competition curves with CP93129 reached a plateau between 100 and 1000nM, and hence a concentration of 300nM was used to represent binding to the 5-HT<sub>1D</sub> receptor (Figure 5.20b). In the striatum the highest proportion of 5-HT<sub>1B</sub> receptors (~80% of specific [ $^3$ H]GR125,743 binding) and 5-HT<sub>1D</sub> receptors (~20% of specific [ $^3$ H]GR125,743 binding) was observed. As GR125,743 had a Hill slope of 1 in all brain areas, it was assumed that GR125,743 was equipotent for both receptor subtypes as reported for the human 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors (Audinot *et al.*, 1997). B<sub>max</sub> values of the total and individual components of specific [ $^3$ H]GR125,743 binding could therefore be calculated with the use of the 5-HT<sub>1B</sub> block (300nM CP93129). This revealed that the highest density of 5-HT<sub>1D</sub> receptors was in the striatum, with much lower densities in the hippocampus and the cortex (see Table 5.11) which is in agreement with the relative proportions obtained in the [ $^3$ H]5-CT binding assays.

The kinetics and pharmacology of [ $^3$ H]GR125,743 binding did not differ between the studies reported above (in Wistar Cobb rats) and those in Sprague Dawley rats (data not shown).

### 5.3 Discussion

In this chapter, membrane radioligand binding assays were characterised for 5-HT<sub>1A</sub> receptors using [<sup>3</sup>H]8-OH DPAT, 5-HT<sub>7</sub> receptors using [<sup>3</sup>H]5-CT and 5-HT<sub>1B/1D</sub> receptors using [<sup>3</sup>H]GR125,743.

[<sup>3</sup>H]8-OH DPAT was used to label 5-HT<sub>1A</sub> receptors. The binding of [<sup>3</sup>H]8-OH DPAT to both cerebral cortex and hippocampus membranes was reversible, saturable and of high affinity. The K<sub>D</sub> and B<sub>max</sub> values were similar in their respective kinetic, saturation and competition studies in both membrane preparations (Figures 5.1 & 5.2). However, a higher density of [<sup>3</sup>H]8-OH DPAT binding sites (approximately 3 fold) was observed in hippocampus compared to cerebral cortex membranes which is in agreement with previous studies (Gozlan *et al.*, 1983). The pharmacology of [<sup>3</sup>H]8-OH DPAT binding was identical in cerebral cortex and hippocampus membranes (Table 5.1; Figure 5.4). The affinities of the drugs tested agreed with the known affinities at 5-HT<sub>1A</sub> receptors from previous studies (Gozlan *et al.*, 1983; Hoyer, 1989; Boess & Martin, 1994; Fletcher *et al.*, 1996). Therefore a binding assay for 5-HT<sub>1A</sub> receptors had been characterised using [<sup>3</sup>H]8-OH DPAT membrane binding.

[<sup>3</sup>H]5-HT has been previously used to label 5-HT<sub>7</sub> receptors in transfected cells (Plassat *et al.*, 1993; Ruat *et al.*, 1993b; Shen *et al.*, 1993; Bard *et al.*, 1993; Lovenberg *et al.*, 1993a). In native brain tissue 5-HT<sub>7</sub> receptors have been demonstrated to be involved in [<sup>3</sup>H]5-HT binding to non-5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors (Gobbi *et al.*, 1996). However the binding of [<sup>3</sup>H]5-HT in the presence of 3µM pindolol was heterogeneous and better fit a two site model of binding, suggesting that receptor sub-types other than 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors were present (Gobbi *et al.*, 1996). [<sup>3</sup>H]5-CT is alternative agonist radioligand, which binds with nanomolar affinity to 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>5A</sub> and 5-HT<sub>7</sub> receptor subtypes (Hoyer *et al.*, 1985a & b; Heuring & Peroutka, 1987; Hoyer, 1991; Nowak *et al.*, 1993). Previous studies with this radioligand in native tissues have required the use of various masking drugs in an attempt to block binding to non-5-HT<sub>7</sub> receptors. Using [<sup>3</sup>H]5-CT in the presence of such masking drugs, previous studies have identified a homogeneous 5-HT<sub>7</sub> receptor population in guinea pig brain membranes (To *et al.*, 1995; Boyland *et al.*, 1995). However in these same studies a

heterogeneous receptor population of [<sup>3</sup>H]5-CT binding sites in rat cerebral cortex homogenates was identified. A recent study was the first to label a homogeneous population of native 5-HT<sub>7</sub> receptors using [<sup>3</sup>H]5-CT binding in the presence of 10μM Pindolol (to block binding to 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors) and 100nM WAY100635 (to block residual binding to 5-HT<sub>1A</sub> receptors) in rat whole brain membranes (Stowe & Barnes, 1998b). These drugs would not block binding to 5-HT<sub>1D</sub> receptors known to be in low abundance throughout the rat brain (Bruinvels *et al.*, 1993). However in specific brain regions such as the striatum, there are relatively high levels of 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors (Bruinvels *et al.*, 1993). Therefore 5-HT<sub>1D</sub> receptors may have accounted for an increase in the non-5HT<sub>7</sub> receptor binding of this radioligand in previous reports in specific brain region homogenates, where only pindolol was used to mask binding to 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptors (Stowe & Barnes, 1996; Stowe & Barnes, 1998a). In this chapter the binding of [<sup>3</sup>H]5-CT was characterised in individual brain areas with variable amounts of 5-HT<sub>1B/1D</sub> expression, which were to be used in drug treatment studies (chapters 6 & 7). Experiments were primarily conducted using rat cortex membranes, and then subsequently with rat hippocampus (similar 5-HT<sub>1B/1D</sub> expression to cortex) and striatal membranes (higher 5-HT<sub>1B/1D</sub> expression).

In the absence of any masking drugs, the binding of [<sup>3</sup>H]5-CT to rat cortex membranes was reversible, saturable and of high affinity. The K<sub>D</sub> and B<sub>max</sub> values were similar in their respective kinetic, saturation and competition studies (Figures 5.4 & 5.5). The Hill slopes generated were not different from 1, which usually suggests binding to one population of receptors, but in this case confirms that [<sup>3</sup>H]5-CT labels multiple 5-HT receptors with approximately equal affinity. In an attempt to develop a [<sup>3</sup>H]5-CT binding assay for 5-HT<sub>7</sub> receptors in native rat brain membranes, it was necessary to mask binding to non-5-HT<sub>7</sub> receptors. [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> receptors was blocked by the use of the selective 5-HT<sub>1A</sub> antagonist, WAY100635. A variety of other 5-HT<sub>1A</sub> selective drugs were used to confirm the block of 5-HT<sub>1A</sub> receptors. Each compound tested inhibited [<sup>3</sup>H]5-CT binding fully, but best fit a two-site model of binding, with the high affinity proportion occupying approximately 60% of specific binding. The affinities of the high affinity sites of these compounds not only agreed with published data at the 5-

HT<sub>1A</sub> receptor (Boess & Martin, 1994), but also with data derived from [<sup>3</sup>H]8-OH DPAT binding in this chapter (Tables 5.4 & 5.5). WAY100635 inhibition binding curves reached an obvious plateau between 100 and 1000nM (Figure 5.6), and best fit a single site logistic model with a mean pK<sub>i</sub> value of 8.81 ± 0.16. This was in agreement with the known affinity of WAY100635 at 5-HT<sub>1A</sub> receptors (Khawaja *et al.*, 1995). 200nM WAY100635, which blocked 61.65 ± 0.99 % of the total specific binding, was therefore used to block [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A</sub> receptors. The pharmacology of all the 5-HT<sub>1A</sub> receptor ligands was identical in the hippocampus, and striatum, though the percentage block by 200nM WAY100635 was greater in the hippocampus, and much less in the striatum (Table 5.9). This is also in good agreement with a reported higher density of 5-HT<sub>1A</sub> receptors in the hippocampus (Deshmukh *et al.*, 1983).

In rat cortex membranes drugs with reported affinities at 5-HT<sub>1B</sub> receptors (eg. CP93129 and cyanopindolol), blocked approximately 60% of WAY100635 insensitive [<sup>3</sup>H]specific binding. This corresponded to 85% of total specific binding with affinities comparable to binding at 5-HT<sub>1B</sub> receptors and with Hill slopes of 1 (Table 5.6; Figure 5.7). Conversely, drugs with reported activities at both 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors (eg. GR125,743 and GR127,935) blocked WAY100635 insensitive [<sup>3</sup>H]specific binding by approximately a further 10% with Hill slopes of 1, confirming the presence of a small proportion of 5-HT<sub>1D</sub> receptors (Table 5.6; Figure 5.7). In rat hippocampus and striatum membranes the pharmacology of WAY100635 insensitive binding was identical to cortex membranes. However the total specific binding was inhibited by varying degrees, by either 300nM CP93129 (to define the proportion of 5-HT<sub>1B</sub> receptors) or 200nM GR125,743 (to define the proportion of 5-HT<sub>1B/1D</sub> receptors) in the presence of 200nM WAY100635 (Table 5.9; Figure 5.7). 200nM GR125,743, a selective 5-HT<sub>1B/1D</sub> antagonist, was used in conjunction with 200nM WAY100635 in subsequent experiments to determine the pharmacology of the remaining binding.

The remaining WAY100635 & GR125,743 [<sup>3</sup>H]5-CT binding was reversible, saturable and of high affinity (Figures 5.8 & 5.9). The K<sub>D</sub> and B<sub>max</sub> values were similar in their respective kinetic, saturation and competition studies. Calculation of B<sub>max</sub> values revealed a binding site density order of: hippocampus > cortex > striatum



(Table 5.9). However the Hill slope of  $<1$  for both competition and saturation experiments is possibly indicative of the presence of more than one binding site. All high and moderate affinity drugs inhibited binding fully to the non-specific level (defined by  $10\ \mu\text{M}$  5-HT), indicating that no non 5-HT sites were labelled. The pharmacology of the remaining [ $^3\text{H}$ ]5-CT binding in the presence of 200nM WAY100635 and 200nM GR125,743 revealed Hill slopes of one for all antagonists, whereas all agonists including 5-CT had Hill slopes of  $<1$  (Table 5.7). The pharmacology of this WAY100635 and GR125,743 insensitive binding site was identical in rat hippocampus and striatum membranes. Comparing the experimental affinities of drugs with the known affinities at recombinant 5-HT<sub>7</sub> receptors revealed a very good correlation ( $r = 0.90$ ; Figure 5.11). The rank order of affinities of the drugs tested was also similar as reported for recombinant 5-HT<sub>7</sub> receptors. This suggests that 5-HT<sub>7</sub> receptors are labelled by [ $^3\text{H}$ ]5-CT in the presence of 200nM WAY100635 and 200nM GR125,743.

The fact that all antagonists inhibited this remaining [ $^3\text{H}$ ]5-CT binding with Hill slopes of 1 suggests that another binding site other than the 5-HT<sub>7</sub> receptor is unlikely. The remaining binding (believed to be 5-HT<sub>7</sub>) is unlikely to include the 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> receptor since the low affinities of pindolol, cyanopindolol and WAY100635 are consistent with binding to 5-HT<sub>7</sub> but not 5-HT<sub>1</sub> receptors, where they all possess near nanomolar affinities (Table 5.2). It was not possible to determine the Hill slopes of these compounds due to the incomplete inhibition binding curves obtained, a consequence of their low affinity. 8-OH DPAT which is known to have sub- nanomolar affinity at the 5-HT<sub>1A</sub> receptor (Table 5.4), had a mean  $\text{pK}_i$  value of  $7.15 \pm 0.15$  at this binding site (Table 5.7) which further suggests that no residual 5-HT<sub>1A</sub> binding was present. 200nM WAY100635 was therefore sufficient to block binding to all 5-HT<sub>1A</sub> receptors.

Binding to residual 5-HT<sub>1D</sub> receptors was ruled out by the use of 200 nM GR125,743, the higher affinity of 5-CT and 8-OH DPAT in this study, compared to their lower affinities at 5-HT<sub>1D</sub> receptors and the low affinities of DP-5-CT and sumatriptan in this study, compared to their higher affinities at 5-HT<sub>1D</sub> receptors (Tables 5.2 & 5.7). Binding to residual 5-HT<sub>1B</sub> receptors was also ruled out by the use of 200nM GR125,743, which inhibited more binding than all of the 5-HT<sub>1B</sub>

selective drugs alone. Furthermore the majority of drugs tested in the presence of 200nM WAY100635 & 200nM GR125,743, also displayed a higher affinity for the remaining binding, than is known for 5-HT<sub>1B</sub> receptors (Tables 5.2 & 5.7).

The other 5-HT<sub>1</sub> receptor subtypes, 5-HT<sub>1E</sub> and 5-HT<sub>1F</sub> were ruled out by the high affinity of 5-CT in this study and would therefore not be labelled using 0.25nM [<sup>3</sup>H]5-CT (Hoyer *et al.*, 1994). The receptor labelled is very unlikely to include 5-HT<sub>2A</sub> or 5-HT<sub>2C</sub> receptors due to the low affinity of 5-CT at these subtypes, compared to the high affinity in this study. Furthermore, sumatriptan was found to have micromolar affinity in this study, whilst it has only millimolar affinity at 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors (Hoyer *et al.*, 1994). The high affinity of 5-CT and 5-MeOT also ruled out the involvement of 5-HT<sub>3</sub> receptors, where these agonists are inactive (Bard *et al.*, 1993, Hoyer *et al.*, 1994). 5-CT also binds to 5-HT<sub>5A</sub> receptors with nanomolar affinity (Hoyer *et al.*, 1994). This is unlikely to account for any of the remaining binding, due to the high affinities of mesulergine, methiothepin and metergoline at 5-HT<sub>7</sub> receptors as in our experimental assay (Table 5.7), but not at 5-HT<sub>5A</sub> receptors (Table 5.2). Furthermore, compounds with known high affinities at 5-HT<sub>5A</sub> receptors, such as ergotamine (Table 5.2), showed much lower affinity in the experimental assay (Table 5.7) in agreement with binding at 5-HT<sub>7</sub> receptors (Table 5.2). The high affinity of mesulergine, not only excluded the binding to 5-HT<sub>5A</sub> receptors, but also to 5-HT<sub>5B</sub> and 5-HT<sub>6</sub> receptors where it has micromolar affinity (Plassat *et al.*, 1993; Erlander *et al.*, 1993; Monsma *et al.*, 1993) and to 5-HT<sub>4</sub> receptors where it is inactive (Hoyer *et al.*, 1994). Further evidence against 5-HT<sub>5B</sub> and 5-HT<sub>6</sub> receptor involvement is the low affinity of 5-CT (pK<sub>i</sub> = 7.4 & 6.6 respectively) for these receptors (Hoyer *et al.*, 1994). While the 5-HT orphan receptor reported by Castro *et al.*, (1997b) has a high affinity for 5-CT, it cannot play any part in the remaining binding, since mesulergine has only a micromolar affinity at this uncharacterised receptor. The presence of other known receptor systems is unlikely for the reasons detailed above and also because all antagonists had Hill slopes of 1. Therefore the remaining [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 and 200nM GR125,743 is likely to represent binding to a 5-HT<sub>7</sub> receptor population in rat brain membranes.

The WAY100635 & GR125,743 [<sup>3</sup>H]5-CT binding to rat cortex, hippocampus or striatum membranes appears however to be heterogeneous due to the agonist hill slopes of <1. Shallow Hill slopes of agonist receptor binding can represent the presence of high and low affinity states of the receptor, reflecting the receptor being coupled to and uncoupled from G proteins (Gilman, 1987; Birnbaumer *et al.*, 1990). The existence of high and low affinity states of agonist 5-HT<sub>7</sub> receptor binding has indeed been observed in rat brain using the non-selective antagonist ligand [<sup>3</sup>H]mesulergine in the presence of masking agents for D<sub>2</sub> receptors, α<sub>1/2</sub>-adrenoreceptors and 5-HT<sub>2A/2C</sub> receptors, which displays a pharmacological profile broadly comparable to 5-HT<sub>7</sub> receptors in rat brain (Hemedah *et al.*, 1999).

Using membranes derived from guinea pig cerebral cortex, the initial binding of [<sup>3</sup>H]5-CT was reanalysed. As in the rat, 200nM WAY100635, was sufficient to block binding to 5-HT<sub>1A</sub> receptors (Table 5.8; Figure 5.12a). The pharmacology of the WAY100635 insensitive binding site, however was different to in rat membranes. Unlike in the rat, CP93129 and cyanopindolol, both displayed very low affinity for this remaining binding (Table 5.8; Figure 5.12b). This however was not surprising as the pharmacology of rodent 5-HT<sub>1B</sub> receptors compared to non-rodent 5-HT<sub>1B</sub> receptors is different due to a single amino acid difference; asparagine in rat and threonine in guinea pig at position 355 (Boess & Martin, 1994; Hoyer *et al.*, 1994). The 5-HT<sub>1B/1D</sub> drugs gave the same profile as in the rat, with 200nM GR125,743 being sufficient to block binding to the 5-HT<sub>1B/1D</sub> receptor population. The remaining binding in the presence of 200nM WAY100635 and 200nM GR125,743 correlated well with affinities at both recombinant and native guinea pig 5-HT<sub>7</sub> receptors (Figures 5.13a & 5.13b). The correlation between affinities at native experimentally determined rat and guinea pig 5-HT<sub>7</sub> receptors was very good (Figure 5.13c), although the agonist DP-5-CT was approximately 1000 fold more potent at guinea pig 5-HT<sub>7</sub> receptors in this study and others (To *et al.*, 1995). There are also other isolated reports of an apparent species difference in the 5-HT<sub>7</sub> receptor. For example, the affinity of clozapine for the rat 5-HT<sub>7</sub> receptor was found to be almost two orders of magnitude greater than the affinity of the compound at the mouse 5-HT<sub>7</sub> receptor (Sleight *et al.*, 1995b). Of particular importance is that agonists and antagonists

inhibited WAY100635 & GR125,743 [ $^3\text{H}$ ]5-CT binding fully with Hill slopes of 1, suggesting the lack of different affinity states of this G-protein coupled receptor in the guinea pig brain.

Another possibility which may help to explain the observed complexity of [ $^3\text{H}$ ]5-CT agonist binding in the rat brain compared to in the guinea pig brain is the presence of multiple 5-HT<sub>7</sub> receptor isoforms in the rat. Alternative splicing of the single receptor gene which encodes the 5-HT<sub>7</sub> receptor (Gelertner *et al.*, 1995) has enabled the identification of four different isoforms of the 5-HT<sub>7</sub> receptor (5-HT<sub>7(a)</sub>, 5-HT<sub>7(b)</sub>; 5-HT<sub>7(c)</sub> & 5-HT<sub>7(d)</sub>; Heidmann *et al.*, 1997), three of which exist in the rat (5-HT<sub>7(a)</sub>, 5-HT<sub>7(b)</sub> and 5-HT<sub>7(d)</sub>; Heidmann *et al.*, 1998). In guinea pig the presence of one receptor isoform (5-HT<sub>7(a)</sub>) has previously been shown not to complicate agonist [ $^3\text{H}$ ]5-CT binding as in this study (Tsou *et al.*, 1994; To *et al.*, 1995; Boyland *et al.*, 1995). In humans, as in rats, three isoforms exist, namely 5-HT<sub>7(a)</sub>, 5-HT<sub>7(b)</sub> and 5-HT<sub>7(d)</sub> (Heidmann *et al.*, 1997), but [ $^3\text{H}$ ]5-CT binding in the presence of blocks that would selectively label 5-HT<sub>7</sub> receptors in guinea pigs where one isoform exists, only appears to label a heterogeneous population of receptors (Barnes *et al.*, 1997). Differential coupling to these isoforms has been suggested by Clemett *et al.*, (1997), who have shown a differential desensitisation of human 5-HT<sub>7(a)</sub> and 5-HT<sub>7(b)</sub> receptor mediated cAMP responses. The possible presence of high and low affinity states of [ $^3\text{H}$ ]5-CT agonist binding in the rat (indicated by low agonist Hill slopes) but not in the guinea pig seen in this study, may therefore be due to differential G protein coupling.

The unsuccessful past development of binding assays for native 5-HT<sub>7</sub> receptor detection in rat brain, has hindered autoradiographic studies in this tissue. It has been unclear if appropriate conditions have been used to detect the 5-HT<sub>7</sub> receptor, possibly due to insufficient blockade of 5-HT<sub>1A</sub> and/or 5-HT<sub>1B/1D</sub> receptors, and a possible involvement of 5-HT<sub>5A</sub> receptors (Waeber & Moskowitz, 1995a; Gustafson *et al.*, 1996). Gustafson *et al.*, (1996) believe that [ $^3\text{H}$ ]5-CT binding in the presence of 30nM PAPP and 160nM pindolol is mainly to 5-HT<sub>7</sub> receptors. They claimed that as [ $^3\text{H}$ ]5-CT was used at the  $K_D$  for 5-HT<sub>7</sub> receptors, there can be no binding to 5-HT<sub>5A/5B</sub> receptors as the affinity of 5-CT for these receptors is three fold lower (Table 5.3). This is not the case, as 5-CT also has

nanomolar affinity for 5-HT<sub>5A</sub> receptors (Table 5.2). Waeber & Moskowitz (1995a), in contrast, claimed that they have no evidence for 5-HT<sub>5A</sub> binding, despite the reported high affinity, but rather the heterogeneous nature of their binding is more likely due to the incomplete blockade of other 5-HT receptor subtypes, particularly 5-HT<sub>1A</sub>. This seems unlikely, as 100nM 8-OH DPAT should be a sufficiently high concentration to block binding to 5-HT<sub>1A</sub> receptors (Figure 5.6a). 100nM 8-OH DPAT would however also inhibit some 5-HT<sub>7</sub> binding. It is more likely that the 100nM GR127,935 is not sufficient to block binding to 5-HT<sub>1B/1D</sub> receptors. GR127,935 (Figure 5.7b), a weaker compound than its structurally similar counterpart GR125,743 (Audinot *et al.*, 1997; Table 5.6) would need to be used at micromolar concentrations to be effective. It is therefore unlikely that GR127,935 blocked all 5-HT<sub>1B/1D</sub> binding and that this actually corresponds to the heterogeneity of binding, apparent in another study using 250nM GR127,935 (Mengod *et al.*, 1996). In the absence of available selective antagonist ligands, it was therefore important to re-evaluate the autoradiographic localisation of 5-HT<sub>7</sub> binding sites using the binding assay conditions developed in this chapter, where there was only evidence of [<sup>3</sup>H]5-CT binding to 5-HT<sub>1A/1B/1D</sub> and 5-HT<sub>7</sub> receptors.

The autoradiographic distribution of 5-HT<sub>7</sub> binding sites in this chapter correlates well with that of previous studies. Relatively high binding site densities were observed in the hippocampus areas, the septum and the dorsal raphé, and moderate to low binding site densities observed in the striatum, cortex and hypothalamus (To *et al.*, 1995; Gustafson *et al.*, 1996; Table 5.10; figures 5.15 to 5.18). The rank order of autoradiographic binding site densities agreed well with the [<sup>3</sup>H]5-CT membrane binding studies with the hippocampus being the most abundant and the cortex/striatum the least. The highest density of 5-HT<sub>7</sub> binding sites was located in the dorsal raphé (Table 5.11; Figure 5.15e). The identification of 5-HT<sub>7</sub> mRNA in a serotonergic cell line derived from embryonic rat raphé neurons (Jackson *et al.*, 1997), as well as detection in the dorsal and paramedian raphé nuclei of mature rats (Ruat *et al.*, 1993b) may indicate the 5-HT<sub>7</sub> receptor as an additional serotonergic cell body autoreceptor.

Binding site densities also correlated well with reported 5-HT<sub>7</sub> receptor mRNA signals, with highest receptor densities being detected in the thalamus,

hippocampus, superficial cortex layers, amygdala and hippocampus (To *et al.*, 1995; Gustafson *et al.*, 1996). Although levels of 5-HT<sub>7</sub> mRNA and binding site densities are generally well correlated, previous studies have revealed differences. Hippocampus mRNA levels were high in the guinea-pig CA3 region, while [<sup>3</sup>H]5-HT binding sites were higher in the CA1 and CA2 regions, suggesting a possible pre-synaptic location of the 5-HT<sub>7</sub> receptor in the CA3 region (To *et al.*, 1995). 5-HT<sub>7</sub> binding sites were also present in areas (central grey, superior colliculus and spinal trigeminal nucleus) lacking 5-HT<sub>7</sub> mRNA, which may also reflect presynaptic 5-HT<sub>7</sub> binding sites (To *et al.*, 1995). In this study in the rat brain, the CA3 region of the hippocampus gave a higher density of 5-HT<sub>7</sub> binding sites compared to the CA1 and CA2 regions of the hippocampus. The high levels of 5-HT<sub>7</sub> mRNA in this structure (Gustafson *et al.*, 1996) suggest a possible pre-synaptic location in this species. Furthermore the superior colliculus also gave a very high density of 5-HT<sub>7</sub> binding sites (not measured), an area which has very low 5-HT<sub>7</sub> mRNA (Gustafson *et al.*, 1996), which may also suggest a pre-synaptic location. The substantia nigra, like the superior colliculus (two structures with high 5-HT<sub>1B</sub> receptor density; Sari *et al.*, 1999), contained relatively high levels of 5-HT<sub>7</sub> receptor density (Table 5.10). The substantia nigra contains no 5-HT<sub>7</sub> mRNA and receives inputs from the striatum (To *et al.*, 1995; Gustafson *et al.*, 1996). The striatum contains no 5-HT<sub>7</sub> mRNA (To *et al.*, 1995; Gustafson *et al.*, 1996) but does contain 5-HT<sub>7</sub> binding sites as shown in this study. This may therefore represent a pre-synaptic location in the substantia nigra or striatum, or even non-5-HT<sub>7</sub> receptor binding. The latter is unlikely as the concentration of blocks used were sufficient to block non-5-HT<sub>7</sub> binding in the membrane binding assays. However the affinity of GR125,743 for 5-HT<sub>1B/1D</sub> binding sites could be different in the substantia nigra, compared to the striatum, and hence some binding may represent incomplete blockade of non-5-HT<sub>7</sub> receptors. Similarly, although the binding to 5-HT<sub>5A</sub> receptors was not detected in hippocampus, cortex or striatum membranes, the distribution of the 5-HT<sub>5A</sub> receptor is widespread throughout the rat brain (Erlander *et al.*, 1993) and so may contribute to non-5-HT<sub>7</sub> binding in other structures. However no binding was detected in the cerebellum (data not shown), where it is known that there is no 5-HT<sub>7</sub> mRNA, but where there is appreciable quantities of 5-HT<sub>5A</sub> mRNA (Plassat *et al.*, 1992; Erlander *et al.*, 1993).

Experiments were also performed in rat adrenal glands where it is believed there is a lack of 5-HT<sub>7</sub> mRNA (Lovenberg *et al.*, 1993a). No specific [<sup>3</sup>H]5-CT autoradiographic binding was observed in any region of the adrenal gland in the presence of 200nM WAY100635 & 200nM GR125,743 (Figure 5.18). This in contrast to a recent study whereby functionally active 5-HT<sub>7</sub> receptors have been demonstrated in the zona glomerulosa (Contesse *et al.*, 1999). The resolution of such a region by autoradiographic experiments or a very low abundance may explain why no 5-HT<sub>7</sub> receptors were detected in the present study. Although no specific [<sup>3</sup>H]5-CT or [<sup>3</sup>H]8-OH DPAT binding could be detected in membranes derived from the rat adrenal medulla (data not shown), 5-HT<sub>1A</sub> and 5-HT<sub>1B/1D</sub> receptors could be autoradiographically demonstrated in the rat adrenal medulla (Figure 5.15). This is in contrast to other 5-HT receptors which are found in the cortex or zona glomerulosa of the adrenal gland and are associated with the release of steroids (Williams *et al.*, 1984; Matsouka *et al.*, 1985; Lefebvre *et al.*, 1992; Contesse *et al.*, 1999). This may suggest a different role for those receptors not previously discovered in the rat adrenal medulla. The lack of 5-HT<sub>7</sub> binding in the adrenal medulla, but presence of 5-HT<sub>1B/1D</sub> binding confirms the ability of 200nM GR125,743 to completely block binding to these 5-HT<sub>1B/1D</sub> receptor subtypes.

The lack of selective 5-HT<sub>7</sub> antagonists precluded the use of [<sup>3</sup>H]5-CT to develop a binding assay to directly study the 5-HT<sub>1B/1D</sub> receptor population. Therefore the selective 5-HT<sub>1B/1D</sub> selective antagonist [<sup>3</sup>H]GR125,743 was used to characterise a membrane binding assay for 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors which has previously only been used in human and guinea pig studies (Audinot *et al.*, 1997; Doménech *et al.*, 1997). The binding of [<sup>3</sup>H]GR125,743 to rat cortex, hippocampus and striatum membranes was of high affinity. The K<sub>D</sub> values were similar in the three different brain areas (cortex, hippocampus, and striatum) from competition studies and the pharmacology identical (Table 5.11). Furthermore similar affinities were obtained compared to studies with [<sup>3</sup>H]5-CT (Table 5.6). The B<sub>max</sub> values were of the rank order; striatum > hippocampus = cortex (Figure 5.20; Table 5.11), confirming the higher expression of 5-HT<sub>1B/1D</sub> binding sites in the striatum (Table 5.11). In the presence of 300nM CP93129 (the selective 5-HT<sub>1B</sub> agonist; Macor *et al.*, 1990) the 5-HT<sub>1B</sub> component of [<sup>3</sup>H]GR125,743 binding was effectively blocked. Only 20, 13

and 8 % of initial binding remained in striatum, cortex, and hippocampus respectively, which correlates with the rank order of 5-HT<sub>1D</sub> expression being highest in the striatum and lowest in the hippocampus. The distribution of these receptors and the fact that most 5-HT<sub>1B/1D</sub> receptors in rat brain are of the 5-HT<sub>1B</sub> type are in agreement with other studies (Pazos & Palacios, 1985a; Sijbesma *et al.*, 1991; Bruinvels *et al.*, 1993a & b; Sari *et al.*, 1997). Hill slopes of GR125,743 were not different from unity, which in the case of the striatum, where there is a higher proportion of 5-HT<sub>1D</sub> receptors, suggests that GR125,743 has the same affinity for both receptor subtypes as is apparent at the equivalent human receptors (Audinot *et al.*, 1997). This enabled calculation of the individual B<sub>max</sub> values for both receptor subtypes (Table 5.11).

At present there are no selective ligands for 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors, although there are reports of two compounds which can differentiate between human/guinea pig 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors in recombinant cell lines (Price *et al.*, 1997) and in functional studies (Schliker *et al.*, 1997). These two compounds, SB216641 and BRL15572 were included in this study. SB216641, inhibited both [<sup>3</sup>H]GR125,743 and [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 with Hill slopes of 1, to the level of the block caused by 300nM CP93129 (Tables 5.6 & 5.12). It would therefore appear that SB216641, is also selective for the 5-HT<sub>1B</sub> receptor subtype in native rat tissue. Moreover the affinity in these rat studies were similar to the reported nanomolar affinity for human cloned 5-HT<sub>1B</sub> receptors (Price *et al.*, 1997). Despite the reported difference in pharmacology between rodent and non-rodent 5-HT<sub>1B</sub> receptors due to a single amino acid difference, there are indeed some compounds that show no such differences in affinity (eg. 5-CT, 5-HT, DHE & methiothepin; see Boess & Martin, 1994). BRL15572 on the other hand completely inhibited WAY insensitive [<sup>3</sup>H]5-CT binding with a Hill slope of 1 and micromolar affinity, suggesting that in the rat, this compound can not differentiate between 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub> or 5-HT<sub>7</sub> receptors.



## 5.4 Summary

In this chapter membrane binding assays were characterised for the 5-HT<sub>1A/1B/1D</sub> and 5-HT<sub>7</sub> receptors, prior to their use for receptor abundance and affinity determinations after drug treatments in chapters 6 & 7.

[<sup>3</sup>H]8-OH DPAT was used to label 5-HT<sub>1A</sub> receptors, whose pharmacology agreed well with previous reports.

[<sup>3</sup>H]5-CT in the presence of 200nM WAY100635 (to block binding to 5-HT<sub>1A</sub> receptors) and 200nM GR125,743 (to block binding to 5-HT<sub>1B/1D</sub> receptors) revealed a pharmacology for the remaining component consistent with 5-HT<sub>7</sub> receptor binding. The Hill slopes of 1 for antagonists suggest specific labelling. The Hill slopes of <1 for agonists (in rat brain only) suggests a heterogeneity of agonist binding, which was not due to incomplete blockade of 5-HT<sub>1A/1B/1D</sub> receptors or due to binding to 5-HT<sub>5A</sub> receptors. It also suggests the presence of high and low affinity states of this G protein coupled receptor. The complex binding of this agonist radioligand in rat brain in this study (and in human brain) may also potentially be due to differential G-protein coupling of the multiple 5-HT<sub>7</sub> receptor isoforms found in these species. This complexity of binding is not observed in guinea pig brain where only one 5-HT<sub>7</sub> receptor isoform exists.

[<sup>3</sup>H]5-CT autoradiographic experiments, revealed a higher density of 5-HT<sub>7</sub> binding sites in the hippocampus than in the cortex or striatum, in agreement with the membrane binding studies. The highest binding was in the dorsal raphe, suggesting a possible role for the 5-HT<sub>7</sub> receptor as an autoreceptor. In the adrenal gland, no 5-HT<sub>7</sub> binding was detected, but binding sites for the 5-HT<sub>1A</sub> and 5-HT<sub>1B/1D</sub> receptors were specifically located in the rat adrenal medulla.

[<sup>3</sup>H]GR125,743 was used to label 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors, whose pharmacology agreed well with both previous reports and [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635. The rank order of 5-HT<sub>1B/1D</sub>, 5-HT<sub>1B</sub> or 5-HT<sub>1D</sub> receptors was: striatum > hippocampus >= cortex which was in agreement with [<sup>3</sup>H]5-CT binding studies.

**CHAPTER 6**

**THE EFFECT OF CHRONIC ANTIDEPRESSANT  
TREATMENTS ON THE DENSITY AND AFFINITY OF THE  
5-HT TRANSPORTER AND 5-HT RECEPTORS**

The hypothesis that disturbances in normal serotonergic transmission play an important role in the aetiology of depression is based predominantly on the clinical efficacy of selective serotonin re-uptake inhibitors (SSRIs) as highly effective antidepressants (Åsberg *et al.*, 1986; Blier *et al.*, 1990; Price, 1990; Delgado *et al.*, 1992). SSRIs are effective inhibitors of the 5-HT transporter (SERT) and bind to the same or closely overlapping site in SERT as 5-HT itself (Bäckström *et al.*, 1989; Graham *et al.*, 1989). The therapeutic action of SSRIs is generally believed to result from their ability to enhance central 5-HT neurotransmission by increasing the synaptic availability of 5-HT (Blier *et al.*, 1987; Chaput *et al.*, 1991). Although SSRIs inhibit SERT within minutes (De Montigny *et al.*, 1984; Stark *et al.*, 1985; Artigas *et al.*, 1996), their full antidepressant action is not apparent until after a therapeutic delay of some 2-3 weeks (Meltzer & Lowy, 1987; Blier & De Montigny, 1994). However the nature of the link between the acute actions of these drugs *in vitro* and the weeks of treatment required for clinical improvement remains unresolved.

SERT is abundantly expressed in the midbrain raphe complex, whereas lower but measurable concentrations are found in the projections areas of the cortex, hippocampus and striatum (Hrdina *et al.*, 1990; Lesch *et al.*, 1993a). 5-HT<sub>1A</sub> receptors are also relatively high in abundance in the midbrain raphe nuclei (Pazos & Palacios, 1985), with lower concentrations in the terminal fields of the hippocampus and cortex (Deshmukh *et al.*, 1983; Gozlan *et al.*, 1983, and Vergé *et al.*, 1986). The majority of these terminal field 5-HT<sub>1A</sub> receptors are putatively postsynaptic (Francis *et al.*, 1992; Lawrence *et al.*, 1993). It is also well established that 5-HT<sub>1B</sub> receptors regulate 5-HT release from terminals of serotonergic neurons ascending from the dorsal and median raphe nuclei to the hippocampus, the striatum and the cortex (Engel *et al.*, 1986; Maura *et al.*, 1986; Limberger *et al.*, 1991).

Acute administration of SSRIs results in a large increase in 5-HT efflux in several sub-cortical brain regions as shown by microdialysis in freely moving rats *in vivo* (see Fuller *et al.*, 1994). This increase, which is dependent on neuronal firing, involves mechanisms in the terminal field since efflux of 5-HT is increased by local infusion of the SSRI via the dialysis probe (Auerbach *et al.*, 1989). However, changes in the concentration of extracellular 5-HT in dialysates of the cerebral cortex

after systemic drug administration are inconsistent (Artigas, 1993) and relatively high doses are required (Invernizzi *et al.*, 1992a). Acute administration of SSRIs preferentially increase extracellular levels of 5-HT in the midbrain raphe nuclei (Aghajanian 1978; Adell & Artigas, 1991; Invernizzi *et al.*, 1992a). This results in a reduction in neuronal firing (Chaput *et al.*, 1986) due to somatodendritic 5-HT<sub>1A</sub> receptor activation (Vandermaelen & Aghajanian, 1983; Adell & Artigas, 1991; Hodgkiss *et al.*, 1992;). This decrease in serotonergic neuronal activity following acute administration limits the ability of SSRIs to increase 5-HT levels in the forebrain (e.g., frontal cortex), as 5-HT synthesis (Carlsson & Lindqvist, 1978) and release (Fuller *et al.*, 1974) are actually reduced. However there is regional variation in this effect (Frankfurt *et al.*, 1994). Increased extracellular 5-HT levels in the terminal fields can also cause activation of 5-HT<sub>1B</sub> or 5-HT<sub>1D</sub> receptors which can cause feedback inhibition of transmitter release in rat forebrain projection areas (Sharp *et al.*, 1989; Auerbach *et al.*, 1991; Hjorth & Tao, 1991). Thus it appears that 5-HT autoreceptors have a restraining influence on pharmacological treatments in the short-term that would otherwise tend to greatly increase 5-HT neurotransmission. This is supported by the ability of 5-HT autoreceptor antagonists to augment the increase in extracellular 5-HT levels produced by acute administration of SSRIs alone (Invernizzi *et al.*, 1992a; Hjorth *et al.*, 1993; Rollema *et al.*, 1996) or in combination with an even greater effect (Gobert *et al.*, 1997; Sharp *et al.*, 1997). In some scenarios, however, the concurrent block of all three 5-HT autoreceptors is required to elevate terminal field 5-HT concentrations and/or to overcome SSRI induced inhibition of 5-HT synthesis (Barton & Hutson, 1999; Roberts *et al.*, 1999).

In contrast to acute treatment, long-term antidepressant treatment causes a sustained increase in the extracellular concentrations of 5-HT (Rutter *et al.*, 1994), even in the cerebral cortex (Bel, & Artigas, 1993). This is believed to be due to the desensitisation of somatodendritic 5-HT<sub>1A</sub> receptors and nerve terminal 5-HT<sub>1B/1D</sub> receptors (De Montigny *et al.*, 1984; Vergé *et al.*, 1985; Chaput *et al.*, 1986 & 1988; Sprouse & Aghajanian, 1987; Blier *et al.*, 1988 a & b; Moret and Briley, 1990; Hjorth, 1993; Le Poul *et al.*, 1995a & b). The desensitisation of the 5-HT<sub>1A</sub> receptor allows serotonergic neurons to resume their normal firing activity in the presence of continued re-uptake inhibition (Chaput *et al.*, 1986; Blier *et al.*, 1990). It has also

been hypothesised that chronic SSRI treatment increases 5-HT release, perhaps by down-regulating terminal autoreceptors (Blier *et al.*, 1987 & 1990; Briley & Moret, 1993a & b; Mansari *et al.*, 1995). The resumption of serotonergic neuronal activity after 5-HT autoreceptor desensitisation is thought to lead to an overall enhancement of serotonin neurotransmission, which, in turn, may mediate the therapeutic effect. The time course of these desensitisations is consistent with the delayed onset of action of these drugs in the clinic. Indeed co-administration of a 5-HT<sub>1A</sub> receptor antagonist (pindolol) with an SSRI (citalopram) has been shown to enhance the therapeutic efficacy and shorten the onset of action of SSRIs in depressive patients (Artigas *et al.*, 1996). Gradual desensitisation of these 5-HT autoreceptors may therefore underlie the delayed onset of action of SSRIs.

However, this is not the only mechanism that could be involved. Sustained blockade of the 5-HT transporter (SERT) located in the terminal field has been shown to produce a desensitisation of the 5-HT re-uptake response. Long-term treatments with paroxetine (a high affinity SSRI) induced a decreased uptake of [<sup>3</sup>H]5-HT into hippocampus slices and a decrease in the density of SERT (Piñeyro *et al.*, 1994). Furthermore, long term administration of antidepressants, which inhibit 5-HT re-uptake such as the SSRI, fluoxetine and the tricyclics, imipramine and chlorimpramine, decrease the steady-state concentrations of brain SERT mRNA (Lesch *et al.*, 1993a). These studies collectively suggest that chronic treatment with re-uptake inhibiting antidepressants may be associated with down-regulation of SERT at the levels of gene and protein expression. SERT is also found in the rat adrenal medulla as shown in chapter 4. The adrenal gland is a primary source of steroids, which have been implicated in the depressive state (see Barden *et al.*, 1995). Therefore part of the mechanism of antidepressant drugs may be due to the inhibition of adrenal SERT.

The identification of a further class of 5-HT receptor, namely the 5-HT<sub>7</sub> receptor, warrants its investigation as to its potential involvement in the mechanism of action of antidepressant drug treatments. Several different classes of antidepressant drugs have been reported to interact with the 5-HT<sub>7</sub> receptor; both amitriptyline and mianserin significantly enhance 5-HT<sub>7</sub>-mediated cAMP accumulation in rat frontocortical astrocytes following chronic exposure (Shimizu *et*

*al.*, 1996). Glucocorticoids have been implicated in the aetiology of depressive illness (Dinan, 1996), and the alteration of circulating glucocorticoid levels alters both 5-HT<sub>7</sub> mRNA levels and functional adenylyl cyclase responses (Yau *et al.*, 1997a; Le Corr e *et al.*, 1997; Shimizu *et al.*, 1997). In rat frontocortical astrocytes, prolonged exposure to dexamethasone reduced both 5-HT<sub>7</sub>-mediated cAMP accumulation and 5-HT<sub>7</sub> mRNA levels (Shimizu *et al.*, 1997). Additionally, blockade of endogenous corticosterone synthesis by chemical adrenalectomy up-regulates 5-HT<sub>7</sub> receptor mRNA expression in rat hippocampal subfields, demonstrating that the 5-HT<sub>7</sub> receptor can be regulated by circulating adrenal steroids (Yau *et al.*, 1997a; Le Corr e *et al.*, 1997). Furthermore the 5-HT<sub>7</sub> receptor has been suggested to be involved in the therapeutic effects of the adrenocortical hormone synthesis blockers, such as aminoglutethimide and metyrapone, which have been used to treat drug-resistant depression (Yau *et al.*, 1997a).

To date there has only been two reports on the effects of an SSRI chronic treatment on 5-HT<sub>7</sub> receptors in native brain tissue. In the first, chronic fluoxetine treatment caused a down-regulation in the density of 5-HT<sub>7</sub> binding sites in the rat hypothalamus (Sleight *et al.*, 1995a). However the conditions of the radioligand binding assay employed have been disputed (Gobbi *et al.*, 1996), and so the results need to be treated with caution. In a second study, using more appropriate binding conditions a significant decrease in 5-HT<sub>7</sub> receptor density in the rat hypothalamus was observed with a variety of chronic antidepressant treatments, including fluoxetine (Mullins *et al.*, 1999). The 5-HT<sub>7</sub> receptor has been implicated in the regulation of circadian rhythms (Lovenberg *et al.*, 1993a) which is controlled in part by the suprachiasmatic nucleus of the hypothalamus (SCN; Ralph *et al.*, 1990). Many researchers have suggested that alterations in circadian rhythm play an important role in the aetiology of depression (see Duncan, 1996). Indeed many classes of antidepressants have been shown to shift established rhythmicity and possess the ability to restore experimentally phase-shifted circadian activity patterns (Wollnick, 1992). c-Fos (a member of the AP-1 family of transcription factors) is significantly induced in the SCN in response to the acute administration of 5-HT<sub>7</sub> agonists (5-CT & 8-OH DPAT) and a variety of antidepressant drugs (fluoxetine, mianserin, imipramine, desipramine, clorgyline, or nefazodone; Mullins *et al.*, 1999). This

response is desensitised following chronic antidepressant treatment with an accompanying neuroadaptive downregulation of 5-HT<sub>7</sub> receptor density in the hypothalamus (Mullins *et al.*, 1999). These recent findings further support a role for the 5-HT<sub>7</sub> receptor in the mechanism of antidepressant action and in the regulation of the circadian rhythms controlled by the SCN.

Factors controlling the delayed mechanism of action of most antidepressants may involve changes in receptor expression throughout the time course of treatment until clinical benefit is observed. Such long-term neuroadaptive mechanisms responsible for the therapeutic effect of antidepressants have been suggested (Briley & Moret, 1993b). These may involve alterations in the density of SERT and/or 5-HT<sub>1A</sub> receptors and/or 5-HT<sub>1B/1D</sub> receptors and/or 5-HT<sub>7</sub> receptors to control the availability of 5-HT in the terminal fields and consequently to increase 5-HT neurotransmission. Potential adaptive changes of SERT density after chronic antidepressant treatment were therefore investigated in the 5-HT projection areas of the cortex, hippocampus and striatum and in the periphery in the rat adrenal medulla, using membrane [<sup>3</sup>H]citalopram binding as previously described (chapters 3 & 4). Using site-directed SERT antibodies characterised in Chapter 2, SERT density was assessed qualitatively in the raphé, a particularly small region of the rat brain that was too small to use in conventional membrane binding studies. Immunohistochemical methods were also used to assess SERT density in the projectional areas of the cortex, striatum and hippocampus to complement radioligand binding methods. Western blot analysis was also included to assess any possible effects in the rat brain and rat blood platelets using a site-directed SERT antibody. The effect of chronic antidepressant treatment on the density of 5-HT<sub>1A</sub> receptors was assessed in the projection areas of the cortex and hippocampus using [<sup>3</sup>H]8-OH DPAT membrane binding. 5-HT<sub>1B/1D</sub> receptors were also investigated in the same projection areas as the more robust 5-HT terminal marker (SERT) using [<sup>3</sup>H]GR125,743 membrane binding. The conditions described in chapter 5 to selectively label the 5-HT<sub>7</sub> receptor were used to investigate the effect of chronic antidepressant treatment on the density of 5-HT<sub>7</sub> receptors in the projection areas of the cortex, hippocampus and striatum.

Three antidepressants were used for chronic treatment studies to investigate potential neuroadaptive changes of SERT and 5-HT receptors, which may further help to explain the mechanism of action of distinct classes or individual antidepressants. These included the two SSRIs fluoxetine and citalopram. Fluoxetine (Prozac®) is the least selective inhibitor of 5-HT uptake (Stanford, 1996) and citalopram (Cipramil®) the most selective inhibitor of 5-HT uptake (Hyttel, 1994). The atypical antidepressant, tianeptine, was also included, as unlike the SSRIs it selectively stimulates the high affinity uptake of [<sup>3</sup>H]5-HT by rat brain synaptosomes without affecting noradrenergic and dopaminergic uptake systems (Mennini *et al.*, 1987; Fattaccini *et al.*, 1990) and without modifying the frequency of 5-HT neuronal firing (Piñeyro *et al.*, 1995c & d).

## **6.1 Methods**

### **6.1.1 Treatment of Animals**

Male Sprague-Dawley rats (Charles River, 200-250g) on the day of arrival, were housed in groups of three to four for three days, given free access to food and water and kept on a 12 hour light cycle at 21°C throughout the study. Following a further two days habituation (which involved frequent handling to reduce experimental stress), all animals were put on a restricted diet of 10g chow per 100g rat body weight per day, but with free access to water and kept on this regime throughout the treatment. Animals were dosed by subcutaneous injection, with twice daily injections between 08:00 and 09:00, and again between 16:00 and 17:00. Each drug used was dissolved in 0.9% saline. For each antidepressant tested, 0.1ml of stock solution was injected subcutaneously (s.c.) per 100g body weight. Control groups represent those animals receiving the equivalent volumes of 0.9% saline vehicle for the duration of the antidepressant treatment. The final dose was followed by a two full day washout period (64-72 hours) before animals were used for neurochemical or immunological experiments. Two sets of independent chronic antidepressant treatments were carried out.

#### **Treatments to Study SERT and 5-HT<sub>1A</sub> Receptors**

The first set of chronic antidepressant treatments involved three independent studies (repeated twice). For each study 20 animals were used in total. Ten were



treated with antidepressant and ten with control. Animals in the first study were injected with either citalopram (10mg/kg bd for 21 days) or control. In a similar chronic study, this dose has previously been shown to desensitise somatodendritic 5-HT<sub>1A</sub> autoreceptors (Invernizzi *et al.*, 1994). Animals in the second study were injected in the mornings only with either fluoxetine (10mg/kg od for 21 days) or control. Once daily dosing with fluoxetine has previously been shown to desensitise somatodendritic 5-HT<sub>1A</sub> autoreceptors through the course of a chronic treatment (Le Poul *et al.*, 1995a & b). Animals in the third study were injected with either (10mg/kg bd for 14 days) tianeptine or control. This dose was used as previous studies have shown an increase in 5-HT uptake in rat platelets and brain synaptosomes (Mennini *et al.*, 1987).

For each independent antidepressant study 6 control and 6 drug treated animals were used for binding studies. Rats for these studies, after the washout period, were killed by a sharp blow to the back of the neck, the head guillotined and membranes immediately made according to the methods previously described in chapter 3. Brain frontal cortex, caudal cortex, hippocampus, and striatum and also adrenal medulla membranes were prepared. From these animals the effect of chronic antidepressant treatments on SERT and 5-HT<sub>1A</sub> receptors was investigated. For each independent antidepressant study, 2 control and 2 drug treated animals were also used for immunohistochemical studies. A further 2 control and 2 treated animals were used for Western blot analysis.

### **Treatments to Study 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> Receptors**

This series of chronic antidepressant treatments involved two independent studies with 6 animals in each study group. In the first study, rats were treated with either citalopram (10mg/kg bd for 21 days), fluoxetine (10mg/kg bd for 21 days) or control. In the second study, rats were treated with either tianeptine (10mg/kg bd for 14 days) or control. Rats for these studies, after the washout period, were killed by a sharp blow to the back of the neck, the head guillotined and membranes immediately made according to the methods previously described in chapter 3. Brain frontal cortex, caudal cortex, hippocampus, and striatum and also adrenal medulla membranes were prepared. From these animals the effect of chronic antidepressant treatments on 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> receptors was investigated. However as

fluoxetine was administered using a twice daily dosing regime the effect on SERT was initially reassessed in comparison to twice daily dosing in citalopram, tianeptine and control treated rats in frontal cortex membranes only.

### 6.1.2 Neurochemical and Immunological Assays

Membrane radioligand binding assays were conducted as described previously (chapters 3, 4 & 5). For each binding assay 6 control and 6 treated animals were compared in membranes from each brain area and the adrenal medulla. However the small size of the striatum and hippocampus only permitted 3 control and 3 treated animals to be compared in both 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> receptor binding assays.

In addition to the calculation of the  $K_D$  and  $B_{max}$  of the 5-HT<sub>7</sub> receptor binding site, the proportion of receptors labelled by 0.25nM [<sup>3</sup>H]5-CT were calculated as described in chapter 5. In the 5-HT<sub>7</sub> receptor binding assays untreated naïve membranes were assayed to ensure that the conditions of the blocks used were sufficient to reveal the 5-HT<sub>7</sub> receptor population. Inhibition curves for WAY100635 against [<sup>3</sup>H]5-CT binding and GR125,743 against WAY100635 insensitive [<sup>3</sup>H]5-CT binding were generated in naïve membranes corresponding to the tissue being assayed.

In addition to the calculation of the  $K_D$  and  $B_{max}$  of the 5-HT<sub>1B/1D</sub> receptor binding sites, the proportion of receptors labelled by 0.25nM [<sup>3</sup>H]GR125,743 were calculated as described in chapter 5. The calculation of the  $K_D$  and  $B_{max}$  values of the individual 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptor components were calculated assuming that GR125,743 had equal affinity for these receptor subtypes as described and discussed in chapter 5. In the 5-HT<sub>1B/1D</sub> receptor binding assays untreated naïve membranes were assayed to ensure that the conditions of the blocks used were sufficient to reveal the 5-HT<sub>1D</sub> receptor population. An Inhibition curve for CP93129 against [<sup>3</sup>H]GR125,743 binding was generated in naïve membranes corresponding to the tissue being assayed.

SERT abundance was qualitatively assessed in the projection areas of the cortex, hippocampus and striatum and also in the raphe nucleus, using the 998 antibody (1 in 2000 dilution) in immunohistochemical studies whose characterisation

and methodology was determined in chapter 2. SERT abundance was also qualitatively assessed in brain cortical and rat platelet homogenates, using the 1001 antibody (1 in 200 dilution) in Western blotting studies whose characterisation and methodology was determined in chapter 2.

## **6.2 Results**

### **6.2.1 Effect of Chronic Antidepressant Treatments on SERT**

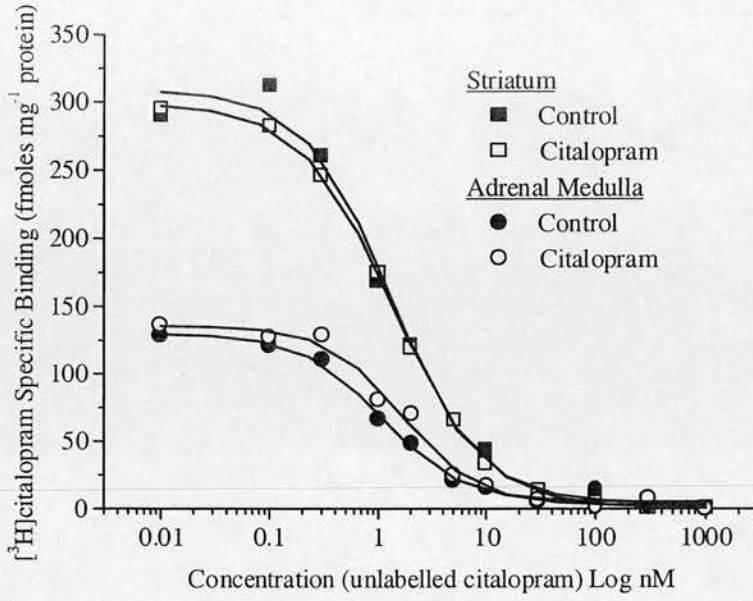
#### **6.2.1.1 [<sup>3</sup>H]Citalopram Binding Studies in Rat Brain and Adrenal Medulla Membranes**

Chronic citalopram (10mg/kg bd s.c. for 21 days), fluoxetine (10mg/kg once daily for 21 days) or tianeptine (10mg/kg bd s.c. for 14 days) treatments caused no difference in either the density or affinity of SERT binding sites labelled with 0.25nM [<sup>3</sup>H]citalopram in frontal or caudal cortex, hippocampus or striatum membranes, when compared to controls (Table 6.1; Figure 6.1a). Each treatment was repeated twice and the same results obtained (data not shown). Similarly there were no differences in the [<sup>3</sup>H]citalopram binding parameters in rat adrenal medulla membranes after chronic SSRI or tianeptine treatments, when compared to controls (Table 6.1; Figure 6.1a). The Hill slopes, whether from brain or adrenal membranes were unaffected by chronic antidepressant treatment (Table 6.1).

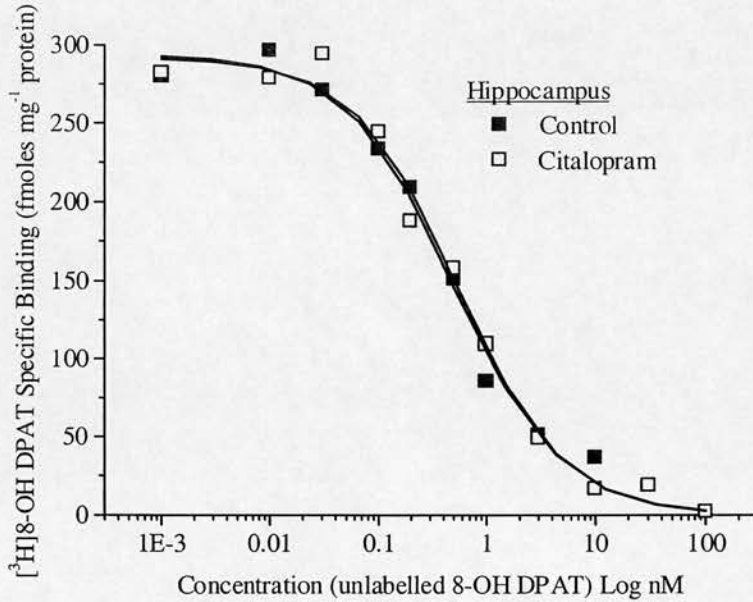
#### **6.2.1.2 Immunohistochemical and Western Blot Studies**

There was no apparent difference in the terminal field staining in regions of the cortex, hippocampus or striatum after each antidepressant treatment compared to controls. There was also no difference in cell body staining in the raphe after any antidepressant treatment compared to controls (Figure 6.2). Qualitative analysis of SERT abundance using Western blots also revealed no differences after chronic antidepressant treatment compared to controls, in cerebral cortex or platelet homogenates. Cerebral cortex immunoreactivity was detected at 76 kDa, and platelet immunoreactivity detected at 94 kDa (Figure 6.3).

(a)



(b)



**Figure 6.1: Example [<sup>3</sup>H]Citalopram & [<sup>3</sup>H]8-OH DPAT Binding Isotherms After Chronic Antidepressant Treatment.**

Data represent typical (a) [<sup>3</sup>H]citalopram (0.25 nM) binding isotherms in striatum and adrenal medulla membranes after chronic citalopram treatment (10 mg/kg bd for 21 days) and (b) [<sup>3</sup>H]8-OH DPAT (0.25 nM) binding isotherms in hippocampus membranes after chronic citalopram treatment (10 mg/kg bd for 21 days). Mean data is in tables 6.1 & 6.2.

Treatment	Fronal Cortex Membranes		Caudal Cortex Membranes		Hippocampus Membranes	
	K <sub>D</sub>	B <sub>max</sub>	nH	K <sub>D</sub>	B <sub>max</sub>	nH
Control	1.11 ± 0.06	2.00 ± 0.10	0.99 ± 0.03	1.16 ± 0.06	1.45 ± 0.07	1.02 ± 0.04
Citalopram	1.03 ± 0.05	2.03 ± 0.15	1.03 ± 0.05	1.16 ± 0.06	1.36 ± 0.08	1.11 ± 0.09
Control	0.91 ± 0.04	1.93 ± 0.26	1.04 ± 0.06	1.06 ± 0.11	1.26 ± 0.16	1.09 ± 0.04
Fluoxetine	0.97 ± 0.11	2.00 ± 0.17	1.13 ± 0.04	1.11 ± 0.03	1.23 ± 0.14	1.11 ± 0.03
Control	0.91 ± 0.06	1.76 ± 0.11	0.92 ± 0.03	1.06 ± 0.08	1.19 ± 0.11	1.07 ± 0.04
Tianeptine	1.06 ± 0.09	1.89 ± 0.18	0.95 ± 0.05	1.08 ± 0.06	1.15 ± 0.08	1.00 ± 0.04

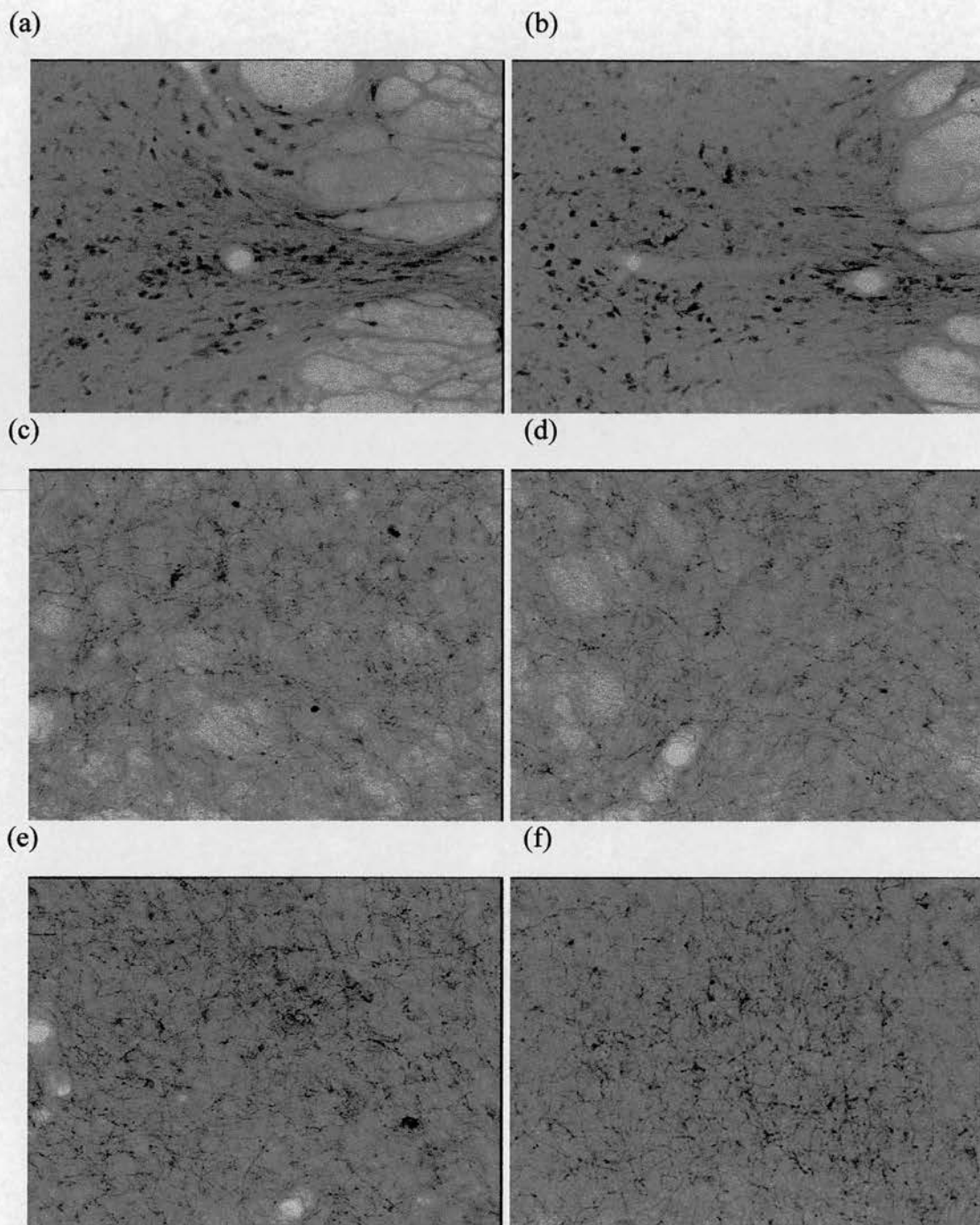
  

Treatment	Striatum Membranes		Adrenal Medulla Membranes	
	K <sub>D</sub>	B <sub>max</sub>	nH	K <sub>D</sub>
Control	1.05 ± 0.06	1.60 ± 0.15	1.05 ± 0.02	0.92 ± 0.11
Citalopram	1.19 ± 0.07	1.61 ± 0.17	1.09 ± 0.05	1.18 ± 0.08
Control	0.95 ± 0.07	1.53 ± 0.13	1.07 ± 0.03	1.02 ± 0.06
Fluoxetine	1.16 ± 0.16	1.69 ± 0.26	1.02 ± 0.05	1.10 ± 0.08
Control	1.18 ± 0.19	1.50 ± 0.22	0.89 ± 0.03	1.15 ± 0.05
Tianeptine	0.91 ± 0.18	1.43 ± 0.12	0.92 ± 0.03	1.06 ± 0.09

**Table 6.1: Effect of Chronic Antidepressant Treatments on 5-HT Transporters.**

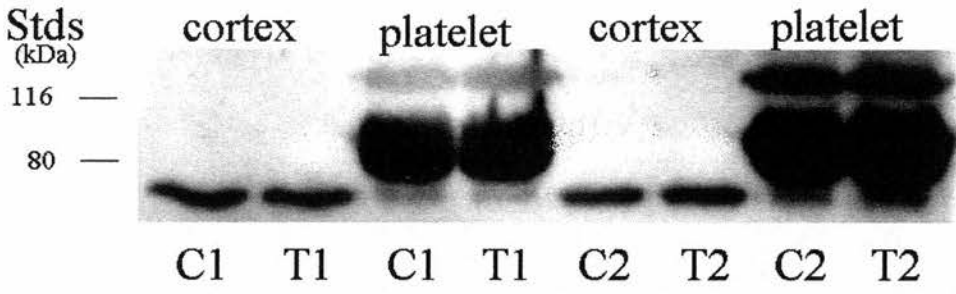
The 5-HT transporter population was labelled with 0.25nM [<sup>3</sup>H]citalopram. Three independent chronic antidepressant treatments are shown: 10mg/kg citalopram twice daily for 21 days, 10mg/kg fluoxetine once daily for 21 days and 10mg/kg twice daily for 14 days tianeptine. The corresponding controls received the equivalent volume of vehicle. Each animal was allowed a 2 full day washout period after the final dose before membranes were made for binding studies. The results are expressed as the mean ± s.e.mean for 6 animals in each group. The K<sub>D</sub> in nM, the B<sub>max</sub> in pmoles mg<sup>-1</sup> protein and the Hill slope (nH) are given.





**Figure 6.2: Immunohistochemistry of SERT Protein After Chronic Antidepressant Treatment.**

Data presented are representative immunohistological rat brain sections processed for SERT immunoreactivity after different chronic antidepressant treatments using the 998 antibody as described in Chapter 2. Each section was processed using a 1 in 2000 dilution of the 998 antibody and images represent (a) control and (b) tianeptine (10mg/kg bd for 14 days) treated in dorsal raphé (magnification x100), (c) control and (d) fluoxetine (10mg/kg once daily for 21 days) treated in striatum (magnification x 200), (e) control and (f) citalopram (10mg/kg bd for 21 days) treated in hippocampus (magnification x 200).



**Figure 6.3: Western Blot Analysis of SERT Protein After Chronic Antidepressant Treatment with 1001 Antibody.**

Example of western blot analysis from rats chronically treated with fluoxetine (10mg/kg once daily for 21 days) using an antibody designed to the 4<sup>th</sup> extracellular loop of SERT as described in Chapter 2 and assigned as 1001. Tissue homogenates were prepared according to the methods described in chapter 2 and loaded to give 50µg of protein per lane. Standard rainbow wide-range colour markers (Sigma) gave the appropriate molecular weight standards, shown to the left of the blot. Cerebral cortex immunoreactivity was observed at 76 kDa and platelet immunoreactivity at 94 kDa. C1= control animal 1, T1= fluoxetine treated animal 1, C2= control animal 2, T2= fluoxetine treated animal 2.



## 6.2.2 Effect of Chronic Antidepressant Treatments on 5-HT<sub>1A</sub> Receptors

Chronic citalopram (10mg/kg bd s.c. for 21 days), fluoxetine (10mg/kg once daily for 21 days) or tianeptine (10mg/kg bd s.c. for 14 days) treatments caused no changes in either the density or affinity of 5-HT<sub>1A</sub> binding sites labelled with 0.25nM [<sup>3</sup>H]8-OH DPAT in frontal cortex, caudal cortex, or hippocampus membranes, when compared to controls (Table 6.2; Figure 6.1b). The Hill slopes were unaffected by chronic antidepressant treatment (Table 6.2). Each treatment was repeated twice and the same results obtained (data not shown).

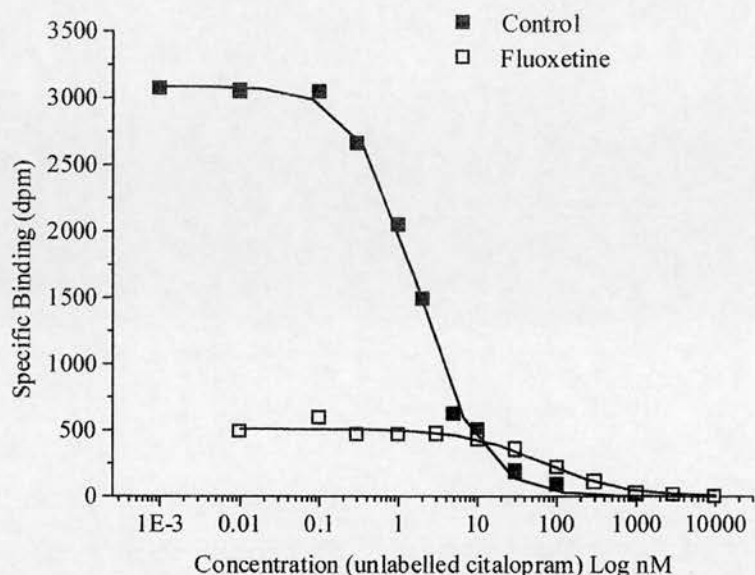
## 6.2.3 Effects of Chronic Fluoxetine Twice Daily Treatments on SERT and 5-HT<sub>1A</sub> Receptors

Chronic fluoxetine treatments using a once daily dosing regime resulted in no change in the binding parameters as measured in the above sections (Tables 6.1 & 6.2). To make this SSRI treatment more comparable to citalopram and tianeptine treatments, chronic fluoxetine treatments were conducted as before but with a twice daily, rather than a once daily dose of 10mg/kg.

[<sup>3</sup>H]citalopram binding studies revealed a significant decrease in the amount of specific binding (93 %) and affinity (45 fold) in frontal cerebral cortex membranes (Table 6.3; Figure 6.4). No change in any binding parameter in frontal cortex membranes was once again observed after chronic citalopram or tianeptine treatments (data not shown). Accompanying these reductions in the frontal cortex after chronic twice daily dosing of fluoxetine was a 2-3 fold increase in the B<sub>max</sub> values (Table 6.3). Using adrenal medulla membranes, there was an 84% reduction in the amount of [<sup>3</sup>H]citalopram binding specific binding ( $93 \pm 7$  and  $15 \pm 7$  fmoles mg<sup>-1</sup> protein in fluoxetine treated and control animals respectively). Due to the low amount of binding in the fluoxetine treated animals, no affinity values could be determined. This effect may have been due to presence of residual fluoxetine competing for [<sup>3</sup>H]citalopram binding sites.

In an attempt to investigate whether or not residual fluoxetine could account for these results, competition [<sup>3</sup>H]citalopram binding assays were constructed in

**Figure 6.4: Effect of Chronic Fluoxetine Treatment on Specific [<sup>3</sup>H]Citalopram Binding (DPM) in Frontal Cortex Membranes.**



Rats were treated with: fluoxetine (10mg/kg bd for 21 days) or the equivalent volume of vehicle (control). Following a two day washout period, animals were sacrificed and membranes made. The 5-HT transporter population was labelled with 0.25nM [<sup>3</sup>H]citalopram. Figure 6.4 shows typical binding isotherms obtained using 147µg of fluoxetine treated protein and 55µg of control treated protein. Combining the data from 6 animals per group is shown in Table 6.3 below.

**Table 6.3: Effect of Chronic Fluoxetine Treatment on [<sup>3</sup>H]Citalopram Binding in Frontal Cortex Membranes.**

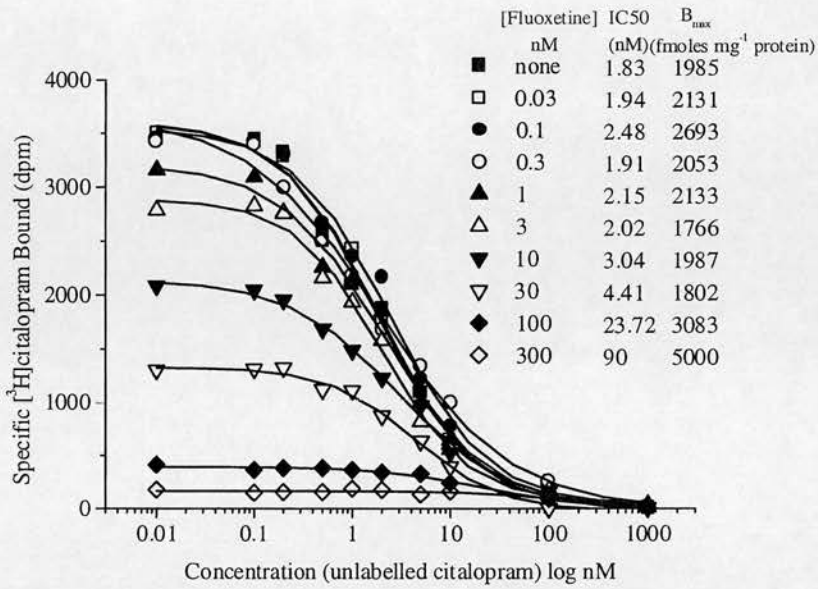
	$K_D$	$B_{max}$	nH	Specific Binding
Control	$1.81 \pm 0.05$	$2.14 \pm 0.08$	$1.07 \pm 0.02$	$256 \pm 8$
Fluoxetine	$83 \pm 17^*$	$5.66 \pm 1.21^*$	$0.99 \pm 0.18$	$19 \pm 5^*$

The results are expressed as the mean  $\pm$  s.e.mean. The  $K_D$  in nM, the  $B_{max}$  in fmoles  $mg^{-1}$  protein, Hill slope (nH), and the amount of specific binding at 0.25nM [<sup>3</sup>H]citalopram in fmoles  $mg^{-1}$  protein are given. \* =  $p < 0.05$  compared to controls.

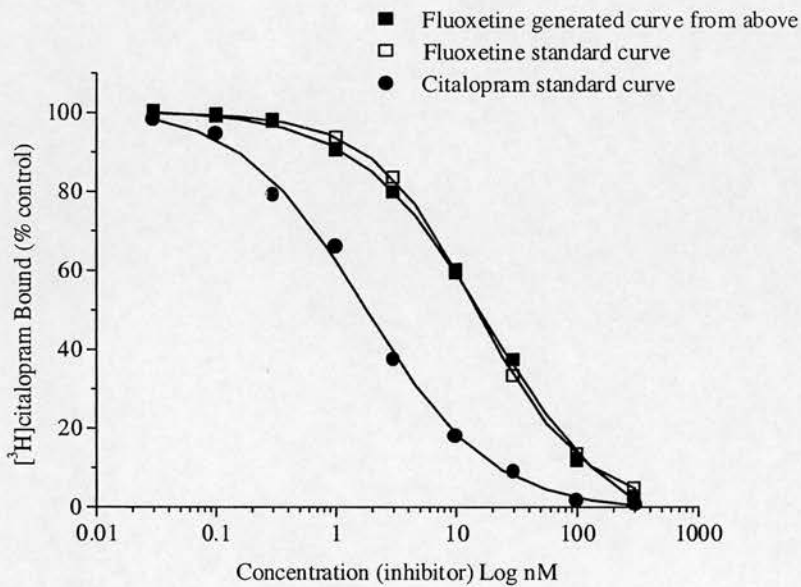
naïve cerebral cortex membranes (non-treated rat membranes), whereby citalopram inhibition curves were conducted in the absence or presence of increasing concentrations of fluoxetine. This was done to estimate the concentration of residual fluoxetine required to cause a 45 fold decrease in affinity compared to control. As expected there were decreasing amounts of specific 0.25nM [<sup>3</sup>H]citalopram binding in the presence of increasing concentrations of fixed concentrations of fluoxetine (Figure 6.5a). In the presence of 10nM or greater fluoxetine the affinity of citalopram was reduced. Concentrations of fluoxetine between 100 and 300nM in the assay caused an approximate 88-97% reduction in specific [<sup>3</sup>H]citalopram binding and a 20 to 100 fold decrease in affinity (Figure 6.5a). At these concentrations the B<sub>max</sub> value was increased by 2 to 3 fold, whereas the B<sub>max</sub> did not change at or below 10nM fluoxetine. This is comparable to that observed in membranes derived from animals dosed twice daily with fluoxetine (Table 6.3), suggesting that residual fluoxetine may be competing for [<sup>3</sup>H]citalopram binding sites. Furthermore, the IC<sub>50</sub> value of the generated fluoxetine curve of 18.99nM is in good agreement with the fluoxetine standard curve of 15nM (Figure 6.5b) and the affinity values determined in chapter 4.

From these frontal cortex membranes from fluoxetine treated animals, binding experiments were also conducted using [<sup>3</sup>H]8-OH DPAT and [<sup>3</sup>H]nisoxetine to investigate any effect on 5-HT<sub>1A</sub> receptors and NET respectively. There was no change in the amount of specific binding using either ligand after chronic fluoxetine twice-daily treatment as compared to controls (Figure 6.6). Fluoxetine has very low affinity for 5-HT<sub>1A</sub> receptors (pK<sub>i</sub> of 4.62 as determined in chapter 5). Therefore if fluoxetine was present in the [<sup>3</sup>H]8-OH DPAT binding assay at concentrations less than 1μM, no difference in specific binding would be expected. Fluoxetine however has reasonable affinity for NET (pK<sub>i</sub> of 6.06 as determined in chapter 4). If there was a high concentration of fluoxetine present in the assay (100-300 nM as described above), there should be a reduction of between 9 and 23% in [<sup>3</sup>H]nisoxetine binding, based on a theoretical model of fluoxetine inhibition of [<sup>3</sup>H]nisoxetine binding (Figure 6.7). No reduction was however observed in the amount of specific [<sup>3</sup>H]nisoxetine binding (Figure 6.6). Therefore residual fluoxetine does not appear to account for the striking differences seen in these studies.

(a)



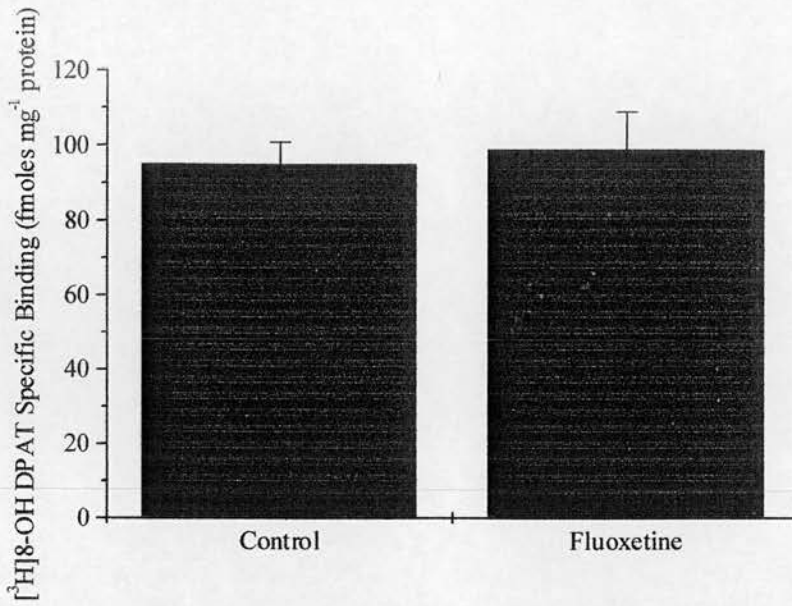
(b)



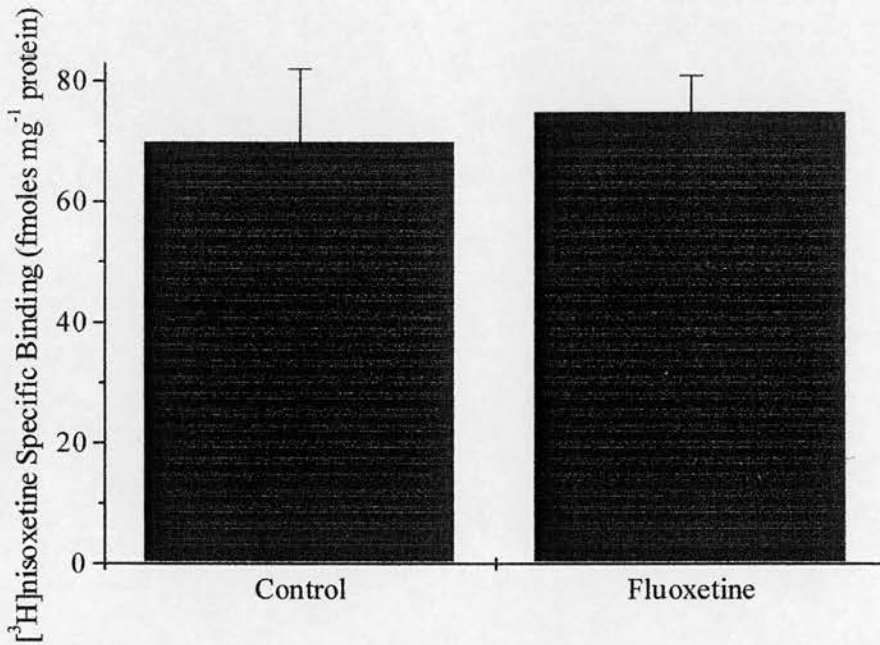
**Figure 6.5: Inhibition of [<sup>3</sup>H]citalopram Binding by Fluoxetine.**

Data shown represents; (a) inhibition of [<sup>3</sup>H]citalopram binding by citalopram in the presence of varying concentrations of fluoxetine (b) inhibition of [<sup>3</sup>H]citalopram by citalopram and fluoxetine and the generated fluoxetine inhibition curve from (a).

(a)

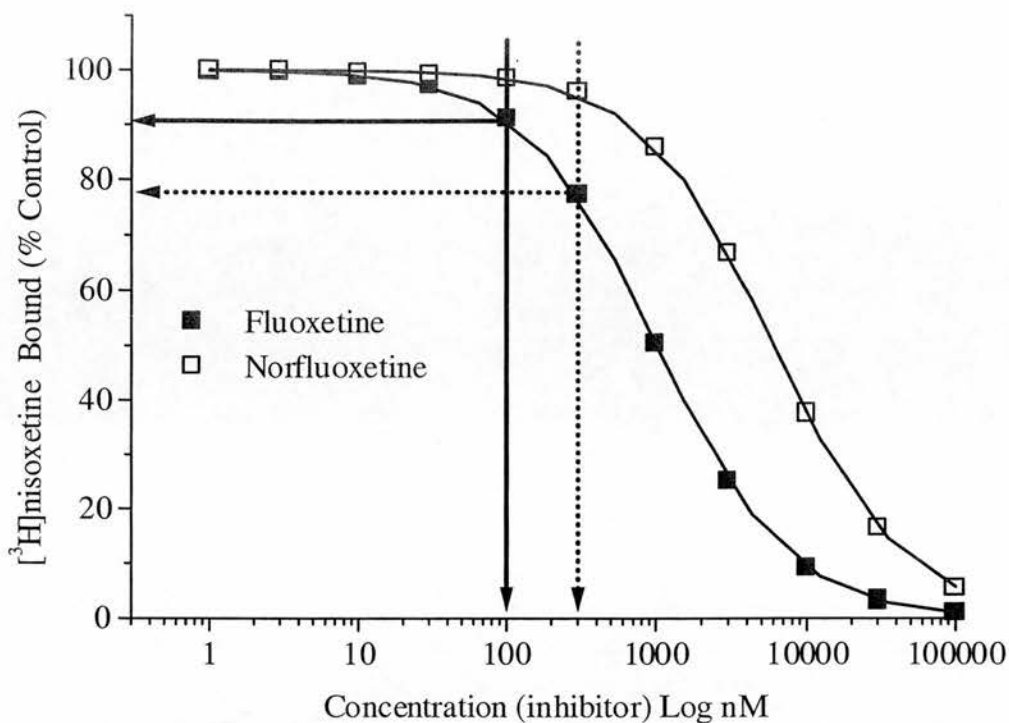


(b)



**Figure 6.6: Effect of Chronic Twice Daily Fluoxetine Treatment on [3H]8-OH DPAT & [3H]nisoxetine Binding.**

Rats were treated with: fluoxetine (10mg/kg bd for 21 days) or the equivalent volume of vehicle (control). The results are expressed as the mean  $\pm$  s.e.m from 6 animals per group in frontal cortex membranes. 5-HT<sub>1A</sub> receptors were labelled with 0.25nM [3H]8-OH DPAT in (a). The noradrenaline transporter was labelled with 0.50nM [3H]nisoxetine in (b).



**Figure 6.7: Theoretical Displacement of  $[^3\text{H}]$ Nisoxetine Binding by Fluoxetine and Norfluoxetine.**

Using the mean  $pK_i$  value of fluoxetine for nisoxetine binding (chapter 4), and assuming approximately 6 fold lower potency of the demethylated metabolite, norfluoxetine, theoretical binding curves are shown. 100nM fluoxetine would account for a 9% decrease in nisoxetine binding. 300nM fluoxetine would account for a 23% reduction in nisoxetine binding. These concentrations of norfluoxetine would account for a negligible decrease in nisoxetine binding.

Fluoxetine is extensively biotransformed by N-demethylation to norfluoxetine (Parli & Hicks, 1974). In animal models, norfluoxetine is a potent and selective inhibitor of serotonin uptake with activity essentially equivalent to fluoxetine (Wong *et al.*, 1993). Despite similar affinities for 5-HT re-uptake, the half-life of norfluoxetine is considerably longer than that of the parent drug (Caccia *et al.*, 1990 & 1992). It is therefore likely that the effects seen after chronic fluoxetine twice daily dosing treatments on [<sup>3</sup>H]citalopram binding could be explained by the presence of residual norfluoxetine.

One study which was similar in methodology to this fluoxetine treatment, albeit by a different route of administration (i.p. compared to s.c. in these studies), caused a final brain concentration of approximately 30µM norfluoxetine and negligible levels of fluoxetine after the same washout period (Gardier *et al.*, 1994). Taking into account the dilution of tissue for use in a [<sup>3</sup>H]citalopram binding assay, this concentration would account for the presence of 250nM norfluoxetine. Norfluoxetine is not commercially available hence it was assumed that both norfluoxetine and fluoxetine would have the same affinity for 5-HT re-uptake sites as previously reported (Wong *et al.*, 1993). 250nM norfluoxetine would therefore account for an approximate 93% reduction in specific [<sup>3</sup>H]citalopram binding when extrapolated from a standard curve (Figure 6.5). This reduction would be comparable to the effects seen after chronic fluoxetine treatment. Furthermore, the affinity of norfluoxetine for NET is known to be approximately 6 fold weaker (Tatsumi *et al.*, 1997), and therefore 250nM norfluoxetine would only account for a 4 % reduction in [<sup>3</sup>H]nisoxetine binding (Figure 6.7). In this study there was no significant reduction in such binding (Figure 6.6). Therefore it is likely that the effects seen with a twice daily treatment of fluoxetine are due to the presence of the residual active metabolite, norfluoxetine.

The remaining tissue from this treatment was to be used to examine the effects of chronic fluoxetine treatment on 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> receptor densities and affinities. Prior to performing these experiments, the interaction of fluoxetine with the 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> receptors was investigated. Fluoxetine had weak affinity (<10µM) for both [<sup>3</sup>H]5-CT binding sites (in the presence of 200nM WAY100635 and 200nM GR125,743) and [<sup>3</sup>H]GR125,743 binding sites in rat cortex membranes

(Table 6.4). Therefore if chronic fluoxetine treatment caused any effect on these receptor subtypes, it would be unlikely to be due to the presence of residual drug.

#### 6.2.4 Effects of Chronic SSRI Treatments on 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> Receptors

In each of the brain areas investigated naïve membranes from non-treated rats revealed that 200nM WAY100635 was sufficient to block the 5-HT<sub>1A</sub> component of [<sup>3</sup>H]5-CT binding described in chapter 5 (data not shown). 200nM WAY100635 & 200nM GR125,743 was sufficient to block the 5-HT<sub>1B/1D</sub> component of [<sup>3</sup>H]5-CT binding described in chapter 5 (data not shown).

Chronic citalopram treatment caused a significant increase in the percentage of 5-HT<sub>1A</sub> receptors (~8 %), and decrease in the percentage of 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors (~9 and 13 % respectively) labelled with 0.25nM [<sup>3</sup>H]5-CT in the frontal cortex (Table 6.5). Chronic fluoxetine treatment similarly caused a significant increase in the percentage of 5-HT<sub>1A</sub> receptors (~10%), and decrease in the percentage of 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors (~11 and 18 % respectively) labelled with 0.25nM [<sup>3</sup>H]5-CT in the frontal cortex (Table 6.5). Chronic citalopram or fluoxetine treatments both revealed no change in the affinity ( $K_D$  in nM) of 5-HT<sub>7</sub> binding, but caused a significant reduction in the density ( $B_{max}$  values in fmoles mg<sup>-1</sup> protein) of these receptors by 14 and 26% respectively in the frontal cortex as compared to controls determined by [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743 (Table 6.5; Figure 6.8a). For the other brain areas investigated, caudal cortex, hippocampus and striatum, there was no change in any of the relative proportions of receptors labelled or in the affinity or density of the 5-HT<sub>7</sub> receptor population after either SSRI chronic treatment (Table 6.5; Figures 6.8b-d). The Hill slopes were unaffected by chronic antidepressant treatment (Table 6.5).

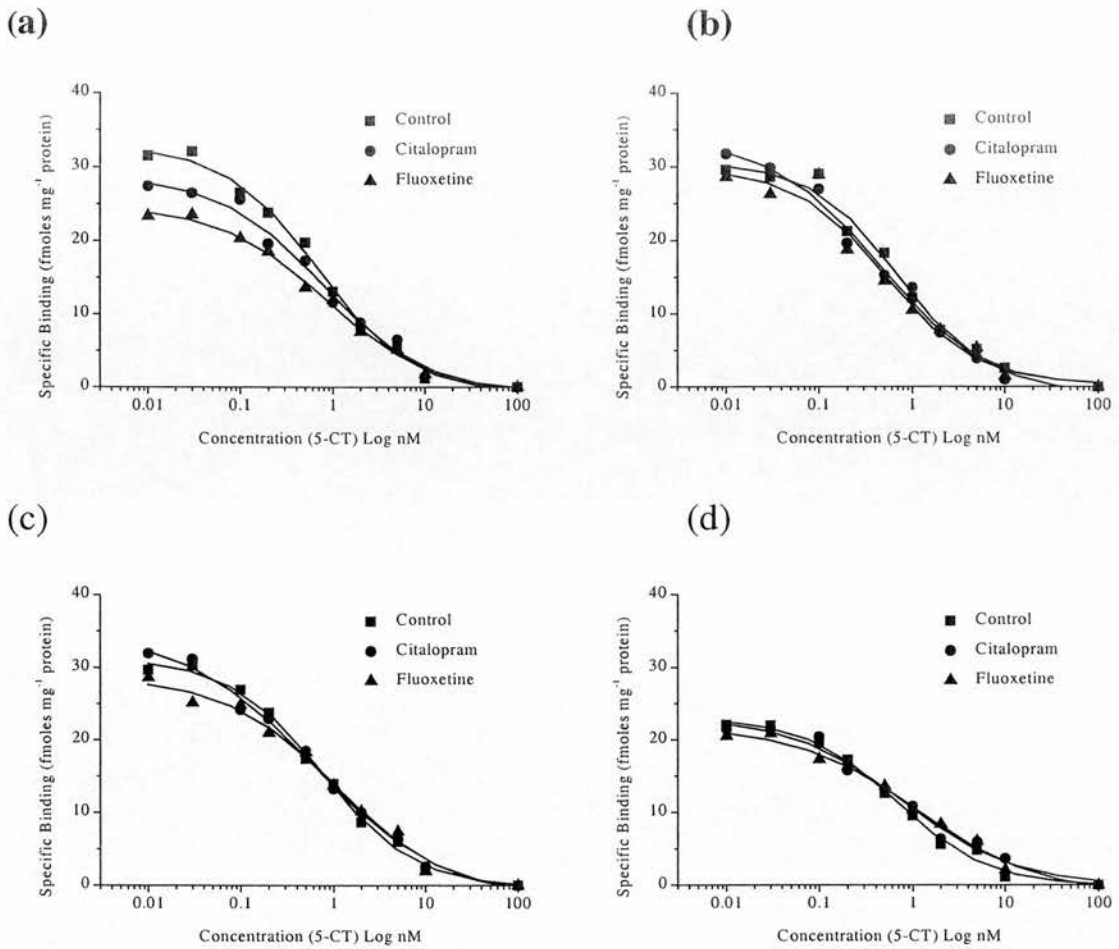
Using 0.25nM [<sup>3</sup>H]GR125,743, both chronic SSRI treatments revealed no change in the density or affinity of the total 5-HT<sub>1B/1D</sub> population or individual components, in the caudal cortex, hippocampus or striatum (Table 6.6; Figures 6.9b-d). The density of 5-HT<sub>1B/1D</sub>, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors was significantly reduced by 17, 18 and 13 % respectively by chronic citalopram treatment and 25, 25 and



Compound	$[^3\text{H}]5\text{-CT}$ Binding 5-HT <sub>7</sub> receptors		$[^3\text{H}]GR125,743$ Binding 5-HT <sub>1B/1D</sub> receptors	
	pK <sub>i</sub>	Hill slope	pK <sub>i</sub>	Hill slope
5-CT	9.31 ± 0.08	0.84 ± 0.01		
GR125,743			8.94 ± 0.18	1.00 ± 0.08
Citalopram	4.82 ± 0.04	0.91 ± 0.04	4.11 ± 0.02	0.90 ± 0.03
Fluoxetine	4.60 ± 0.08	1.20 ± 0.11	4.83 ± 0.02	1.16 ± 0.14
Tianeptine	No Inhibition up to 1mM		No Inhibition up to 1mM	
MDMA	4.57 ± 0.05	0.92 ± 0.03	4.46 ± 0.07	1.02 ± 0.03

**Table 6.4: Inhibition of  $[^3\text{H}]5\text{-CT}$  and  $[^3\text{H}]GR125,743$  Binding by Antidepressants Drugs and MDMA.**

The affinity values were determined from the inhibition of 0.25nM  $[^3\text{H}]5\text{-CT}$  (in the presence of 200nM WAY100635 & 200nM GR125,743) or 0.25nM  $[^3\text{H}]GR125,743$  binding to rat cerebral cortex membranes. The results are expressed as pK<sub>i</sub> values and Hill slope (nH) for a one site logistic fit. Data is expressed as the mean ± s.e.mean from at least three independent experiments.



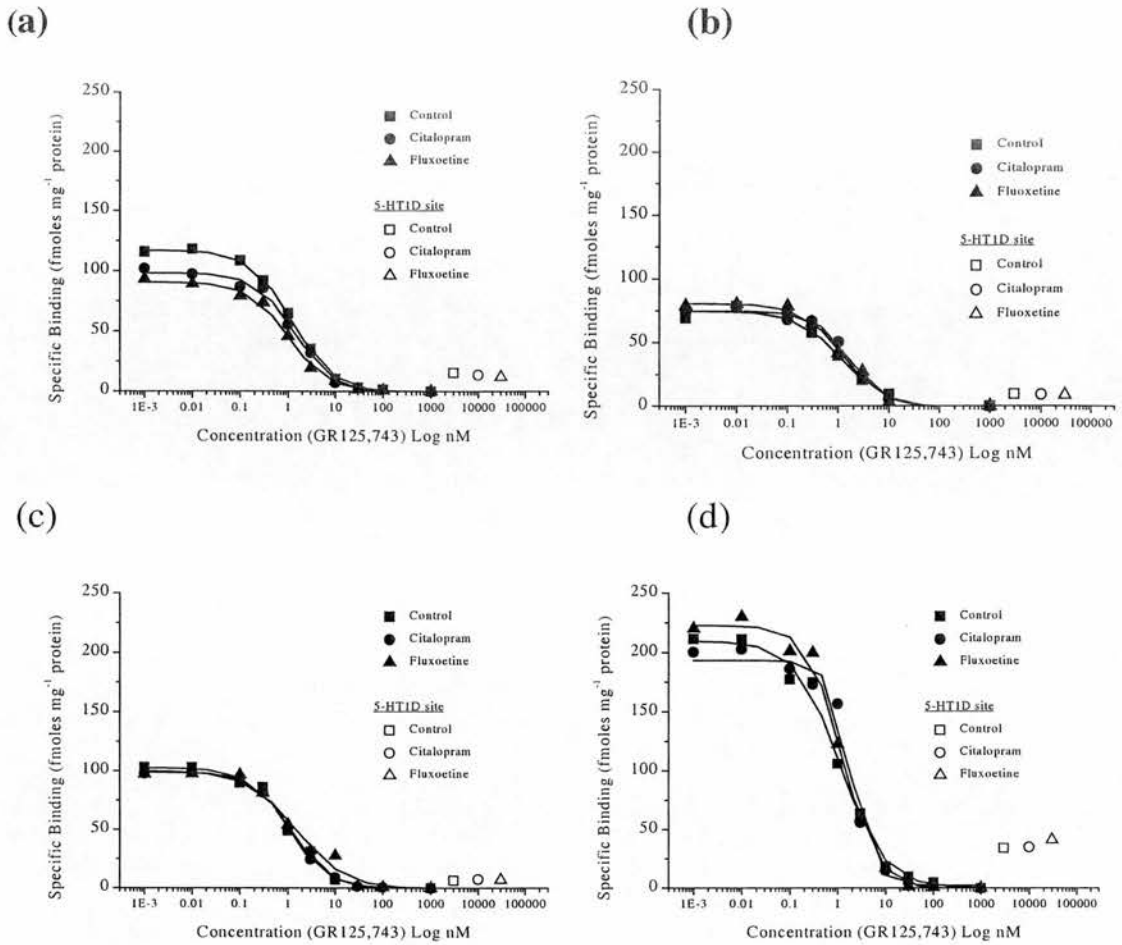
**Figure 6.8: Inhibition of  $[^3\text{H}]5\text{-CT}$  Binding to  $5\text{-HT}_7$  Receptors after Chronic SSRI Treatment.**

$[^3\text{H}]5\text{-CT}$  (0.25nM) binding was conducted in the presence of 200nM WAY100635 and 200nM GR125,743 to reveal the  $5\text{-HT}_7$  receptor population as shown in chapter 5. The data represent typical binding isotherms for animals chronically treated with 10mg/kg, bd, s.c., for 21 days with either citalopram, fluoxetine or equivalent control volume in (a) frontal cortex, (b) caudal cortex, (c) hippocampus and (d) striatum membranes. Mean data is in Table 6.5.

**Table 6.5: Effect of Chronic SSRI Treatment on 0.25nM [<sup>3</sup>H]5-HT Binding.**

	Control	Citalopram	Fluoxetine	Control	Citalopram	Fluoxetine
<u>Frontal Cortex</u>						
<u>Hippocampus</u>						
% 5-HT <sub>1A</sub>	54.49 ± 1.37	58.99 ± 0.97*	60.15 ± 1.47*	77.44 ± 1.29	76.45 ± 0.27	76.78 ± 1.06
% 5-HT <sub>1B/1D</sub>	36.19 ± 1.36	32.91 ± 0.80*	32.26 ± 1.44*	17.99 ± 1.58	19.16 ± 0.08	19.54 ± 1.02
% 5-HT <sub>7</sub>	9.31 ± 0.21	8.10 ± 0.36*	7.59 ± 0.37*	4.57 ± 0.36	4.40 ± 0.26	4.68 ± 1.21
Hill slope	0.89 ± 0.02	0.87 ± 0.03	0.82 ± 0.04	0.86 ± 0.01	0.80 ± 0.04	0.82 ± 0.06
K <sub>D</sub>	0.44 ± 0.03	0.43 ± 0.05	0.41 ± 0.06	0.45 ± 0.01	0.47 ± 0.80	0.49 ± 0.09
B <sub>max</sub>	87.53 ± 2.51	75.03 ± 1.41*	64.37 ± 3.12*	88.37 ± 5.06	84.68 ± 7.97	93.44 ± 4.79
<u>Caudal Cortex</u>						
<u>Striatum</u>						
% 5-HT <sub>1A</sub>	64.08 ± 0.61	63.54 ± 0.75	65.37 ± 1.10	18.18 ± 3.30	14.26 ± 2.26	13.65 ± 3.79
% 5-HT <sub>1B/1D</sub>	27.71 ± 1.01	28.71 ± 0.56	26.94 ± 0.90	76.56 ± 3.59	80.45 ± 1.22	81.18 ± 4.02
% 5-HT <sub>7</sub>	8.21 ± 0.46	7.75 ± 0.25	7.69 ± 0.23	5.26 ± 0.07	5.29 ± 0.36	5.17 ± 0.25
Hill slope	0.82 ± 0.03	0.82 ± 0.04	0.85 ± 0.03	0.87 ± 0.08	0.85 ± 0.04	0.83 ± 0.06
K <sub>D</sub>	0.31 ± 0.06	0.34 ± 0.03	0.34 ± 0.07	0.57 ± 0.16	0.49 ± 0.03	0.52 ± 0.02
B <sub>max</sub>	67.04 ± 4.86	67.95 ± 5.28	68.86 ± 2.65	75.77 ± 2.22	78.67 ± 3.25	74.09 ± 2.65

The results are expressed as the mean ± s.e.mean for 6 animals in frontal and caudal cortex, and 3 animals in hippocampus and striatum membranes. The proportions of 5-HT<sub>1A</sub>, the mixed 5-HT<sub>1B/1D</sub> and the 5-HT<sub>7</sub> receptors occupied by 0.25nM [<sup>3</sup>H]5-HT are reported (%). The K<sub>D</sub> in nM, the B<sub>max</sub> in fmoles mg<sup>-1</sup> protein and the Hill slope (nH) of the 5-HT<sub>7</sub> binding site determined by [<sup>3</sup>H]5-HT binding in the presence of 200nM WAY100635 & 200nM GR125,743 are given. \* = P < 0.05 compared to controls.



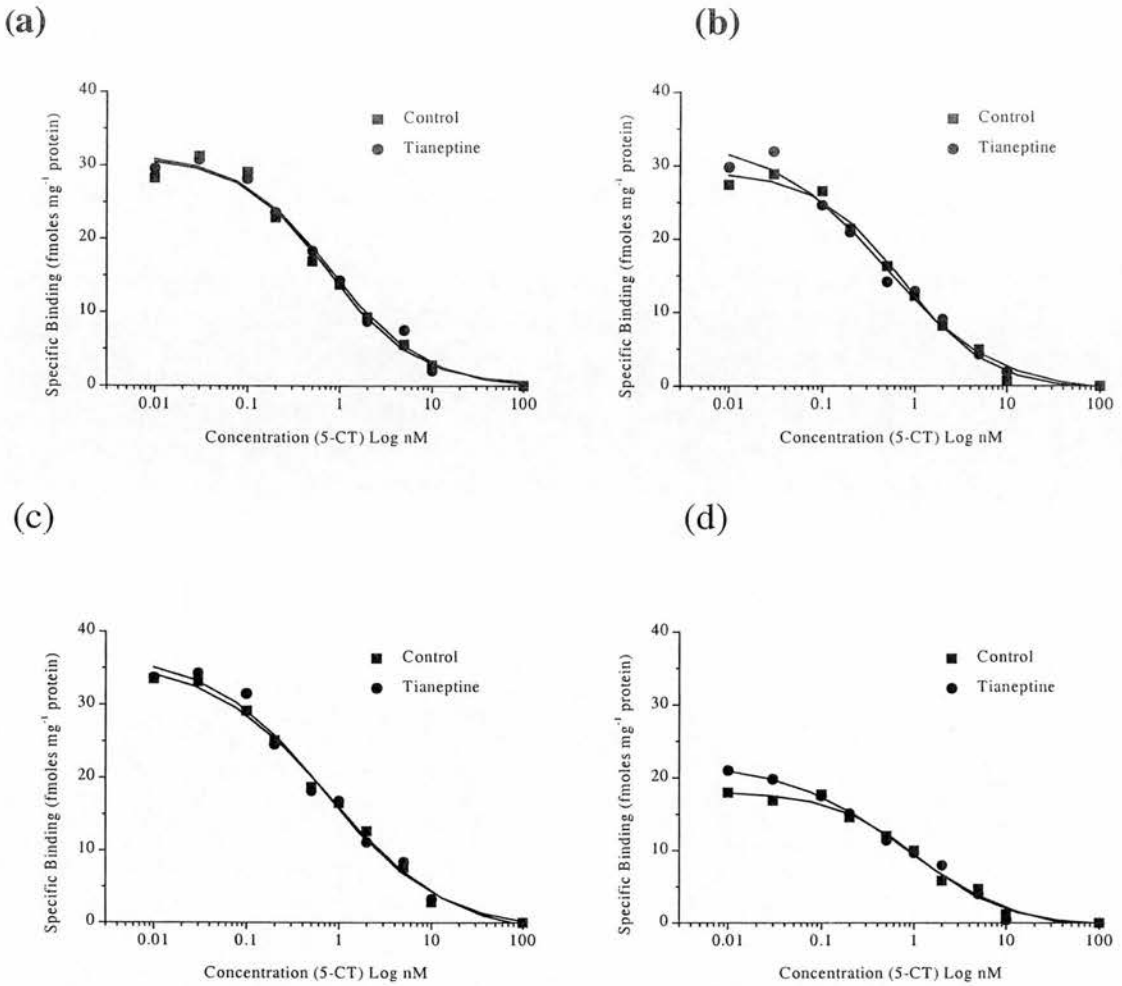
**Figure 6.9: Inhibition of  $[^3\text{H}]\text{GR125,743}$  Binding to  $5\text{-HT}_{1\text{B}/1\text{D}}$  Receptors after Chronic SSRI Treatment.**

The data represent typical  $[^3\text{H}]\text{GR125,743}$  (0.25nM) binding isotherms for animals chronically treated with 10mg/kg, bd, s.c., for 21 days with either citalopram, fluoxetine or equivalent control volume in (a) frontal cortex, (b) caudal cortex, (c) hippocampus and (d) striatum membranes. The amount of specific binding corresponding to  $5\text{-HT}_{1\text{D}}$  binding as determined in the presence of 300nM CP93129 (to block binding to  $5\text{-HT}_{1\text{B}}$  receptors) is shown as the corresponding hollow figures. Mean data is in Table 6.6.

**Table 6.6: Effect of Chronic SSRI Treatment on 0.25nM [<sup>3</sup>H]GR125,743 Binding.**

	Control	Citalopram	Fluoxetine	Control	Citalopram	Fluoxetine
<u>Frontal Cortex</u>						
<u>Hippocampus</u>						
Hill slope	1.13 ± 0.06	1.12 ± 0.03	1.10 ± 0.03	1.13 ± 0.07	1.07 ± 0.04	1.08 ± 0.13
K <sub>D</sub>	1.03 ± 0.06	1.04 ± 0.09	0.95 ± 0.08	0.87 ± 0.08	0.89 ± 0.07	1.02 ± 0.11
B <sub>max</sub> 5-HT <sub>1B/1D</sub>	583.51 ± 7.76	482.86 ± 32.16*	436.06 ± 26.11*	437.09 ± 40.98	480.78 ± 29.16	470.68 ± 59.64
B <sub>max</sub> 5-HT <sub>1B</sub>	508.61 ± 8.07	417.08 ± 27.20*	378.73 ± 22.08*	396.11 ± 39.68	433.60 ± 34.62	432.97 ± 56.20
B <sub>max</sub> 5-HT <sub>1D</sub>	75.25 ± 0.63	65.78 ± 3.29*	57.33 ± 4.88*	40.98 ± 6.97	47.19 ± 5.46	37.71 ± 9.58
<u>Striatum</u>						
Hill slope	1.08 ± 0.06	1.03 ± 0.05	1.10 ± 0.07	0.99 ± 0.03	1.06 ± 0.04	1.10 ± 0.06
K <sub>D</sub>	1.09 ± 0.09	1.24 ± 0.12	1.06 ± 0.12	0.94 ± 0.04	0.95 ± 0.08	0.98 ± 0.03
B <sub>max</sub> 5-HT <sub>1B/1D</sub>	407.20 ± 34.52	409.86 ± 40.42	377.90 ± 31.11	1001.69 ± 31.21	1024.57 ± 11.43	1032.62 ± 55.72
B <sub>max</sub> 5-HT <sub>1B</sub>	359.37 ± 35.51	362.71 ± 36.67	335.98 ± 31.93	823.32 ± 23.18	841.57 ± 6.43	840.82 ± 48.42
B <sub>max</sub> 5-HT <sub>1D</sub>	47.83 ± 9.75	47.15 ± 15.44	41.93 ± 10.21	178.36 ± 8.23	183.00 ± 9.04	191.80 ± 9.36

The results are expressed as the mean ± s.e.mean for 6 animals in frontal and caudal cortex, and 3 animals in hippocampus and striatum membranes. The B<sub>max</sub> in fmoles mg<sup>-1</sup> protein, the K<sub>D</sub> in nM and the Hill slope (nH) for the 5-HT<sub>1B/1D</sub> receptor population labelled by 0.25nM [<sup>3</sup>H]GR125,743 binding are given. The B<sub>max</sub> (fmoles mg<sup>-1</sup> protein) of the 5-HT<sub>1D</sub> population was calculated based on the percentage block of specific [<sup>3</sup>H]GR125,743 binding by 300nM CP93129 and using the assumption that GR125,743 has the same affinity at 5-HT<sub>1D</sub> or 5-HT<sub>1B</sub> or 5-HT<sub>1B/1D</sub> mixed populations (see chapter 5). The B<sub>max</sub> (fmoles mg<sup>-1</sup> protein) of the 5-HT<sub>1B</sub> receptor population was calculated as the difference between the 5-HT<sub>1D</sub> and the combined 5-HT<sub>1B/1D</sub> receptor population.



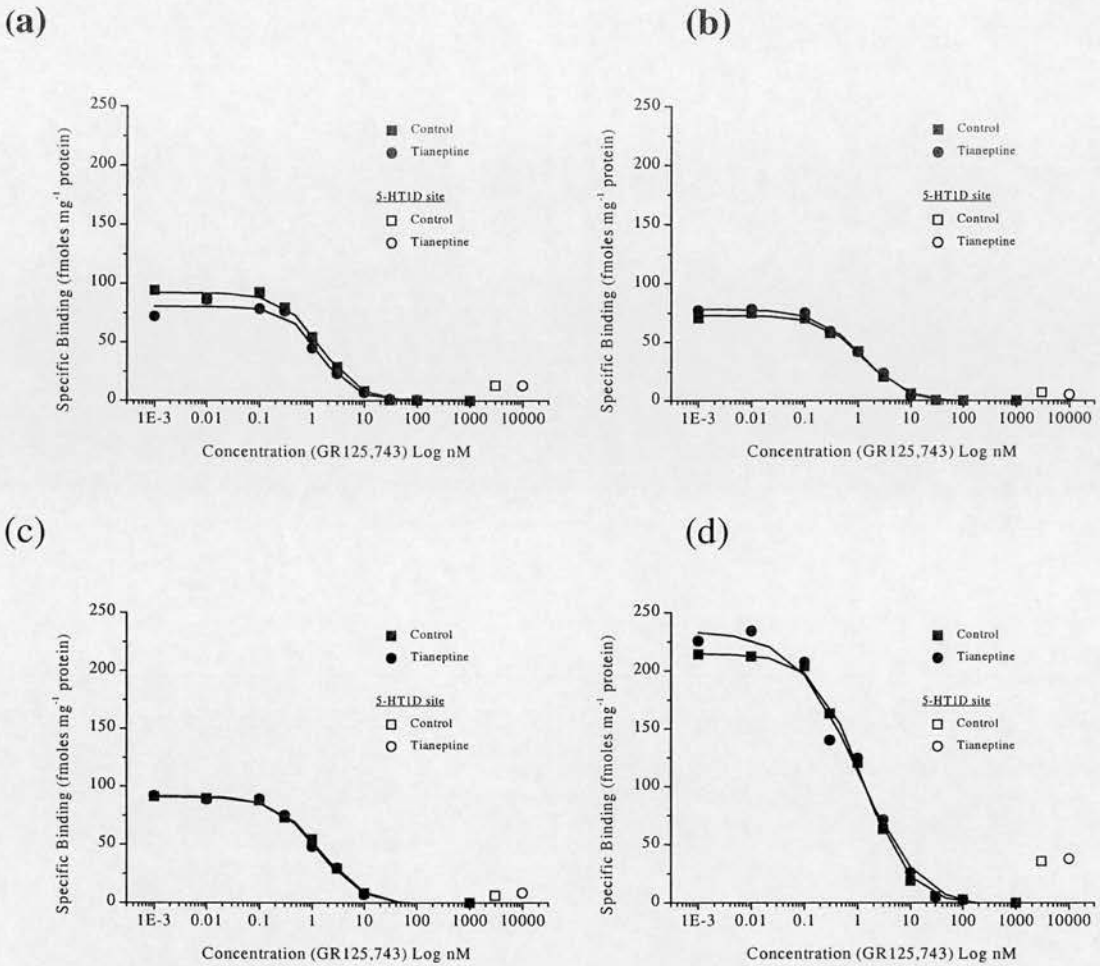
**Figure 6.10: Inhibition of [<sup>3</sup>H]5-CT Binding to 5-HT<sub>7</sub> Receptors after Chronic Tianeptine Treatment.**

[<sup>3</sup>H]5-CT (0.25nM) binding was conducted in the presence of 200nM WAY100635 and 200nM GR125,743 to reveal the 5-HT<sub>7</sub> receptor population as shown in chapter 5. The data represent typical binding isotherms for animals chronically treated with 10mg/kg, bd, s.c., for 14 days with either tianeptine or equivalent control volume in (a) frontal cortex, (b) caudal cortex, (c) hippocampus and (d) striatum membranes. Mean data is in text Table 6.7.

**Table 6.7: Effect of Chronic Tianeptine Treatment on 0.25nM [<sup>3</sup>H]5-CT Binding.**

	Control	Tianeptine	Control	Tianeptine
<u>Frontal Cortex</u>				
% 5-HT <sub>1A</sub>	52.09 ± 1.34	54.07 ± 1.55	75.16 ± 0.59	74.98 ± 0.75
% 5-HT <sub>1B/1D</sub>	39.79 ± 1.28	38.74 ± 1.50	20.22 ± 0.54	21.29 ± 0.63
% 5-HT <sub>7</sub>	8.12 ± 0.26	8.49 ± 0.11	4.62 ± 0.46	3.73 ± 0.61
Hill slope	0.90 ± 0.02	0.87 ± 0.04	0.89 ± 0.12	0.81 ± 0.05
K <sub>D</sub>	0.52 ± 0.04	0.49 ± 0.03	0.62 ± 0.12	0.54 ± 0.15
B <sub>max</sub>	85.73 ± 3.82	89.68 ± 5.58	106.05 ± 10.54	102.98 ± 13.60
<u>Caudal Cortex</u>				
% 5-HT <sub>1A</sub>	62.79 ± 1.28	62.42 ± 1.44	10.87 ± 4.75	10.89 ± 2.07
% 5-HT <sub>1B/1D</sub>	29.37 ± 1.40	28.75 ± 1.34	82.26 ± 5.57	83.40 ± 2.33
% 5-HT <sub>7</sub>	7.84 ± 0.37	8.83 ± 0.87	6.87 ± 0.84	5.71 ± 0.46
Hill slope	0.82 ± 0.02	0.79 ± 0.03	0.88 ± 0.10	0.89 ± 0.12
K <sub>D</sub>	0.31 ± 0.06	0.35 ± 0.03	0.62 ± 0.11	0.71 ± 0.14
B <sub>max</sub>	74.66 ± 5.45	74.51 ± 6.32	88.07 ± 7.66	71.22 ± 7.09
<u>Hippocampus</u>				
% 5-HT <sub>1A</sub>				
% 5-HT <sub>1B/1D</sub>				
% 5-HT <sub>7</sub>				
Hill slope				
K <sub>D</sub>				
B <sub>max</sub>				
<u>Striatum</u>				
% 5-HT <sub>1A</sub>				
% 5-HT <sub>1B/1D</sub>				
% 5-HT <sub>7</sub>				
Hill slope				
K <sub>D</sub>				
B <sub>max</sub>				

The results are expressed as the mean ± s.e.mean for 6 animals in frontal and caudal cortex, and 3 animals in hippocampus and striatum membranes. The proportions of 5-HT<sub>1A</sub>, the mixed 5-HT<sub>1B/1D</sub> and the 5-HT<sub>7</sub> receptors occupied by 0.25nM [<sup>3</sup>H]5-CT are reported (%). The K<sub>D</sub> in nM, the B<sub>max</sub> in fmoles mg<sup>-1</sup> protein and the Hill slope (nH) of the 5-HT<sub>7</sub> binding site determined by [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743 are given. \* = P < 0.05 compared to controls.



**Figure 6.11: Inhibition of  $[^3\text{H}]\text{GR125,743}$  Binding to 5-HT<sub>1B/1D</sub> Receptors after Chronic Tianeptine Treatment.**

The data represent typical  $[^3\text{H}]\text{GR125,743}$  (0.25nM) binding isotherms for animals chronically treated with 10mg/kg, bd, s.c., for 14 days with either tianeptine or equivalent control volume in (a) frontal cortex, (b) caudal cortex, (c) hippocampus and (d) striatum membranes. The amount of specific binding corresponding to 5-HT<sub>1D</sub> binding as determined in the presence of 300nM CP93129 (to block binding to 5-HT<sub>1B</sub> receptors) is shown as the corresponding hollow figures. Mean data is in Table 6.8.



**Table 6.8: Effect of Chronic Tianeptine Treatment on 0.25nM [<sup>3</sup>H]GR125,743.**

	Control	Tianeptine	Control	Tianeptine
<u>Frontal Cortex</u>				
Hill slope	1.07 ± 0.04	1.09 ± 0.04	1.04 ± 0.04	1.02 ± 0.04
K <sub>D</sub>	1.11 ± 0.07	1.04 ± 0.02	1.21 ± 0.23	1.06 ± 0.23
B <sub>max</sub> 5-HT <sub>1B/1D</sub>	507.62 ± 13.92	532.62 ± 21.24	467.18 ± 31.57	496.36 ± 39.69
B <sub>max</sub> 5-HT <sub>1B</sub>	451.59 ± 16.09	472.81 ± 20.21	426.30 ± 33.46	453.60 ± 29.60
B <sub>max</sub> 5-HT <sub>1D</sub>	56.17 ± 6.05	59.81 ± 2.76	40.88 ± 0.99	42.76 ± 0.70
<u>Hippocampus</u>				
Hill slope				
K <sub>D</sub>				
B <sub>max</sub> 5-HT <sub>1B/1D</sub>				
B <sub>max</sub> 5-HT <sub>1B</sub>				
B <sub>max</sub> 5-HT <sub>1D</sub>				
<u>Striatum</u>				
Hill slope	1.10 ± 0.05	1.07 ± 0.03	1.03 ± 0.05	0.97 ± 0.10
K <sub>D</sub>	0.88 ± 0.05	0.85 ± 0.05	1.20 ± 0.16	0.94 ± 0.17
B <sub>max</sub> 5-HT <sub>1B/1D</sub>	301.41 ± 10.90	291.78 ± 11.93	1131.86 ± 166.74	880.79 ± 116.41
B <sub>max</sub> 5-HT <sub>1B</sub>	272.41 ± 9.78	261.94 ± 11.99	939.52 ± 131.93	732.31 ± 92.23
B <sub>max</sub> 5-HT <sub>1D</sub>	28.99 ± 1.80	29.77 ± 2.94	192.34 ± 35.11	148.48 ± 24.20

The results are expressed as the mean ± s.e.mean for 6 animals in frontal and caudal cortex, and 3 animals in hippocampus and striatum membranes. The B<sub>max</sub> in fmoles mg<sup>-1</sup> protein, the K<sub>D</sub> in nM and the Hill slope (nH) for the 5-HT<sub>1B/1D</sub> receptor population labelled by 0.25nM [<sup>3</sup>H]GR125,743 binding are given. The B<sub>max</sub> (fmoles mg<sup>-1</sup> protein) of the 5-HT<sub>1D</sub> population was calculated based on the percentage block of specific [<sup>3</sup>H]GR125,743 binding by 300nM CP93129 and using the assumption that GR125,743 has the same affinity at 5-HT<sub>1D</sub> or 5-HT<sub>1B</sub> or 5-HT<sub>1B/1D</sub> mixed populations (see chapter 5). The B<sub>max</sub> (fmoles mg<sup>-1</sup> protein) of the 5-HT<sub>1B</sub> receptor population was calculated as the difference between the 5-HT<sub>1D</sub> and the combined 5-HT<sub>1B/1D</sub> receptor population.

24 % respectively by chronic fluoxetine treatment in frontal cortex membranes, as compared to controls (Table 6.6; Figure 6.9a). For each SSRI chronic treatment, the affinity of [<sup>3</sup>H]GR125,743 binding to 5-HT<sub>1B/1D</sub> receptors remained unchanged in frontal cortex membranes as compared to controls (Table 6.6). The Hill slopes were unaffected by chronic antidepressant treatment (Table 6.6).

### **6.2.5 Effects of Chronic Tianeptine Treatment on 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> Receptors**

In each of the brain areas investigated naïve membranes from non-treated rats revealed that 200nM WAY100635 was sufficient to block the 5-HT<sub>1A</sub> component of [<sup>3</sup>H]5-CT binding described in chapter 5 (data not shown). 200nM WAY100635 & 200nM GR125,743 was sufficient to block the 5-HT<sub>1B/1D</sub> component of [<sup>3</sup>H]5-CT binding described in chapter 5 (data not shown).

Chronic treatment with the atypical antidepressant drug, tianeptine, did not reveal any changes in any of the relative proportions of receptors labelled using 0.25nM [<sup>3</sup>H]5-CT, or in the affinity or density of the 5-HT<sub>7</sub> receptor population ([<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743) in any of the brain areas investigated, as compared to controls (Table 6.7; Figures 6.10a-d). Using 0.25nM [<sup>3</sup>H]GR125,743, tianeptine caused no change in the density or affinity of the total 5-HT<sub>1B/1D</sub> population or individual components as compared to controls, in any of the brain areas investigated (Table 6.8; Figures 6.11a-d). The Hill slopes of both these radioligand binding assays were unaffected by chronic antidepressant treatment (Tables 6.7 & 6.8).

## **6.3 Discussion**

Chronic treatment with citalopram (10mg/kg bd), fluoxetine (10mg/kg once daily) or tianeptine (10mg/kg bd) resulted in no change in SERT binding site density or affinity, labelled with [<sup>3</sup>H]citalopram in frontal cortex, caudal cortex, hippocampus or striatum as compared to controls (Table 6.1; Figure 6.1). The abundance of SERT was also unchanged after the same treatments in cerebral cortex, hippocampus, striatum, raphe nuclei or blood platelets as qualitatively assessed by immunohistochemistry and SDS-PAGE Western blots (Figures 6.2 & 6.3).

Increasing the dose of fluoxetine by means of a twice daily dosing regime in a separate study resulted in a significant decrease in the amount of [<sup>3</sup>H]citalopram specific binding and affinity in frontal cortex membranes compared to the equivalent controls (Table 6.3; Figure 6.4). Unlike other SSRIs, one of the metabolites of fluoxetine, norfluoxetine, retains high affinity for [<sup>3</sup>H]citalopram binding sites with a much greater half life than that of the parent drug (Caccia *et al.*, 1990 & 1992; Wong *et al.*, 1993; Tatsumi *et al.*, 1997; Sanchez & Hyttel, 1999). It is therefore likely that the effects seen with twice daily dosing of fluoxetine was due to the presence of residual norfluoxetine competing with cold citalopram for SERT binding sites in the [<sup>3</sup>H]citalopram binding assays and not because of an adaptational change. The half life of citalopram in rats is only 3 hours (Fredrickson *et al.*, 1982) and hence no residual drug would be present after chronic treatment with a two day washout period as shown by no change in citalopram binding affinity (Table 6.1). Tianeptine also has a short half life (Royer *et al.*, 1988) and more importantly has very low affinity for SERT (>1mM as determined in chapter 4), which would explain why no affinity change was also apparent after this chronic treatment (Table 6.1).

Controversial results have been published regarding the effect of chronic SSRI treatments on the density of SERT in rat brain regarding a possible neuroadaptive mechanism underlying their therapeutic benefit (see Piñeyro & Blier, 1999). In agreement with the findings in this thesis some authors indicate that chronic treatment of rats with SSRIs (i.e. citalopram, fluoxetine, paroxetine and sertraline) does not result in adaptive modification of SERT, by measuring [<sup>3</sup>H]5-HT uptake in rat brain synaptosomes (Hyttel *et al.*, 1984), or by measuring 5-HT re-uptake sites with specific ligands (Graham *et al.*, 1987; Hrdina *et al.*, 1990; Brunswick *et al.*, 1991; Cheetham *et al.*, 1991; Kovachich *et al.*, 1992; Cheetham *et al.*, 1993; Dewar *et al.*, 1993; Gobbi *et al.*, 1997). It should be noted at this point that despite a common inhibitory action at SERT, SSRIs might have different individual mechanisms of action attributed to their different pharmacology. For example, fluoxetine is approximately 60 times more potent at 5-HT<sub>2A</sub> receptors than paroxetine (Owens *et al.*, 1997), which also in part explains why fluoxetine is the least selective of the SSRIs. A paroxetine-induced down-regulation (Piñeyro *et al.*, 1994) and a fluoxetine-induced up-regulation (Hrdina & Vu, 1993) of [<sup>3</sup>H]paroxetine

binding sites in rat frontal cortex and hippocampus after chronic treatment have been observed. The large reduction in [<sup>3</sup>H]paroxetine binding in cortex and hippocampus membranes involved the administration of paroxetine via subcutaneously inserted mini-osmotic pumps (Piñeyro *et al.*, 1994). The authors of this paper and Lesch *et al.*, (1993a) believe that this dosing route was essential for the induction of an adaptive response at the level of SERT.

Studies on SERT mRNA after SSRI chronic treatment in rats are also controversial. For example whereas chronic citalopram treatment had no effect on the mRNA for 5-HT uptake sites (Spurlock *et al.*, 1994), chronic fluoxetine reduced it in the dorsal raphe nucleus (Lesch *et al.*, 1993; Neumaier *et al.*, 1996) or had no effect (Koed & Linnet, 1997). The study by Neumaier *et al.* (1996) reduced SERT mRNA in the dorsal raphe nucleus but not in several other regions detectable after 7 days of treatment, but was not detected after drug washout or after 21 days treatment and/or washout. It should be mentioned however that alternative post-translational regulation of protein levels has been well documented (Latchman, 1990). Therefore despite discrepancies in reported SERT mRNA levels after chronic antidepressant treatments, these differences may not necessarily account for the same differences in SERT protein levels.

The effect of chronic tianeptine treatment on SERT is also controversial. In this thesis tianeptine was administered at a total dose of 20mg/kg/day which is comparable to work done by other groups (Mennini *et al.*, 1987; Mennini & Garattini 1991; Ortitz *et al.*, 1991; Whitton *et al.*, 1991; Bertorelli *et al.*, 1992; Datla & Curzon 1993; Piñeyro *et al.*, 1995c & d). Within the first 24 hours of tianeptine administration 80% of its metabolites are eliminated (Royer *et al.*, 1988). It has been suggested that the effect of tianeptine on 5-HT uptake disappears after a 48 washout period using mini osmotic pumps (Piñeyro *et al.*, 1995c & d). This suggests that tianeptine or its metabolites must be present to enhance *in vivo* 5-HT uptake activity (Piñeyro *et al.*, 1995a) although this effect is “not always reproducible” (Mennini *et al.*, 1987). Indeed the same group showed that administration of tianeptine (10mg/kg twice daily for 15 days i.p.) enhances 5-HT uptake *ex vivo*, following a longer 72 hour washout period (Mennini *et al.*, 1987).

In the studies described within this chapter a dosing regime of 10mg/kg twice-daily s.c. with a 2-day washout period is therefore consistent with the enhancing effects of tianeptine. Using a similar dosing regime and washout period as in this thesis, chronic tianeptine treatments have revealed no changes in SERT density or affinity using [<sup>3</sup>H]imipramine (Mennini & Garattini, 1991) which binds to heterogeneous sites (Reith *et al.*, 1983 and Marcusson *et al.*, 1985). However using [<sup>3</sup>H]cyanoimipramine which is more selective than [<sup>3</sup>H]imipramine for SERT (Kovachich *et al.*, 1988), tianeptine delivered using mini-osmotic pumps, caused a significant increase in cortical B<sub>max</sub> values and a non significant increase in hippocampus B<sub>max</sub> values (Piñeyro *et al.*, 1995d). In this study the K<sub>D</sub> values were unaffected, which would be in agreement with the enhancing effect of tianeptine upon [<sup>3</sup>H]5-HT uptake by increasing V<sub>max</sub> without affecting K<sub>m</sub> (Mennini *et al.*, 1987). However the large 25% error surrounding the K<sub>D</sub> values could inherently account for the 22% increase in B<sub>max</sub> values in the cortex. Using [<sup>3</sup>H]paroxetine as a marker for SERT, which is much more comparable to citalopram than cyanoimipramine, tianeptine had no effect on hippocampus and cerebral cortex membrane binding parameters when given at 10mg/kg/day i.p. for 21 days (Frankfurt *et al.*, 1993). Interestingly the same group did however report decreases of SERT density in the same brain membranes labelled with [<sup>3</sup>H]paroxetine when tianeptine was administered at a higher dose of 30mg/kg/day i.p. for 14 days (Watanabe *et al.*, 1993). This latter effect is very unlikely to be due to the presence of residual drug; not only because of the drugs short half-life, but also that it has no affinity (>1mM) in inhibiting [<sup>3</sup>H]citalopram binding (chapter 4). One study however in the raphé nuclei showed no changes in [<sup>3</sup>H]paroxetine binding sites or SERT mRNA in the median raphé, but reported decreases in SERT protein and mRNA in the rat dorsal raphé nucleus after tianeptine treatment (10mg/kg bd i.p. for 14 days ; Kuroda *et al.*, 1994).

The detection of rat adrenal SERT (chapter 4) suggests another therapeutic site for antidepressant action. Antidepressants have been shown to inhibit the uptake of [<sup>3</sup>H]serotonin (Holzwarth *et al.*, 1984) or [<sup>14</sup>C]cocaine (Delarue *et al.*, 1992) or block p-chloroamphetamine-induced serotonin depletion in adrenals (Som *et al.*, 1994) suggesting an active function of adrenal SERT. In adrenal medulla

membranes, there was no change in the binding density or affinity of SERT after chronic citalopram, fluoxetine (once daily) or tianeptine treatments as in the cortex, hippocampus or striatum of the brain (Table 6.1; Figure 6.1). Chronic fluoxetine treatment using a twice-daily dosing regime reduced the specific [<sup>3</sup>H]citalopram binding by a similar degree as that seen in the brain. This was possibly due to the presence of norfluoxetine for reasons discussed above. Indeed fluoxetine and norfluoxetine have been shown to accumulate in other regions than the brain such as in the blood, liver and body hair (Lefebvre *et al.*, 1999). It is therefore not surprising that accumulation occurs in the adrenals where there is also a high density of antidepressant sensitive SERT protein (chapter 4). Though no adaptive changes of adrenal SERT protein density was observed after chronic SSRI or tianeptine treatments, consequences of SERT blockade may help to understand the involvement of the adrenal gland in the regulation of the HPA axis in depression and its management with antidepressant drugs.

The lack of effect on SERT density or affinity after chronic SSRI (citalopram and fluoxetine) and tianeptine in both the brain and adrenal medulla suggest that adaptive changes of SERT at the protein level may not be central to the mechanism of action of these antidepressants. However differences between the findings in this chapter and some of those cited in the literature may be due to a variety of reasons. These include the route of drug administration, the length of treatment, the brain areas considered for biochemical measurements, the biochemical measurement itself and the drug washout period as previously suggested (Gobbi *et al.*, 1997). Indeed the latter effect was apparent in the twice-daily dose study using fluoxetine. In order to remove active residual drug a 7 day washout period would have to be used (Gardier *et al.*, 1994). Such studies using this extended washout period have been performed, showing no change in the both density or affinity of SERT after chronic fluoxetine treatment in the raphé or terminal fields (Gobbi *et al.*, 1997).

Studies using mini-osmotic pumps are the only treatments that have consistently demonstrated an adaptive change in SERT density in response to chronic antidepressant administration (Piñeyro *et al.*, 1994 & Piñeyro *et al.*, 1995d). More recently using the same mini-osmotic pump procedure, chronic treatment with paroxetine or sertraline have been shown to downregulate SERT binding sites

throughout the rat brain (Benmansour *et al.*, 1999). It may therefore be that in order to produce adaptations in SERT density, stable serum concentrations of drug are necessary throughout the chronic treatment as previously suggested (Lesch *et al.*, 1993a; Piñeyro *et al.*, 1994). However stable serum concentrations have been reported not to be essential for the antidepressant effect in humans (Artigas *et al.*, 1996). In the rat the half-lives of most antidepressants are generally much shorter than in humans (Sanchez & Hyttel, 1999). Therefore treatments in rats by any other method other than using mini-osmotic pumps may produce a greater spiked or transient drug exposure compared to the steadier exposure to drug achieved in human patients. This may suggest that a more continual blockade of SERT is indeed required to produce a down regulation of SERT, which in the clinic results in elevation of depressive symptoms. Mini osmotic pumps in experimental animals may therefore closer resembles the pharmacokinetics observed in human patients and hence be a more relevant model to understand antidepressant action than that used in this thesis and currently by a large number of other researchers. From these limited osmotic mini-pump studies it also interesting to see that SSRIs produce a down regulation of SERT density (Piñeyro *et al.*, 1994; Benmansour *et al.*, 1999) whereas tianeptine, which has opposing effects on uptake actually produces an upregulation of SERT density (Piñeyro *et al.*, 1995d). Furthermore those antidepressant drugs that do not directly alter 5-HT uptake (e.g. NA uptake inhibitors or MAOIs), do not alter SERT density or SERT mRNA levels (Lesch *et al.*, 1993a; Bensamour *et al.*, 1999). The use of mini-osmotic pumps in future studies may therefore help to remove some of the controversy surrounding antidepressant mechanisms of action and indeed may help to answer many of the questions that remain unanswered with a closer resemblance to what occurs in the clinic.

Chronic treatment with citalopram (10mg/kg bd), fluoxetine (10mg/kg once daily) or tianeptine (10mg/kg bd) resulted in no change in the 5-HT<sub>1A</sub> receptor binding site density or affinity labelled with [<sup>3</sup>H]8-OH DPAT in frontal cortex, caudal cortex or hippocampus membranes as compared to controls (Table 6.1; Figure 6.1). This is agreement with other binding studies using this ligand (Hensler *et al.*, 1991; Newman *et al.*, 1993; Watanabe *et al.*, 1993; Wieland *et al.*, 1993; Le Poul *et al.*, 1995a & b; Carli *et al.*, 1996). Work has also been extended to 5-HT<sub>1A</sub> mRNA

showing no change in concentration after chronic antidepressant treatment (Spurlock *et al.*, 1994) or no changes in post mortem brain tissue of antidepressant users (Yates & Ferrier, 1990). It would therefore appear that despite a gradual desensitisation of 5-HT<sub>1A</sub> receptors located somatodendritically after chronic SSRI treatment (see Piñeyro & Blier, 1999), no adaptive changes of the receptor number or affinity are associated with this desensitisation process in the terminal field. The ligand used in these studies ([<sup>3</sup>H]8-OH DPAT) is an agonist ligand and therefore will only label 5-HT<sub>1A</sub> receptors functionally coupled to G-proteins (Emerit *et al.*, 1990). Previous studies have suggested that alterations in G proteins might account for changes after long term antidepressant treatment (Lesch *et al.*, 1991; Lesch & Manji, 1992). Therefore it is important to use an antagonist ligand (e.g. [<sup>3</sup>H]WAY100635) to label all 5-HT<sub>1A</sub> receptor binding sites (i.e. coupled and uncoupled with regard to G proteins; Gozlan *et al.*, 1995). However, after chronic fluoxetine or paroxetine treatments, no changes were observed in the density or affinity of the 5-HT<sub>1A</sub> receptors labelled by either [<sup>3</sup>H]8-OH DPAT or [<sup>3</sup>H]WAY100635 in the dorsal raphe nucleus, the dentate gyrus or the hippocampus (Le Poul *et al.*, 1995a & b). It is therefore unlikely that the delay of onset of action, of particularly the SSRIs, is due to adaptive changes of the 5-HT<sub>1A</sub> receptor population located in the post-synaptic regions of the terminal field. Similarly adaptive changes of the number or affinity of 5-HT<sub>1A</sub> receptors located somatodendritically in the raphe nuclei are unlikely to account for the desensitisation of these receptors after chronic SSRI treatment.

Another possible receptor subtype involved in the mechanism of action of antidepressant drugs is the 5-HT<sub>7</sub> receptor. The effect of chronic antidepressant treatments on the density and affinity of 5-HT<sub>7</sub> receptors was investigated in the terminal field areas of the frontal and caudal cortex, hippocampus and striatum using [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743 (characterised in chapter 5). Chronic citalopram and fluoxetine treatments (both 10mg/kg bd for 21 days) caused a 14 and 26 % reduction respectively in the density of 5-HT<sub>7</sub> receptors in frontal cortex membranes as compared to controls, with no change in the affinity (Table 6.5; Figure 6.8). For this study, the low affinity of fluoxetine for the 5-HT<sub>7</sub> receptor (Table 6.4) and no change in affinity after chronic treatment, suggests that residual drug did not account for the effect seen. Tianeptine,



which has no affinity for this receptor subtype (Table 6.4), unlike the SSRIs, did not cause any such differences in this brain area (Table 6.7; Figure 6.10). All the antidepressants tested also showed no change in the density or affinity of 5-HT<sub>7</sub> receptors in the caudal cortex, hippocampus or striatum as compared to controls (Tables 6.5 & 6.7; Figures 6.8 & 6.10). Each chronic antidepressant treatment failed to change the Hill slope of the generated binding isotherms from <1 in each brain area investigated (Tables 6.5 & 6.7). This suggests that in the frontal cortex the affinity state of the functionally coupled G protein receptor is not important for a neuroadaptive change to occur.

Previous studies on this receptor subtype are very limited, though a neuroadaptive downregulation of 5-HT<sub>7</sub> receptor density in the SCN following chronic antidepressant treatments, including citalopram and fluoxetine, has been reported (Mullins *et al.*, 1999). If 5-HT<sub>7</sub> receptors are pre-synaptic they may exert a negative influence on the firing of serotonergic neurons or on 5-HT release and therefore a neuroadaptive downregulation may reduce this inhibitory action. The identification of 5-HT<sub>7</sub> binding sites in the raphe nucleus (chapter 5) suggests that this receptor may indeed be a new autoreceptor that can control 5-HT neurotransmission at the somatodendritic level. It will therefore be of interest to study the effects of such chronic drug treatments on the function and density of this receptor subtype in the raphe nucleus. This receptor subtype is however believed to be postsynaptic in not only the hypothalamus (Clemett *et al.*, 1999), but in many other brain regions including the cortex due to the correlation of 5-HT<sub>7</sub> mRNA and binding sites (To *et al.*, 1995; Gustafson *et al.*, 1996). The downregulation of 5-HT<sub>7</sub> postsynaptic receptors after chronic SSRI administration may therefore be a consequence of SSRI treatment or a mechanism by which SSRIs have a delayed onset of action in the clinic.

5-HT<sub>1B/1D</sub> receptors are known to exist pre-synaptically on serotonergic nerve terminals, exerting an inhibitory action on 5-HT release following acute SSRI administration (Sharp *et al.*, 1989; Auerbach *et al.*, 1991; Hjorth & Tao, 1991). Following chronic administration these receptors are believed to be desensitised in a time dependent manner associated with the delayed onset of action of such antidepressants in the clinic (see Piñeyro & Blier, 1999). In this chapter the density

and affinity of 5-HT<sub>1B/1D</sub> receptors was investigated after chronic antidepressant treatments in the terminal field regions of the frontal and caudal cortex, hippocampus and striatum. Similar to the effects seen with the 5-HT<sub>7</sub> receptor after chronic SSRI treatment, citalopram and fluoxetine (both 10mg/kg bd for 21 days) decreased the joint 5-HT<sub>1B/1D</sub> receptor population density. The density of [<sup>3</sup>H]GR125,743 binding sites was decreased by 17 and 25% respectively (without affecting the affinity) in frontal cortex membranes as compared to controls (Table 6.6; Figure 6.9). This would be in agreement with the decrease in the proportion of 5-HT<sub>1B/1D</sub> receptors labelled using 0.25nM [<sup>3</sup>H]5-CT after SSRI treatment (Table 6.5). Using [<sup>3</sup>H]GR125,743 in the presence of 300nM CP93129 the individual binding site densities of both the 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors could be determined, as GR125,743 has the same affinity for both these receptor subtypes (as discussed in chapter 5). Within this population there was an 18 and 17 % decrease in 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptor density respectively after chronic citalopram treatment. There was also a 25 and 24 % decrease in 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptor density respectively after chronic fluoxetine treatment (Table 6.6). The binding parameters in the other brain areas investigated were unchanged after chronic SSRI treatments as compared to controls (Table 6.6; Figure 6.9). The low affinity of fluoxetine and citalopram for the 5-HT<sub>1B/1D</sub> receptor population (Table 6.4) and no change in affinity after chronic treatment, suggests that residual drug did not account for the effects seen. In contrast to the SSRIs, chronic tianeptine administration resulted in no changes in [<sup>3</sup>H]GR125,743 binding parameters in the frontal cortex (Table 6.8; Figure 6.11). However, a similarity of chronic tianeptine treatment effects, which has no affinity for 5-HT<sub>1B/1D</sub> receptors (Table 6.4), compared to the effect of the SSRIs treatments, was the lack of any change of binding parameters in the caudal cortex, hippocampus and striatum as compared to controls (Tables 6.7 & 6.8; Figures 6.10 & 6.11).

Previous radioligand binding studies looking directly at the possible regulation of particularly 5-HT<sub>1B/1D</sub> receptors after chronic antidepressant treatments are limited, due to the lack of selective ligands available. One study in which a [<sup>125</sup>I]cyanopindolol binding assay (Hoyer *et al.*, 1985a & b) for 5-HT<sub>1B</sub> receptors was used, showed no changes in the receptor density in the rat frontal cortex as in this study with a chronic tianeptine treatment (Montero *et al.*, 1991). In the same study,

although no SSRIs were investigated, chlorimipramine, and iprinole (the prototype of atypical antidepressants without any significant blocking effect on monoamine reuptake) also caused no changes in the density or affinity of [<sup>125</sup>I]cyanopindolol binding in the frontal cortex (Montero *et al.*, 1991). On the otherhand, imipramine reduced RU24969-displaceable [<sup>3</sup>H]5-HT binding density of rat brain 5-HT<sub>1B</sub>-like binding sites by 19% (Johanning *et al.*, 1992). This is comparable to the effects seen in this study with citalopram and to a greater extent fluoxetine. In two animal models of depression: the forced swimming test (Cervo *et al.*, 1989) and the learned helplessness paradigm (Martin & Peulch, 1991), 5-HT<sub>1B</sub> receptor agonist stimulation blocks the antidepressant-like effects of SSRIs, such as citalopram and fluoxetine. This suggests an involvement of these receptors in the mechanism of action of these antidepressants. In the dorsal raphé nucleus, 5-HT<sub>1B</sub> mRNA is increased in the learned helplessness animals (Neumaier *et al.*, 1997), which is significantly reduced by chronic fluoxetine treatment of naïve animals (Neumaier *et al.*, 1996). This suggests that 5-HT<sub>1B</sub> receptors are potentially downregulated by SSRIs. Interestingly the specific binding of [<sup>125</sup>I]iodocyanopindolol to 5-HT<sub>1B</sub> sites has been shown to be increased in the cortex, hippocampus and septum of learned helplessness rats (Edwards *et al.*, 1991). No studies have however been done on the blocking effects of antidepressants on increased 5-HT<sub>1B</sub> receptors in this depressive model. In this chapter, the reduction in 5-HT<sub>1B</sub> binding in the frontal cortex after chronic SSRI treatment, may be a site specific effect that may also help to explain the delayed onset of action of these drugs in the clinic. This site specific effect is further suggested by the inability of a chronic citalopram treatment through any part of a 32 day timecourse to change the levels of 5-HT<sub>1B</sub> mRNA in rat whole brains (Spurlock *et al.*, 1994). In this case the effect on 5-HT<sub>1B</sub> receptor density seen in this chapter may be masked by the presence of other brain areas. Furthermore it should be noted that no changes in mRNA, does not necessarily mean no adaptive changes in receptor number, and *vice versa*.

Antidepressant studies on 5-HT<sub>1D</sub> receptor numbers are even more limited than 5-HT<sub>1B</sub> receptors. Chronic studies using the SSRI, paroxetine, have been shown to desensitise 5-HT<sub>1D</sub> but not 5-HT<sub>1B</sub> autoreceptors in the rat lateral geniculate nucleus (Davidson & Stamford, 1997), showing that SSRIs can modulate 5-HT<sub>1D</sub>

activity. The downregulation of 5-HT<sub>1B/1D</sub> receptor density in the frontal cortex of chronically treated rats may be of importance in the mechanism of action of these antidepressant drugs. However, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors also exist on non-serotonergic neurons as a heteroreceptor affecting both noradrenaline and acetylcholine release (see Barnes & Sharp, 1999). Chronic citalopram treatment has been shown to desensitise 5-HT<sub>1B</sub> heteroreceptors by reducing the efficacy of CGS12066B (5-HT<sub>1B</sub> receptor agonist) to inhibit the release of [<sup>3</sup>H]acetylcholine induced by K<sup>+</sup> depolarisation (Bolaños-Jimenez *et al.*, 1994). It is therefore possible that accompanying this desensitisation there is a downregulation in 5-HT<sub>1B</sub> heteroreceptor density. These heteroreceptors may also have been labelled by [<sup>3</sup>H]GR125,743. To understand the importance of the findings in the frontal cortex in this chapter, it will be important to study only the 5-HT<sub>1B/1D</sub> autoreceptor population on serotonergic nerve terminals. The antibodies characterised in chapter 2, may provide the tools necessary for such experiments, but until then it can not be concluded whether the downregulation of 5-HT<sub>1B/1D</sub> receptors after chronic SSRI treatment is either the serotonergic autoreceptor or non-serotonergic heteroreceptor population or both.

In light of the recent findings upon SERT modulation by antidepressants delivered by mini-osmotic pumps, it will be interesting to see if such adaptations can occur at the different 5-HT receptor sub-types to further elucidate mechanisms that may explain the delay onset of action of SSRIs in the clinic. Such studies may ultimately improve antidepressant drug design for more faster acting drugs with even less side effects.

## 6.4 Summary

In this chapter the effects of chronic SSRI (citalopram and fluoxetine) and tianeptine treatments on the density and affinity of a variety of 5-HT receptors and SERT was investigated in an attempt to try and understand the delayed onset of antidepressant action in the clinic.

Chronic citalopram (10mg/kg bd for 21 days), fluoxetine (10mg/kg once daily for 21 days) and tianeptine (10mg/kg bd for 14 days) treatments following a two day washout period caused no changes in the density or affinity of SERT in

frontal or caudal cortex, hippocampus, striatum or adrenal medulla membranes as measured by [<sup>3</sup>H]citalopram binding. Using antibodies raised to SERT no immunohistochemical differences were observed in the terminal field staining in any of these brain areas or cell body staining in the raphé after any of the antidepressant treatments as compared to controls. Similarly no differences were observed in the abundance of SERT in cortex or platelet homogenates after chronic SSRI or tianeptine treatments compared to controls, as assessed by Western blot analysis. The density and affinity of 5-HT<sub>1A</sub> receptors (frontal and caudal cortex and hippocampus) were also unaffected by these treatments. Therefore the mechanism of action of these antidepressant drugs is unlikely to involve neuroadaptational changes in the abundance or affinity of these components of the 5-HT system, that may account for the delay in onset of their therapeutic effectiveness.

Chronic administration of a higher dose of fluoxetine (10mg/kg bd for 21 days) with an identical washout period similarly did not affect the density or affinity of 5-HT<sub>1A</sub> receptors or NET. Significant reductions in the amount and affinity of specific [<sup>3</sup>H]citalopram binding, after such a treatment suggested the presence of residual drug, possibly norfluoxetine, rather than any adaptational change.

Chronic administration of citalopram or fluoxetine (both 10mg/kg bd for 21 days) resulted in site-specific decreases in the density of 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> receptors in frontal cortex membranes as compared to controls. Chronic tianeptine treatment by contrast resulted in no such changes.

It would therefore appear that the mechanism of action underlying the delayed onset of action of SSRIs does not involve neuroadaptational changes (at the protein level) of SERT or 5-HT<sub>1A</sub> receptors in the terminal fields, but may involve changes in the pre-synaptic 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptor population in the frontal cortex. Tianeptine, which enhances 5-HT reuptake rather than block it, though effective in treating depression, does not appear to have any effect on these receptors/transporter at the protein level in the terminal field projection areas of the raphé nuclei. However it must be stated that the delivery of drugs via a mini-osmotic pump system in the future may be more comparable to studies in humans.

**CHAPTER 7**

**THE EFFECT OF SUB-CHRONIC MDMA TREATMENTS ON  
THE DENSITY AND AFFINITY OF THE 5-HT TRANSPORTER  
AND 5-HT RECEPTORS**

The popular recreational drug of abuse, 3,4 methylenedioxy-methamphetamine (Ecstasy, MDMA), is known to selectively reduce the number of radiolabelled SERT binding sites in the rat brain (Battaglia *et al.*, 1987). This reduction in SERT density is a marker for the neurotoxic destruction of serotonergic nerve terminals by MDMA. Initially it was thought that this toxicity required multiple exposure to relatively high doses of MDMA, however subsequent studies have found that a single exposure to a high dose, or several exposures to lower doses can induce the same profile of toxicity (Colado *et al.*, 1993 & 1997a; O'Shea *et al.*, 1998). Reductions in SERT labelling after repeated exposure to MDMA, are clearly detectable in the rat brain within a relatively short time period of between 2 and 14 weeks (Battaglia *et al.*, 1987, 1988 & 1991; Sharkey *et al.*, 1991). However there is evidence of near full recovery within a year after such treatments (Scanzello *et al.*, 1993; Fischer *et al.*, 1995; Lew *et al.*, 1996; Sabol *et al.*, 1996). The mechanism of MDMA uptake by the 5-HT terminal is carrier mediated (see chapter 1, section 1.9). The results which show SERT in the rat adrenal gland to have an identical pharmacological profile to brain SERT (see chapter 4) suggest that the adrenal gland may also be sensitive to MDMA.

Although the mechanism by which MDMA damages 5-HT nerve terminals remains elusive, different drugs interfering with central serotonergic or dopaminergic systems prevent the depletion of brain 5-HT following MDMA administration (Stone *et al.*, 1988; Schmidt *et al.*, 1990a & b; Hewitt & Green, 1994; Sprague *et al.*, 1998; Colado *et al.*, 1999a). However other drugs with no direct action on these two systems also prevent the neurotoxic effects of MDMA (Finnegan *et al.*, 1990; Colado *et al.*, 1993; Colado & Green, 1994). Glutamate, the most abundant excitatory amino acid in the CNS (Stone & Burton, 1988), is capable of producing neuronal damage in a variety of paradigms (for a review, see Olney, 1990). Glutamate has been implicated in the neurotoxicity of MDMA as the NMDA receptor antagonist, MK-801, provides some degree of protection against the decrease in 5-HT levels induced by MDMA treatment (Finnegan *et al.*, 1990; Farfel *et al.*, 1992; Colado *et al.*, 1993; Finnegan & Taraska, 1996). The macrolide tacrolimus, FK506, a powerful immunosuppressive drug, which is widely used to prevent graft rejection following organ transplantation (for a review see Hooks,

1994), has neuroprotective qualities, in particular the ability to protect against glutamate neurotoxicity (Dawson *et al.*, 1993; Kikuchi *et al.*, 1998). Therefore it is possible that FK506 may protect against MDMA induced depletion of SERT. Another mechanism that may be involved in MDMA induced neurotoxicity is the formation of free radicals. Free radicals are highly reactive species capable of independent existence that contains one or more unpaired electrons which can disturb biological systems by damaging a variety of their constituent molecules such as lipids (see Maxwell & Lip, 1997). The metabolism of dopamine in the 5-HT terminal by MAO-B, has been suggested to produce hydrogen peroxide which could lead to lipid peroxidation and general oxidative stress (Sprague & Nichols *et al.*, 1995; Cadet & Brannock *et al.*, 1998; Sprague *et al.*, 1998). Indeed just like metamphetamine which is neurotoxic to both serotonergic and dopaminergic terminals, MDMA exposure may also produce free radicals (Giovanni *et al.*, 1995; Colado & Green, 1995; Colado *et al.*, 1997b; Colado *et al.*, 199a & b; Figure 1.11 in chapter 1). There it is possible that the use of a free radical scavenger may prevent MDMA neurotoxicity.

One consequence of MDMA treatment is an increased initial release of 5-HT (Rudnick & Wall, 1992), followed by a sustained decrease in 5-HT levels in different terminals of the 5-HT system (O'Hearn *et al.*, 1988). The limited availability of 5-HT following sub-chronic MDMA treatment may affect pre- or post-synaptic 5-HT receptors as reported for the 5-HT<sub>1A</sub> receptor after MDMA treatment (Aguirre *et al.*, 1995, 1997 & 1998). 5-HT<sub>1A</sub> receptors in the terminal field are mainly post-synaptic (Francis *et al.*, 1992; Lawrence *et al.*, 1993) whereas 5-HT<sub>1B/1D</sub> receptors are known to exist both pre- and post-synaptically (Middlemiss & Hutson, 1990; Bruinvels *et al.*, 1994a & b; Doucet *et al.*, 1995; Buhlen *et al.*, 1996). At present the location of 5-HT<sub>7</sub> receptors in relation to serotonergic nerve terminals is poorly defined. Only one study has attempted to address this question using the neurotoxin 5, 7-dihydroxytryptamine, which destroys serotonergic neurons and hence reduces 5-HT content (5, 7-DHT; Clemett *et al.*, 1999). In this study, involving only the hypothalamus, an increase in density of pindolol-insensitive [<sup>3</sup>H]5-HT binding was observed following 5,7-DHT treatment, suggesting a post-synaptic location of 5-HT<sub>7</sub> receptors. However, this binding assay did not label a homogeneous population of



receptors and so the confidence as to the location of 5-HT<sub>7</sub> receptors in this brain area is questionable.

In this chapter the effects of two independent sub-chronic MDMA treatments after 2 and 13 week recovery periods in both the brain and rat adrenal gland were investigated. The effect of the 2 week recovery period study was extended to examine the potential protective qualities of FK506, in both the brain and adrenal gland. A third sub-chronic MDMA treatment was included to directly compare the effects of FK506 and a free radical scavenger in the brain (FR122175; personal communication, Dr. J. Sharkey, Fujisawa Institute of Neuroscience, Edinburgh). In this third study, the effect on 5-HT<sub>7</sub> receptors was investigated using the [<sup>3</sup>H]5-CT binding assay developed in chapter 5. In all three treatments, [<sup>3</sup>H]citalopram binding was used to assay the extent of the MDMA lesion of serotonergic nerve terminals using SERT as a marker. All three treatments were also used to examine any effects on 5-HT<sub>1A</sub> receptors using [<sup>3</sup>H]8-OH DPAT binding.

## **7.1 Methods**

### **7.1.1 MDMA Treatment of Rats**

Three separate studies were performed. For each study, rats on the day of arrival, were housed in groups of four to five for three days, given free access to food and water and kept on a 12 hour light cycle at 21°C throughout the study (unless stated otherwise). Following habituation, animals were dosed by subcutaneous injection, with twice daily injections between 08:00 and 09:00, and again between 16:00 and 17:00. Each drug used was dissolved in 0.9% saline. Control groups represent those animals receiving the equivalent volumes of saline vehicle.

#### **Treatment 1**

The first treatment was a collaborative study with Dr. J. Sharkey (Fujisawa Institute of Neuroscience, Edinburgh). Three study groups totalling 15 animals, were used. Male Sprague-Dawley rats (50-70g; Charles River), housed at 19°C, were dosed using a ramped treatment protocol. The first study group was injected with MDMA (10mg/kg bd on day 1, 15mg/kg bd on day 2 & 20mg/kg bd on day 3, n=5). The second study group was injected with FK506 (10mg/kg bd) 20 mins before each of the MDMA injections as above (n=5). The third study group was control animals

(n=5). In each study group, the final dose was followed by a 2 week washout period, whereupon rats (now 150-200g) were killed by a sharp blow to the back of the neck, the head guillotined and membranes immediately made according to the methods previously described in chapter 3. Brain frontal cortex, caudal cortex, hippocampus, and striatum and also adrenal medulla membranes were prepared.

### **Treatment 2**

The second treatment was a collaborative study with Dr. P.A.T. Kelly (Department of Clinical Neurosciences, University of Edinburgh) and Prof. J. Seckl (Department of Medicine, University of Edinburgh). Two study groups totalling 16 animals were used. Adult male Wistar Cobb rats (50-70g; bred in-house, Department of Neuroscience) were injected for 4 consecutive days with either MDMA (20mg/kg bd; n=8) or the equivalent volume of control (n=8). In each study group the final dose was followed by a 13 week washout period, whereupon rats (now 300-400g) were sacrificed and the brains and adrenal glands immediately frozen in isopentane at  $-45^{\circ}\text{C}$  (temperature was maintained by cooling in a dry ice/acetone mixture). Following no less than one week at  $-70^{\circ}\text{C}$ , tissue was thawed in 0.9% saline on ice and membranes made as described in chapter 3. Binding assays carried out on the same day as membrane preparation. Brain whole cortex, hippocampus, and striatum and also adrenal medulla membranes were prepared.

### **Treatment 3**

The third treatment was a collaborative study with Dr. J. Sharkey. Five study groups totalling 25 animals were used. Male Sprague-Dawley rats (100-150g; Charles River) were injected for 3 consecutive days as detailed below. The FK506 (10mg/kg bd) or FR122175 (10mg/kg bd) injections were performed 20 minutes before the MDMA injections (20mg/kg bd). In each study group, the final dose was followed by a 2 week washout period, whereupon rats (now 250-350g) were killed by a sharp blow to the back of the neck, the head guillotined and membranes immediately made according to the methods previously described in chapter 3. Brain frontal cortex, caudal cortex and hippocampus membranes were prepared.

<u>Group</u>	<u>Injection</u>
1	Control
2	MDMA
3	MDMA + FK506
4	MDMA + FR122175
5	FR122175

### 7.1.2 Neurochemical Assays

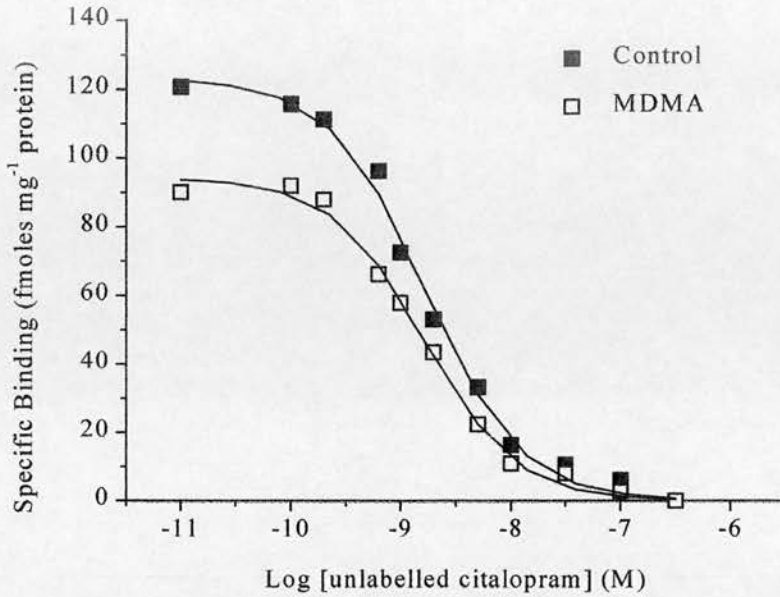
Membranes were prepared as described in chapter 3. Radioligand binding assays were performed as described in chapters 3-5. [<sup>3</sup>H]Citalopram binding was used to assay the extent of the MDMA lesion, [<sup>3</sup>H]8-OH DPAT binding to label 5-HT<sub>1A</sub> receptors and [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY10635 and 200nM GR125,743 to label 5-HT<sub>7</sub> receptors. In addition to the calculation of the K<sub>D</sub> and B<sub>max</sub> of the 5-HT<sub>7</sub> receptor binding site, the proportion of receptors labelled by 0.25nM [<sup>3</sup>H]5-CT were calculated as described in chapter 5. In the 5-HT<sub>7</sub> receptor binding assays untreated naïve membranes were assayed to ensure that the conditions of the blocks used were sufficient to reveal the 5-HT<sub>7</sub> receptor population. Inhibition curves for WAY100635 against [<sup>3</sup>H]5-CT binding and GR125,743 against WAY100635 insensitive [<sup>3</sup>H]5-CT binding were generated in naïve membranes corresponding to the tissue being assayed.

## 7.2 Results

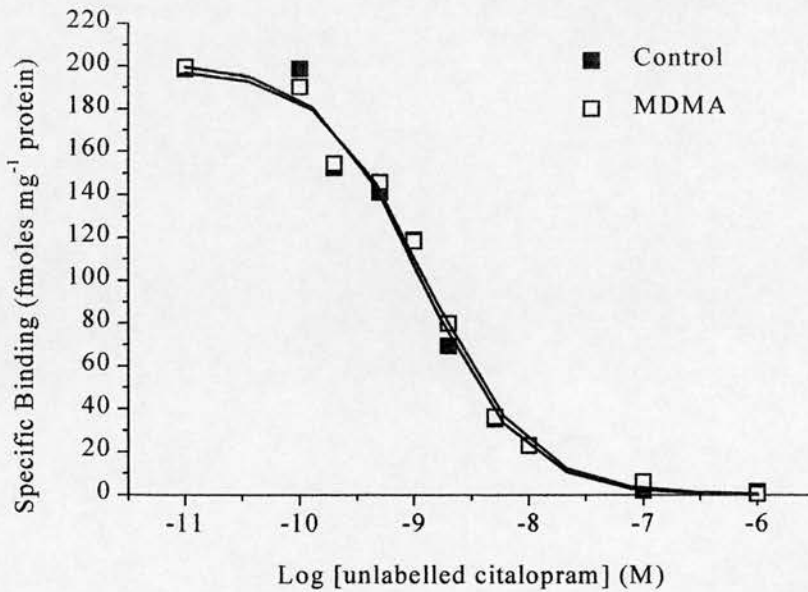
### Treatment 1

In the ramped drug treatment, MDMA (10mg/kg bd on day 1, 15mg/kg bd on day 2 & 20mg/kg bd on day 3) caused a significant 29% decrease in the density of [<sup>3</sup>H]citalopram binding sites in adrenal medulla membranes, without affecting the affinity (Table 7.1a; Figure 7.1a). In brain, MDMA also caused significant reductions in the B<sub>max</sub> values of [<sup>3</sup>H]citalopram binding sites by 33, 36 and 41% in frontal cortex, caudal cortex and hippocampus membranes respectively, without affecting the affinity (Table 7.1a). In striatum, no changes were found in either the affinity or in the binding density of [<sup>3</sup>H]citalopram binding sites (Table 7.1a). In brain regions where MDMA decreased the binding density of [<sup>3</sup>H]citalopram binding sites, no decrease was seen in the presence of FK506 (Table 7.1a). In adrenal medulla

(a)



(b)



**Figure 7.1: Effect of MDMA Treatments on [<sup>3</sup>H]Citalopram Binding Sites in Rat Adrenal Medulla Membranes.**

Data represent typical cold saturations for 0.25nM [<sup>3</sup>H]citalopram binding after; (a) ramped MDMA treatment (treatment 1; 10mg/kg bd on day 1, 15mg/kg bd on day 2 & 20mg/kg bd on day 3) following a 2 week recovery period, and (b) MDMA treatment (treatment 2; 10mg/kg bd for 4 consecutive days) following a 13 week recovery period. See Tables 7.1 and 7.2 respectively for mean data.

(a) [<sup>3</sup>H]citalopram Binding

	Adrenal Medulla		Frontal Cortex		Caudal Cortex		Hippocampus		Striatum	
	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>
Control	0.69 ± 0.06	1.22 ± 0.14	1.53 ± 0.11	0.94 ± 0.07	1.21 ± 0.10	0.99 ± 0.13	1.16 ± 0.05	0.94 ± 0.08	1.40 ± 0.14	0.93 ± 0.17
MDMA	0.49 ± 0.03*	1.14 ± 0.09	1.02 ± 0.10*	1.00 ± 0.16	0.77 ± 0.16*	1.33 ± 0.11	0.69 ± 0.08*	0.99 ± 0.05	1.40 ± 0.19	1.00 ± 0.13
MDMA + FK506	0.48 ± 0.04*	1.13 ± 0.08	1.55 ± 0.08	1.10 ± 0.11	1.18 ± 0.04	0.95 ± 0.11	1.17 ± 0.02	1.10 ± 0.02	1.39 ± 0.15	0.93 ± 0.11

(b) [<sup>3</sup>H]8-OH DPAT Binding

	Adrenal Medulla		Frontal Cortex		Caudal Cortex		Hippocampus		Striatum	
	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>
Control	-	-	0.34 ± 0.02	0.31 ± 0.05	0.41 ± 0.02	0.65 ± 0.03	0.83 ± 0.02	0.31 ± 0.05	-	-
MDMA	-	-	0.30 ± 0.05	0.31 ± 0.04	0.36 ± 0.05	0.53 ± 0.15	0.77 ± 0.14	0.34 ± 0.02	-	-
MDMA + FK506	-	-	0.31 ± 0.02	0.31 ± 0.05	0.36 ± 0.04	0.56 ± 0.08	0.80 ± 0.08	0.34 ± 0.03	-	-

Table 7.1: Effect of Ramped MDMA Treatment (2 Week Recovery Period) on [<sup>3</sup>H]Citalopram and [<sup>3</sup>H]8-OH

DPAT Binding Sites.

Animals were injected subcutaneously (treatment 1; 10mg/kg bd. on day 1, 15mg/kg bd. on day 2 and 20 mg/kg bd. on day 3 with MDMA) for MDMA treated animals, or with 10mg/kg FK506 and MDMA as in the MDMA treated group, or with the equivalent volume of saline for controls. Animals were sacrificed two weeks after the final dose, before binding assays performed as described in the text. Data represents the binding site density (B<sub>max</sub> in pmol mg<sup>-1</sup> protein) and affinity (K<sub>D</sub> in nM) for (a) [<sup>3</sup>H]citalopram (SERT) and (b) [<sup>3</sup>H]8-OH DPAT (5-HT<sub>1A</sub> receptors) binding sites. Data is represented as the mean ± s.e.mean (n=5 per group, \* = P<0.05).

membranes, however, FK506, did not prevent the MDMA induced depletion of [<sup>3</sup>H]citalopram binding sites (Table 7.1a). There were no changes in the binding parameters of [<sup>3</sup>H]8-OH DPAT after treatment with MDMA in any brain area investigated (Table 7.1b).

### **Treatment 2**

In this study where animals were allowed to recover for 13 weeks after the final dose of MDMA (20mg/kg bd for 4 days), significant reductions in the density of [<sup>3</sup>H]citalopram binding sites were observed by 25, 30 and 21 % in cerebral cortex, hippocampus and striatum membranes respectively without affecting affinity in MDMA treated rats (Table 7.2a). However in adrenal medulla membranes, MDMA administration did not have any effect on [<sup>3</sup>H]citalopram binding parameters (Figure 7.2b). As in the ramped treatment, MDMA administration caused no changes in the binding parameters of [<sup>3</sup>H]8-OH DPAT in the brain areas studied (Table 7.2b).

### **Treatment 3**

Following a 2 week recovery period after MDMA administration (20mg/kg bd. for 3 days) a significant reduction of 66, 79 and 79% in [<sup>3</sup>H]citalopram binding sites was observed in frontal cortex, caudal cortex and hippocampus membranes respectively with no change in affinity as compared to controls (Table 7.3). FK506 prevented 50, 27 and 29% of this decrease in [<sup>3</sup>H]citalopram binding site density in frontal, caudal cortex and hippocampus membranes respectively without affecting the affinity as compared to controls (Table 7.3). FR122175 at the same dose as FK506 (10mg/kg bd), prevented 72, 74 and 54% of the decrease in [<sup>3</sup>H]citalopram binding site density in frontal, caudal cortex and hippocampus membranes respectively without affecting the affinity as compared to controls (Table 7.3). FR122175 treatment in the absence of MDMA administration caused no changes in [<sup>3</sup>H]citalopram binding parameters as compared to controls. (Table 7.3). There was no significant change in the density or affinity of 5-HT<sub>1A</sub> receptors as measured using [<sup>3</sup>H]8-OH DPAT binding after any of the drug treatments compared to controls (Table 7.4).

The 5-HT<sub>7</sub> binding assay was only conducted in frontal cortex and caudal cortex membranes, as insufficient hippocampal membranes were available after the [<sup>3</sup>H]citalopram and [<sup>3</sup>H]8-OH DPAT binding assays. In each of the brain areas

(a) [<sup>3</sup>H]citalopram Binding

	Adrenal Medulla		Cerebral Cortex		Hippocampus		Striatum	
	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>
Control	0.99 ± 0.07	1.29 ± 0.07	1.47 ± 0.04	1.31 ± 0.07	1.27 ± 0.03	1.43 ± 0.09	1.93 ± 0.05	1.37 ± 0.05
MDMA	1.04 ± 0.04	1.32 ± 0.06	1.10 ± 0.10*	1.23 ± 0.06	0.89 ± 0.08*	1.36 ± 0.09	1.53 ± 0.04*	1.32 ± 0.05

(b) [<sup>3</sup>H]8-OH DPAT Binding

	Adrenal Medulla		Cerebral Cortex		Hippocampus		Striatum	
	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>	B <sub>max</sub>	K <sub>D</sub>
Control	-	-	0.28 ± 0.02	0.43 ± 0.05	0.79 ± 0.12	0.47 ± 0.08	-	-
MDMA	-	-	0.26 ± 0.04	0.39 ± 0.04	0.85 ± 0.06	0.43 ± 0.05	-	-

**Table 7.2: Effect of MDMA Treatment (13 Week Recovery Period) on [<sup>3</sup>H]Citalopram and [<sup>3</sup>H]8-OH DPAT Binding Sites.**

Animals were injected subcutaneously (treatment 2; 20mg/kg bd. for four consecutive days with MDMA) for MDMA treated animals or with the equivalent volume of saline for controls. Animals were sacrificed thirteen weeks after the final dose, before binding assays performed as described in the text. Data represents the binding site density (B<sub>max</sub> in pmol mg<sup>-1</sup> protein) and affinity (K<sub>D</sub> in nM) for (a) [<sup>3</sup>H]citalopram (SERT) and (b) [<sup>3</sup>H]8-OH DPAT (5-HT<sub>1A</sub> receptors) binding sites. Data is represented as the mean ± s.e.mean (n=8 per group, \* = P < 0.05).

**Table 7.3: Effect of MDMA Treatment on [<sup>3</sup>H]Citalopram and [<sup>3</sup>H]8-OH DPAT Binding Sites**

	Frontal Cortex			Caudal Cortex			Hippocampus		
	[ <sup>3</sup> H]citalopram	[ <sup>3</sup> H]8-OH DPAT	[ <sup>3</sup> H]citalopram	[ <sup>3</sup> H]8-OH DPAT	[ <sup>3</sup> H]citalopram	[ <sup>3</sup> H]8-OH DPAT	[ <sup>3</sup> H]citalopram	[ <sup>3</sup> H]8-OH DPAT	
Control	B <sub>max</sub> 1603.18 ± 254.02 K <sub>D</sub> 1.10 ± 0.17 Hill slope 1.03 ± 0.08	253.15 ± 26.21 0.42 ± 0.05 0.93 ± 0.04	1117.63 ± 142.78 1.04 ± 0.05 0.96 ± 0.06	223.33 ± 38.52 0.52 ± 0.12 0.85 ± 0.04	1402.77 ± 133.80 1.14 ± 0.10 0.98 ± 0.03	810.25 ± 40.27 0.45 ± 0.04 1.01 ± 0.03			
MDMA	B <sub>max</sub> 544.21 ± 52.28* K <sub>D</sub> 1.04 ± 0.06 Hill slope 0.98 ± 0.05	260.55 ± 42.22 0.48 ± 0.08 0.94 ± 0.06	229.97 ± 33.81* 1.07 ± 0.04 1.11 ± 0.18	265.28 ± 22.25 0.60 ± 0.12 0.81 ± 0.07	288.08 ± 45.75* 0.98 ± 0.03 1.17 ± 0.10	799.25 ± 28.56 0.45 ± 0.08 1.02 ± 0.03			
MDMA + FK506	B <sub>max</sub> 1069.12 ± 156.02 K <sub>D</sub> 1.11 ± 0.08 Hill slope 0.95 ± 0.08	280.12 ± 12.65 0.48 ± 0.05 0.96 ± 0.04	470.48 ± 121.66 1.08 ± 0.06 1.03 ± 0.04	223.35 ± 35.61 0.45 ± 0.06 0.90 ± 0.08	610.21 ± 40.67 0.93 ± 0.03 1.04 ± 0.04	800.01 ± 50.25 0.48 ± 0.08 1.05 ± 0.12			
FR122175	B <sub>max</sub> 1468.44 ± 191.61 K <sub>D</sub> 1.01 ± 0.02 Hill slope 1.12 ± 0.09	258.25 ± 7.51 0.59 ± 0.12 0.91 ± 0.03	953.84 ± 50.55 0.98 ± 0.07 1.21 ± 0.07	245.12 ± 12.76 0.48 ± 0.08 0.86 ± 0.04	1504.75 ± 227.37 1.02 ± 0.13 1.01 ± 0.06	770.55 ± 60.25 0.45 ± 0.05 0.95 ± 0.05			
MDMA + FR122175	B <sub>max</sub> 1309.98 ± 192.32 K <sub>D</sub> 1.20 ± 0.18 Hill slope 0.92 ± 0.03	249.28 ± 12.85 0.43 ± 0.04 0.95 ± 0.04	890.49 ± 63.12 1.18 ± 0.13 1.17 ± 0.09	229.54 ± 25.61 0.46 ± 0.08 0.88 ± 0.04	892.29 ± 125.51 1.15 ± 0.07 0.94 ± 0.08	750.25 ± 25.25 0.41 ± 0.08 1.01 ± 0.07			

Animals were injected subcutaneously (treatment 3; 20mg/kg bd. for three consecutive days with MDMA) for MDMA treated animals, with MDMA and FK506 (10mg/kg bd for 3 days), with FR122175 (10mg/kg bd for 3 days), with MDMA and FR122175, or with the equivalent volume of saline for controls. Animals were sacrificed two weeks after the final dose, before binding assays performed as described in the text. Data represents the B<sub>max</sub> in fmol mg<sup>-1</sup> protein, K<sub>D</sub> in nM and Hill slope (nH) for (a) [<sup>3</sup>H]citalopram (SERT) and (b) [<sup>3</sup>H]8-OH DPAT (5-HT<sub>1A</sub> receptors) binding sites. Data is represented as the mean ± s.e.mean (n=5 per group, \* = P < 0.05).



**Table 7.4: Effect of MDMA Treatment on [<sup>3</sup>H]5-CT Binding.**

		Frontal Cortex	Caudal Cortex
Control	% 5-HT <sub>1A</sub>	55.05 ± 2.26	60.21 ± 3.52
	% 5-HT <sub>1B/1D</sub>	37.12 ± 1.25	29.21 ± 3.25
	% 5-HT <sub>7</sub>	7.83 ± 1.10	10.58 ± 2.21
	Hill slope	0.83 ± 0.03	0.82 ± 0.06
	K <sub>D</sub>	0.51 ± 0.12	0.34 ± 0.06
	B <sub>max</sub>	83.12 ± 4.25	62.01 ± 4.89
MDMA	% 5-HT <sub>1A</sub>	60.23 ± 3.26	65.21 ± 2.21
	% 5-HT <sub>1B/1D</sub>	28.65 ± 2.28*	20.18 ± 3.41*
	% 5-HT <sub>7</sub>	11.12 ± 2.10*	14.61 ± 1.26*
	Hill slope	0.83 ± 0.03	0.81 ± 0.07
	K <sub>D</sub>	0.44 ± 0.08	0.37 ± 0.09
	B <sub>max</sub>	118.25 ± 3.87*	85.81 ± 1.25*
MDMA + FK506	% 5-HT <sub>1A</sub>	58.25 ± 2.65	60.75 ± 3.24
	% 5-HT <sub>1B/1D</sub>	35.25 ± 1.25	28.25 ± 2.21
	% 5-HT <sub>7</sub>	6.50 ± 2.01	11.00 ± 2.12
	Hill slope	0.89 ± 0.02	0.81 ± 0.04
	K <sub>D</sub>	0.48 ± 0.06	0.39 ± 0.09
	B <sub>max</sub>	85.12 ± 4.56	63.21 ± 2.25
FR122175	% 5-HT <sub>1A</sub>	57.25 ± 5.25	60.85 ± 3.75
	% 5-HT <sub>1B/1D</sub>	36.25 ± 2.25	28.59 ± 2.85
	% 5-HT <sub>7</sub>	6.50 ± 2.21	10.56 ± 1.25
	Hill slope	0.85 ± 0.02	0.79 ± 0.07
	K <sub>D</sub>	0.45 ± 0.05	0.44 ± 0.08
	B <sub>max</sub>	88.85 ± 8.21	68.25 ± 6.25
MDMA + FR122175	% 5-HT <sub>1A</sub>	54.51 ± 2.26	62.21 ± 2.51
	% 5-HT <sub>1B/1D</sub>	36.25 ± 2.51	26.25 ± 2.21
	% 5-HT <sub>7</sub>	9.24 ± 1.21	11.54 ± 0.79
	Hill slope	0.85 ± 0.05	0.89 ± 0.02
	K <sub>D</sub>	0.48 ± 0.06	0.41 ± 0.07
	B <sub>max</sub>	89.00 ± 8.25	61.70 ± 5.20

Animals were dosed according to the method described in treatment 3. The results are expressed as the mean ± s.e.mean for 5 animals in frontal and caudal cortex membranes. The proportions of 5-HT<sub>1A</sub>, the mixed 5-HT<sub>1B/1D</sub> and the 5-HT<sub>7</sub> receptors occupied by 0.25nM [<sup>3</sup>H]5-CT are reported (%). The K<sub>D</sub> in nM, the B<sub>max</sub> in fmoles mg<sup>-1</sup> protein and the Hill slope (nH) of the 5-HT<sub>7</sub> binding site determined by [<sup>3</sup>H]5-CT binding in the presence of 200nM WAY100635 & 200nM GR125,743 are given. \* = P < 0.05 compared to controls.

investigated naïve membranes from non-treated rats revealed that 200nM WAY100635 was sufficient to block the 5-HT<sub>1A</sub> component of [<sup>3</sup>H]5-CT binding described in chapter 5 (data not shown). 200nM WAY100635 & 200nM GR125,743 was sufficient to block the 5-HT<sub>1B/1D</sub> component of [<sup>3</sup>H]5-CT binding described in chapter 5 (data not shown).

An increase in the 5-HT<sub>7</sub> binding site density of 42 and 38% in frontal and caudal cortex membranes after MDMA treatment respectively without a change in the affinity was observed. (Table 7.4). The proportion of 5-HT<sub>7</sub> receptors labelled using 0.25nM [<sup>3</sup>H]5-CT were similarly increased by 42 and 38 % in the two respective brain areas after repeated MDMA administration compared to controls. The proportion of 5-HT<sub>1B/1D</sub> receptors labelled using 0.25nM [<sup>3</sup>H]5-CT revealed a significant decrease in both frontal and caudal cortex membranes by 23 and 31% respectively in rats treated with MDMA compared to controls (Table 7.4). There was no change in the proportion of 5-HT<sub>1A</sub> receptors labelled after any drug treatment group in these brain areas (Table 7.4). MDMA administration in the presence of either FK506 or FR122175 resulted in no change in the proportion of all 5-HT receptors labelled using 0.25 nM [<sup>3</sup>H]5-CT as compared to controls (Table 7.4).

### 7.3 Discussion

The selective neurotoxic actions of MDMA in serotonergic nerve terminals can be detected not only by histochemical changes but also by the reduction of radiolabelled SERT binding sites (Battaglia *et al.*, 1987, 1988 & 1991 & Sharkey *et al.*, 1991). In this chapter the effect of repeated MDMA administration on the density and affinity of [<sup>3</sup>H]citalopram binding sites was investigated in both the rat brain and adrenal gland. The potential neuroprotective effects of FK506 and FR122175 and the effect of such treatments on 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors were also examined

Following a two week recovery period after a ramped MDMA treatment (“treatment 1”; 10mg/kg bd. on day 1, 15 mg/kg bd. on day 2 and 20mg/kg bd. on day 3), a significant reduction of 29% in SERT binding site densities in adrenal medulla membranes with no change in affinity was observed (Table 7.1a; Figure 7.1a). MDMA has the same affinity for SERT in the CNS and adrenal medulla (see chapter 4, Table 4.1), and also significantly reduced SERT binding site densities by

41% in hippocampus, 36% in caudal cortex and 33% in frontal cortex membranes, without affecting the affinity (Table 7.1a). Previous studies using a similar repeated dosing schedule, have shown a much greater reduction in SERT density in hippocampus and cortex brain regions of > 70% (Battaglia *et al.*, 1987; Battaglia *et al.*, 1988; Sharkey *et al.*, 1991). However it is now apparent that ambient temperature not only has a pronounced effect on core temperature and thermoregulation of rats, but also on MDMA neurotoxicity, which can cause a persistent loss of thermoregulation (Dafters & Lynch, 1998). Increases in core temperature, caused by increased ambient temperatures, of MDMA-treated animals, increase neurotoxicity whereas decreases in core temperature decrease neurotoxicity. These pronounced changes can be caused by as little as a 2°C fluctuation (Malberg & Sneinden, 1998). In animals treated at or above ambient temperatures, MDMA causes a hyperthermic response (Gordon *et al.*, 1991; Colado *et al.*, 1999d). It is not surprising therefore that in humans, whose users commonly frequent crowded raves, the most common and fatal side effects associated with MDMA use is hyperthermia (Henry *et al.*, 1992). However if the ambient temperature is lowered, a hypothermic response occurs resulting in a dampening of the MDMA induced neurotoxicity (Gordon *et al.*, 1991). In this study, rats were housed at slightly lower than normal ambient temperatures (19°C compared to 22°C), which may explain the lesser neurotoxic action of MDMA observed in the brain regions studied. This temperature effect may also help to explain why no damage was caused in the striatum (Table 7.1a) and hence some brain regions may be less or more susceptible to temperature-dependent neurotoxicity than others.

FK506 prevented the damage caused by MDMA in those brain areas where there was a reduction in SERT binding density, but did not block the MDMA induced depletion of SERT in the adrenal gland (Table 7.1a). The action of FK506 neuroprotection in the brain against MDMA neurotoxicity could involve both glutamate and nitric oxide (NO). The role of glutamate and/or NO in MDMA-induced neurotoxicity at present is rather ambiguous (Sprague *et al.*, 1998). In the brain, MDMA neurotoxicity is prevented by glutamate receptor antagonists (Finnegan *et al.*, 1990; Farfel *et al.*, 1992; Colado *et al.*, 1993; Finnegan & Tarraska, 1996) and by the nitric oxide synthase (NOS) inhibitor, L-NAME, but not by other

NOS inhibitors (Tarasaka & Finnegan, 1997). FK506 is thought to inhibit glutamate neurotoxicity by inhibiting NOS (Kikuchi *et al.*, 1998). The complex caused by FK506 binding to the FK506 binding protein, inhibits calcineurin dephosphorylation of protein-kinase C mediated phosphorylation of NOS, thereby inactivating the enzymes catalytic activity and hence decreases production of NO (Dawson *et al.*, 1993; Zhang & Steiner, 1995). FK506 is also known to produce a hypothermic response in reducing focal cerebral ischaemic damage (Ide *et al.*, 1996; Bochelen *et al.*, 1999). The production of a hypothermic response is a suggested mechanism for other drugs that protect against MDMA induced neurotoxicity (see Malberg *et al.*, 1996; Colado *et al.*, 1999b & c). However this is not a common mechanism of action for drugs that prevent MDMA neurotoxicity. For example, fluoxetine has consistently been shown to block MDMA induced neurotoxicity without preventing MDMA induced hyperthermia (see Malberg *et al.*, 1996). By blocking SERT, fluoxetine therefore prevents entry of MDMA into the serotonergic terminal emphasising that MDMA neurotoxicity involves a carrier mediated process (Rudnick & Wall, 1992).

The present study is the first to investigate the effects of MDMA treatment on SERT in the adrenal gland. In an earlier report MDMA stimulates corticosterone secretion through 5-HT release (Nash *et al.*, 1988). More recently MDMA was shown to potentiate the direct action of 5-HT on aldosterone secretion *in vitro* from adrenals (Burns *et al.*, 1996). The mechanism underlying this increased aldosterone secretion, could be associated with SERT. For example, it is known that MDMA stimulates 5-HT efflux (Rudnick & Wall, 1992) and increased 5-HT levels are known to increase steroid production in the adrenal gland (Rocco *et al.*, 1990 & 1992). The outcome of such increases in corticosterone and 5-HT is a site-specific regulation of corticosteroid receptor expression in the brain, but only in adrenal intact animals suggesting some form of corticosterone control (Yau *et al.*, 1994 & 1997a). In the brain it is believed that the interactions between 5-HT and corticosterone are mediated by 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors (Le Corr e *et al.*, 1997). Recently corticosterone has been shown to regulate not only 5-HT<sub>1A</sub> receptor expression, but also SERT expression in the ageing brain (Maines *et al.*, 1999).

Despite a similar MDMA induced depletion of SERT in the adrenal medulla compared to in cortex and hippocampus brain areas, it can not be concluded if this was due to the destruction of adrenal chromaffin cells. If MDMA also has a toxic effect in the adrenal gland as in the brain, one would expect a similar decrease in SERT and NET binding sites in the adrenal medulla as both transporters are co-localised on adrenal chromaffin cells (Schroeter *et al.*, 1997). An attempt was made to address this question by initially developing a [<sup>3</sup>H]nisoxetine binding assay in both brain and adrenal medulla membranes (chapter 4). However the low amount of specific [<sup>3</sup>H]nisoxetine binding characterised for NET in adrenals, meant that from one animal there was not enough tissue to study the comparative effects of MDMA on both SERT and NET in the adrenal medulla in the same animals. The failure of FK506 to prevent the MDMA depletion of SERT in the adrenal medulla, in no way provides conclusive evidence that the action of MDMA is not a toxic one as in the brain. FK506 may simply exert its effects through site specific mechanisms. For example, although FK506 inhibits glutamate neurotoxicity in the brain, glutamate binding sites in the rat adrenal medulla have been shown to be different to those in the CNS (Yoneda & Ogita *et al.*, 1986) and therefore may be less susceptible to FK506 protection. Future directives must therefore be driven towards answering this MDMA toxicity question in the adrenal medulla. For example, 5-HT, 5-HIAA and dopamine levels may be monitored after MDMA treatment in the adrenal glands. A neurotoxic event may be evident if 5-HT and 5-HIAA levels fall and dopamine levels rise, as previously reported in the brain (Battaglia *et al.*, 1988; Colado *et al.*, 1999a). The ability of the Chemicon monoclonal antibody to recognise SERT in both adrenal and brain tissue may also help to understand the action of MDMA in the adrenal gland (chapter 4). Using this antibody in conjunction with commercially available antibodies raised against proteins co-localised with SERT, such as SNAP-25 (a t-SNARE protein associated in neurotransmitter release; Pfeffer, 1996) or catecholamine biosynthetic enzymes such as phenyl-ethanolamine N-methyltransferase (PMNT; Ubink *et al.*, 1995; Schroeter *et al.*, 1997), an answer may be revealed. It is probably more appropriate to use immunological or autoradiographic techniques to investigate the effects of repeated MDMA administration on the co-localisation of SERT with DAT or NET which are

unaffected by MDMA treatment in the brain (Stone *et al.*, 1986; Schimdt, 1987; Battaglia *et al.*, 1988). This latter method will also overcome the need for the large number of animals needed for membrane binding studies, as multiple sections can be generated from one animal.

Following a longer recovery period of 13 weeks after a slightly different treatment regime ("treatment 2"; 20mg/kg bd. for 4 days), MDMA caused a reduction in SERT binding site density of between approximately 20 and 30 % in cortex, hippocampus and striatum membranes (Table 7.2a). Although it is known that re-innervation of 5-HT terminal regions does occur in rodents and primates it is by no means "normal" or full (Scanzello *et al.*, 1993; Fischer *et al.*, 1995; Lew *et al.*, 1996; Sabol *et al.*, 1996). This has been shown in monkey studies using a 13 month or even seven year recovery after the final dose of a repeated MDMA dosing schedule (Scheffel *et al.*, 1998; Hatzidimitriou *et al.*, 1999). In that respect it is not surprising that MDMA damage is still present after 13 weeks recovery as previously reported (Battaglia *et al.*, 1991; Sharkey *et al.*, 1991). No experimental animals after a two week recovery period were however available in this independent study to assess any possible 5-HT nerve terminal regeneration. As in the previous study a lower than expected serotonergic lesion was apparent, despite the injected animals being maintained at a higher temperature. This may be due to the use of a different strain of rat in this particular study; i.e. Wistar Cobb compared to Sprague Dawley. However, unlike in the previous treatment, this Wistar Cobb rat treatment at the higher temperature of 21°C did induce a reduction in striatum SERT binding sites. This further suggests a temperature-site specific regulation of MDMA neurotoxicity. In contrast to the ramped treatment allowed to recover for two weeks, there was no MDMA induced depletion of SERT in the adrenal medulla after 13 weeks recovery, despite significant decreases in all brain areas (Table 7.2a; Figure 7.2b). The significance of this finding is hard to imagine without knowing whether or not the initial MDMA induced depletion of adrenal medulla SERT in the ramped study was due to MDMA toxicity. However if adrenal SERT depletion is a toxic consequence of MDMA exposure as in the brain, there are two possible explanations to explain this recovery. One is that MDMA has a different mechanism of toxicity in the adrenal gland compared to the brain, which is supported by the inability of FK506 to

protect adrenal SERT depletion. Another possible explanation may lie in the morphology of adrenal chromaffin cells. Cell bodies in the dorsal raphe nuclei and thick beaded axons from the median raphe nuclei are relatively spared in comparison to the fine projections arising from the dorsal raphe nuclei after MDMA treatment (Molliver *et al.*, 1990). Raphe cell bodies are a longer distance away from SERT containing nerve terminals in comparison to the relatively short distance of the nucleus of adrenal chromaffin cells from their SERT-containing plasma membrane. Therefore freshly synthesised SERT, near the nucleus of these cell bodies has a long distance to travel before incorporation into the plasma membrane. This may explain the long period of recovery needed to negate MDMA neurotoxicity in the brain. In the adrenals, however, such a distance is much shorter, and hence may explain why the MDMA induced depletion of SERT is reversed following longer recovery periods. Understanding the trafficking of such transporters in both the brain and adrenal chromaffin cell may therefore help to understand the long term consequences of MDMA exposure in potentially regulating steroid and 5-HT levels.

In both these MDMA treatments (“treatments 1 & 2”), the density of [<sup>3</sup>H]8-OH DPAT binding to 5-HT<sub>1A</sub> receptors was also assessed in some brain areas. Differential regional regulation of this receptor subtype has been previously reported after MDMA treatment (Aguirre *et al.*, 1995, 1997, & 1998). However there were no changes in the density of 5-HT<sub>1A</sub> receptors after either treatment in frontal or caudal cortex or hippocampus membranes (Tables 7.1b & 7.2b). The different survival times (7 days vs. 14 days and 13 weeks in the studies in this thesis) may explain these differences. Indeed another study after two weeks MDMA treatment has shown no change in the level of 5-HT<sub>1A</sub> mRNA (Yau *et al.*, 1994). The longer recovery study only looked at the whole cortex receptor population and therefore may have diluted the region specific regulation of the frontal cortex as reported by Aguirre *et al.*, (1995, 1997 & 1998).

In a third series of treatments (“treatment 3”) the comparative effects of repeated MDMA administration (20mg/kg bd for 4 days) in the presence of FK506 or the free radical scavenger FR122175 was investigated on brain SERT and 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors. The neurotoxicity of MDMA was confirmed by a significant reduction in the number of radiolabelled SERT sites (Battaglia *et al.*, 1987; Table

7.3a), confirming the specific neurotoxic lesioning of serotonergic nerve terminals. This reduction in SERT density unlike the previous treatments, was more comparable to previous studies with a 66, 79 and 79% reduction in radiolabelled SERT binding sites in the frontal cortex, caudal cortex and hippocampus (Battaglia *et al.*, 1987; Sharkey *et al.*, 1991). This may reflect the use of slightly older rats (as reflected by their starting weight) at room temperature compared to the other treatments in this chapter. Indeed it has been shown that there is an age dependent increase in MDMA induced SERT depletion in the rat brain (Broening *et al.*, 1994 & 1995). Such age dependency correlates with the development of the dopaminergic system (Aguirre *et al.*, 1998). It is therefore possible that the 21-25 day old rats used in treatments 1 & 2, may not be as susceptible to MDMA neurotoxicity as their older counterparts used in this third treatment. Both FK506 and FR122175 acted as neuroprotective drugs by preventing the MDMA induced reduction in [<sup>3</sup>H]citalopram binding sites in the brain areas studied, with no effect produced alone (Table 7.3). FR122175 had a greater neuroprotective power than FK506 by returning the number of SERT binding sites closer to controls (Table 7.3). FR122175 is a free radical scavenger (Dr. J. Sharkey, Fujisawa Institute of Neuroscience, Edinburgh), and therefore it would seem that free radicals do play a role in MDMA neurotoxicity as recently reported by others (Colado & Green, 1995; Colado *et al.*, 1997b; Colado *et al.*, 1999a & b; Yeh *et al.*, 1999).

The thermoregulatory effect of FK506 is believed not to be solely responsible for reductions in neurotoxicity (Yagita *et al.*, 1996). The mitochondrial electron transport chain is an intracellular source of free radical production (Dykens, 1997). Mitochondrial dysfunction can cause free radical mediated cell damage (Raddi *et al.*, 1997), which in turn can lead to cell death (Murphy & Bredesen, 1997). FK506 has recently been shown to decrease *in vitro* oxidative phosphorylation of mitochondria from rat forebrain at complex III of the mitochondrial electron transport chain, which is particularly involved with the formation of reactive oxygen species (Zinni *et al.*, 1998). Therefore the mechanism by which FK506 prevents MDMA induced neurotoxicity may also be associated with free radicals by preventing mitochondrial dysfunction.



Mechanisms other than free radical scavenging involved in the neuroprotective attributes of FR122175 can not be ruled out. For example, it is not known if FR122175 has any effects on thermoregulation. Indeed the action of both pentobarbitone and clomethiazole in protecting MDMA induced neurotoxicity was originally thought to occur through a free radical scavenging process. However it is now believed that free radical formation is actually prevented by reductions in MDMA induced hyperthermia caused by the hypothermic responses of these two drugs (Colado *et al.*, 1999b& c). Fluoxetine has recently been shown to protect against MDMA neurotoxicity by reducing free radical production (Shankaran *et al.*, 1999) without altering MDMA induced hyperthermia confirming that the MDMA-induced generation of hydroxyl radicals, and, ultimately, the long term depletion of 5-HT, is dependent in part on the activation of SERT.

In agreement with “treatments 1 and 2”, no changes in the binding parameters for [<sup>3</sup>H]8-OH DPAT binding to 5-HT<sub>1A</sub> receptors was observed in treatment 3. Within this chapter, studies on the density and affinity of 5-HT<sub>7</sub> receptors after repeated MDMA treatment were used in an attempt to ascertain the location of this receptor sub-type in membranes derived from the cortex. After repeated exposure to MDMA (“treatment 3”), the binding density of 5-HT<sub>7</sub> receptors was significantly increased in both the frontal and caudal cortex (Table 7.4). Although this does not rule out the existence of some pre-synaptic receptors (the up-regulation could be an underestimate), these results indicate that the 5-HT<sub>7</sub> receptor is mainly located post-synaptic to 5-HT nerve terminals in the rat cortex, where it appears to be upregulated by a reduction in synaptic levels of 5-HT. A similar experimental set-up, has shown this to be the case in the rat hypothalamus, whereby animals treated with the neurotoxin 5,7-DHT also up-regulates 5-HT<sub>7</sub> receptor binding sites (Clemett *et al.*, 1999). In agreement with this postulated post-synaptic location in these brain areas, is the similar distribution of [<sup>3</sup>H]5-CT binding sites and 5-HT<sub>7</sub> mRNA in these structures (Gustafson *et al.*, 1996). In contrast some brain areas such as in the raphé nuclei as described in chapter 5, may have 5-HT<sub>7</sub> receptors with a pre-synaptic location. If this were the case, a decrease in specific 5-HT<sub>7</sub> receptor binding in conjunction with a decrease in [<sup>3</sup>H]citalopram binding would be expected. In the future it would be interesting to autoradiographically assess the density of 5-HT<sub>7</sub>

receptors (using the methodology in chapter 5) after MDMA treatment to determine the location of these binding sites in all brain areas as to whether they are predominantly pre or post-synaptic. In the presence of FK506 or FR122175, which could only partially prevent the MDMA induced depletion of SERT (Table 7.3), there was no increase in 5-HT<sub>7</sub> binding sites observed after MDMA administration (Table 7.4). This may suggest that there is a threshold of MDMA neurotoxicity that modulates specific receptor changes and may explain why no changes were observed for the 5-HT<sub>1A</sub> receptor subtype. A significant decrease in the proportion of 5-HT<sub>1B/1D</sub> receptors labelled was also observed after repeated MDMA treatment (Table 7.4). This would suggest that 5-HT<sub>1B/1D</sub> receptors are indeed present on 5-HT nerve terminals (Engel *et al.*, 1986; Maura *et al.*, 1986; Limberger *et al.*, 1991). This however would need to be confirmed using a [<sup>3</sup>H]GR125,743 binding assay as developed in chapter 5. The decrease in the % of 5-HT<sub>1B/1D</sub> receptors labelled after MDMA exposure may just be a consequence of the increase in the % of 5-HT<sub>7</sub> receptors radiolabelled. The lack of available tissue meant that this could not be investigated in this study. Furthermore despite being a marker for the pre-synaptic 5-HT nerve terminals, 5-HT<sub>1B/1D</sub> receptors are also found on non-serotonergic neurons acting as heteroreceptors (Bolanós-Jiménez & Fillion, 1989; Molderings *et al.*, 1990). These receptors may therefore mask any reductions in 5-HT<sub>1B/1D</sub> receptors found on 5-HT neurons as MDMA neurotoxicity is selective for 5-HT containing neurons (Battaglia *et al.*, 1988). MDMA administration in combination with the use of a purified serotonergic synaptosomal preparation, as discussed in chapter 2, may help to further elucidate the consequences of MDMA exposure and to determine the proportion of those receptors that are pre- and/or postsynaptic in their location.

#### **7.4 Summary**

In this chapter the effects of repeated MDMA administration on SERT in the brain and adrenal gland and 5-HT receptors in the brain was investigated in the presence or absence of the immunosuppressant drug, FK506 or a free radical scavenger (FR122175).

Repeated MDMA treatment, with a 2 week recovery period, resulted in a significant reduction in adrenal medulla and brain cortex and hippocampus SERT.

However it is not known if the results in the adrenal medulla were caused by the neurotoxicity of MDMA as known in the brain. The immunosuppressant drug, FK506, prevented brain MDMA neurotoxicity, but had no effect on the MDMA induced depletion of SERT in the rat adrenal medulla. Another MDMA treatment with a longer 13 week recovery period, caused a significant reduction in brain SERT density, but had no effect on adrenal medulla SERT.

In a third study, repeated MDMA administration caused a more substantial reduction in the density of SERT. FR122175, a free radical scavenger was more efficient at preventing such neurotoxicity compared to FK506.

5-HT<sub>7</sub> receptor binding site densities were increased in the frontal and caudal cortex whereas no changes in any of the three MDMA treatments were observed for the 5-HT<sub>1A</sub> receptor.

## **GENERAL CONCLUSIONS**

In this thesis the effects of repeated administration of a number of drugs, which interact with the 5-HT transporter, on the rat serotonergic system have been examined. This has included the validation and development of a variety of immunological and radioligand binding techniques for the detection and quantification of particular components of the serotonergic system. This has involved the characterisation of site directed SERT antibodies, the immunological detection of native SERT, the pharmacological characterisation and localisation of SERT in the brain and adrenal medulla, and the establishment of radioligand binding assays capable of selectively labelling 5-HT<sub>1A</sub>, 5-HT<sub>7</sub> and 5-HT<sub>1B/1D</sub> in brain membranes. These methodologies have been used in combination to investigate the effect of chronic antidepressant treatments on SERT and 5-HT<sub>1A</sub>, 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors. They have also been used to study the effects of repeated MDMA administration on the modulation of ligand binding to SERT and 5-HT<sub>1A</sub> and 5-HT<sub>7</sub> receptors in the presence and absence of potentially neuroprotective drugs.

### **Characterisation of Site Directed SERT Antibodies**

A library of 6 site directed antibodies were characterised as to their specificity to recognise SERT. SDS-PAGE Western blots revealed two antibodies that recognised a single immunoreactive species in rat neocortex at 76 kDa and two immunoreactive species in rat blood platelets, one at 94 kDa and one between 54-76 kDa in rat blood platelets. The latter probably represents different degrees of SERT deglycosylation (Qian *et al.*, 1995). No immunoreactivity was detected in rat liver. The rank order of intensity of immunoreactive signal was platelets > cortex > cerebellum > liver. This is consistent with the known electrophoretic mobility and distribution of SERT (Qian *et al.*, 1995). One of these antibodies was raised to a conserved sequence in the 4<sup>th</sup> extracellular loop of SERT (in-house 1001) and the other to the N-terminus (Chemicon mouse monoclonal). Immunohistochemical studies demonstrated SERT immunoreactivity in the cell bodies of the raphe nuclei. In this brain area, SERT immunoreactivity was similar to 5-HT immunoreactivity, consistent with the idea that SERT in the CNS is found specifically on 5-HT containing neurons. SERT specific staining was also present in the dendrites and terminal fields of the raphe nuclei projection areas of the cortex, hippocampus &

striatum. This location of SERT in the rat brain is consistent with previous reports using other SERT directed antibodies (Sur *et al.*, 1996). The antibodies with the best quality of staining were one raised to conserved sequences of the SERT COOH-tail (in house 998), and again the Chemicon mouse monoclonal antibody. The in-house 1001 and 998 antibodies were selected for the immunological qualitative assessment of SERT abundance after drug treatments using SDS-PAGE Western blots and immunohistochemistry respectively.

### **Immunological Detection of Native SERT**

Several methods were used to identify antibodies that recognise the native form of SERT. Using an ELISA based assay no specific signal was detected due to the low abundance of SERT in membranes compared to using the antigenic peptide in a 96 well plate assay. Increasing the abundance of SERT using a centrifugation ELISA was also unsuccessful due to the incorporation of the secondary antibodies in the membrane fraction following centrifugation. Native Western blotting revealed specific SERT immunodetection using the Chemicon mouse monoclonal and guinea pig antibodies. These two antibodies which recognise different SERT epitopes (N-terminus and COOH-tail respectively), were used in combination whereby the mouse antibody immunoprecipitated SERT from native membrane extracts and the guinea pig antibody then used for SERT immunodetection in SDS-PAGE Western blots. The detection of native SERT protein is an important prerequisite to isolate pure, viable and functional serotonergic nerve terminals. The immunoprecipitation procedure demonstrates that the immunomagnetic separation of 5-HT containing nerve terminals may well be possible. The mouse monoclonal antibody can be used to label native SERT, and in conjunction with a magnetic secondary antibody can specifically extract the serotonergic nerve terminal population. The guinea pig antibody may then be used to assess the purity of such a separation. Alternatively a combination of [<sup>3</sup>H]citalopram binding to SERT and radioligand binding to non-serotonergic markers (eg. NET) can be used to monitor the purification to avoid the possible species cross reactivity that is apparent with these two antibodies. This is the key step for preparing serotonergic nerve terminals, which can subsequently be used by neuropharmacologists and electrophysiologists, to identify and determine the

characteristics and influence of the receptor subtypes present on only serotonergic nerve terminals.

### **Pharmacological Characterisation of Adrenal SERT**

An *In vitro* radioligand binding assay was used to specifically detect SERT in the medulla of the rat adrenal gland. The affinities of a number of amine uptake inhibitors and substrates at [<sup>3</sup>H]citalopram binding sites in adrenal medulla, cerebral cortex and platelet membranes were essentially identical. [<sup>3</sup>H]Paroxetine autoradiography and SDS-PAGE Western blots using the Chemicon mouse monoclonal antibody confirmed this specific localisation. Adrenal chromaffin SERT is therefore indistinguishable from neuronal or platelet SERT and may similarly serve as the uptake machinery for 5-HT into the chromaffin cells of the adrenal gland where 5-HT *de novo* synthesis is unclear (Holzwarth *et al.*, 1984; Vandenberg *et al.*, 1991). SERT has recently been shown to exhibit polarity in rat adrenal chromaffin cells (Schroeter *et al.*, 1997). The identical pharmacology and size of adrenal SERT identified in this thesis warrants the use of this relatively simple system to try and increase our current knowledge of SERT trafficking in neurones.

In the adrenal gland, 5-HT uptake has also been proposed to take place through the adrenal chromaffin noradrenaline transporter (NET; Michael-Hepp *et al.*, 1992). An *in vitro* radioligand binding assay was used to detect NET in the rat adrenal medulla. The pharmacology of NET was identical to [<sup>3</sup>H]nisoxetine binding in the cerebral cortex, completely different to the pharmacology of SERT in these tissues and had a density of approximately 8 times less than that of SERT in the adrenal medulla. Together with the low affinity of 5-HT for NET and the significant quantities of 5-HT in chromaffin granules (Brownfield *et al.*, 1985), it is therefore more likely that 5-HT is taken up by SERT present on adrenal chromaffin cells. The discovery of SERT in the rat adrenal medulla may also help explain the regulation of steroid production and function of the HPA axis in various pathophysiological conditions such as depression (see Barden *et al.*, 1995).

## Development of Receptor Binding Assays

Receptor binding assays were validated or developed, minimising the amount of tissue required after drug treatments, to allow the maximum number of receptor binding assays to be studied in parallel. The pharmacological profile of [<sup>3</sup>H]8-OH DPAT binding was consistent with rat 5-HT<sub>1A</sub> receptor binding (Gozlan *et al.*, 1983).

Previous attempts at specifically binding to native rat brain 5-HT<sub>7</sub> receptors have been difficult due to the lack of selective ligands available. Non-selective radioligands in the presence of masking drugs to prevent binding to non-5-HT<sub>7</sub> receptors have failed to label a homogeneous population of binding sites in individual rat brain regions. [<sup>3</sup>H]5-CT in the presence of 200nM WAY100635 (selective 5-HT<sub>1A</sub> antagonist) was sufficient to remove the 5-HT<sub>1A</sub> component of [<sup>3</sup>H]5-CT binding in cortex, striatum and hippocampal membranes. The remaining WAY insensitive binding was inhibited by a variety of non-selective drugs with Hill slopes of <1, suggesting the presence of multiple remaining receptor subtypes. The WAY100635 insensitive component of [<sup>3</sup>H]5-CT binding was only partially inhibited by the selective 5-HT<sub>1B</sub> agonist, CP 93129. The 5-HT<sub>1B/1D</sub> antagonist, GR125,743, also partially and selectively inhibited this binding but to a slightly greater extent than CP93129. This small difference in inhibition between the CP93129 and GR125,743 sensitive components of WAY100635 insensitive [<sup>3</sup>H]5-CT binding presumably represented binding to 5-HT<sub>1D</sub> receptors and varied between brain areas: striatum > hippocampus >= cortex. This would be consistent with the notion that the majority of 5-HT<sub>1B/1D</sub> receptors in the rat brain are 5-HT<sub>1B</sub> receptors and that a higher density are located in the striatum (Bruinvels *et al.*, 1993a & b, 1994a & b). 200nM GR125,743 was subsequently used to block the 5-HT<sub>1B/1D</sub> component of WAY100635 insensitive [<sup>3</sup>H]5-CT binding.

The remaining WAY100635 and GR125,743 insensitive [<sup>3</sup>H]5-CT binding revealed a pharmacological profile that was highly correlated to binding at recombinant rat 5-HT<sub>7</sub> receptors. All antagonist inhibition curves gave Hill slopes of 1 consistent with binding to one population of binding sites. All agonists, including unlabelled 5-CT, gave Hill slopes of <1. Based on the affinities obtained, there was no evidence of [<sup>3</sup>H]5-CT binding to yet another known receptor sub-type. The shallow Hill slopes of the agonists may represent binding to more than one affinity



state of the receptor. The [ $^3\text{H}$ ]5-CT binding assay was used to recharacterise 5-HT $_7$  receptors in guinea pig brain where only one receptor isoform exists (Tsou *et al.*, 1994) unlike the three in rat (Heidmann *et al.*, 1997). The same conditions as used in the rat were sufficient to block binding to non-5-HT $_7$  receptors and also revealed a pharmacological profile consistent with binding to 5-HT $_7$  receptors (Boyland *et al.*, 1995; To *et al.*, 1995). However, the Hill slopes of 1 for all compounds whether agonists or antagonists in the guinea pig brain, suggest that multiple 5-HT $_7$  receptor isoforms may account for the complexity of agonist binding to rat 5-HT $_7$  receptors.

The conditions used to isolate the 5-HT $_7$  receptor component of [ $^3\text{H}$ ]5-CT binding, were used to assess the anatomical distribution of this receptor subtype. These autoradiographic experiments gave the same the rank order of binding site densities as in the membrane binding studies: hippocampus > cortex >= striatum. The highest density was in the dorsal raphe nucleus (DRN), an area that is too small to use for membrane binding studies. It is therefore possible that the 5-HT $_7$  receptor may also be an autoreceptor like its somatodendritic 5-HT $_{1A}$  receptor counterpart in this brain area. In the absence of commercially available radiolabelled antagonists at present, the development of this assay will aid further investigation of the pharmacology, distribution and modulation of expression of the 5-HT $_7$  receptor.

Conditions for using [ $^3\text{H}$ ]5-CT as a radioligand for labelling the 5-HT $_{1B/1D}$  receptor population could not be established largely because of the interference of the 5-HT $_7$  receptor component of binding. No drugs were suitable to block the 5-HT $_7$  component without inhibiting binding to the 5-HT $_{1B/1D}$  receptors. Therefore binding to rat 5-HT $_{1B}$  and 5-HT $_{1D}$  receptors was achieved using the relatively new selective 5-HT $_{1B/1D}$  antagonist ligand, [ $^3\text{H}$ ]GR125,743. The pharmacology of [ $^3\text{H}$ ]GR125,743 binding was consistent with binding to a mixed population of 5-HT $_{1B}$  and 5-HT $_{1D}$  receptors. The 5-HT $_{1B}$  component of [ $^3\text{H}$ ]GR125,743 was blocked using 300nM CP93129 (selective 5-HT $_{1B}$  agonist) and was used to monitor the levels of 5-HT $_{1B}$  and 5-HT $_{1D}$  receptor binding. The  $B_{\text{max}}$  values of both components of [ $^3\text{H}$ ]GR125,743 binding could be calculated based on the valid assumption that GR125,743 had the same affinity for both receptor subtypes (Doménech *et al.*, 1997). The rank order of the binding site densities of the components of the 5-HT $_{1B/1D}$  receptors was: striatum > hippocampus >= cortex. This was consistent with

the WAY100635 insensitive proportion of [<sup>3</sup>H]5-CT binding defined by CP93129 or GR125,743 and the known distribution of these receptor subtypes (Bruinvels *et al.*, 1993a & b, 1994 a & b).

## **Chronic Antidepressant Treatment Studies**

SERT is the primary initial target for many classes of antidepressants, including selective serotonin reuptake inhibitors. SSRIs rapidly inhibit the uptake of 5-HT. However, maximal antidepressant effects are apparent only after 2-3 weeks of repeated treatment, suggesting that in addition to the inhibition of 5-HT reuptake, other longer-term adaptive changes occur that contribute to therapeutic efficacy. To try and produce future antidepressant drugs that have a faster onset of action, a higher clinical efficacy and a more tolerable side effect profile, it is important to pinpoint the mechanism of action of the current drugs used to treat affective disorders. The initial consequence of most antidepressant medication is an increase in extracellular 5-HT levels (Fuller, 1994). 5-HT levels may be affected in many ways including the blockade of SERT and also by blockade/desensitisation of 5-HT autoreceptors, which appear to have an inhibitory influence over 5-HT neurotransmission in the early stages of treatment.

In this thesis potential neuroadaptive changes of SERT, 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub> and 5-HT<sub>7</sub> receptors were investigated using the binding and immunological methods developed above. Three antidepressants were investigated; citalopram and fluoxetine (both SSRIs), and tianeptine which unlike the SSRIs apparently enhances 5-HT reuptake (Fattaccini *et al.*, 1990). Rats were treated with doses comparable to those that have previously been shown to produce such desensitisation or enhancing uptake responses (Mennini *et al.*, 1987; Invernizzi *et al.*, 1994; Le Poul *et al.*, 1995a).

No changes in the density or affinity of SERT were observed in any brain area with any of the antidepressant drugs examined. This would suggest that adaptive changes of SERT at the protein level do not play a role in the mechanism of action of various antidepressant drugs. This agrees with the majority of the literature available (for a review see Piñeyro & Blier, 1999). Adrenal medulla SERT was similarly unaffected by such treatments suggesting that this peripheral SERT does not play a

role in the mechanism of action of antidepressants which are known to normalise the hyperactivity of the HPA axis seen in depression (see Barden *et al.*, 1995).

5-HT<sub>1A</sub> receptors were also unaffected in the terminal field areas of the raphe cell body projections, suggesting that this post-synaptic population of receptors is unlikely to account for the delayed onset of action of antidepressant treatment. This agrees with previous studies (see chapter 6). In this thesis the effect of these chronic treatments were not investigated in the DRN, although it is apparent that the adaptive processes associated with chronic antidepressant drug in this brain area are not manifested by changes in receptor density or affinity (Le Poul *et al.*, 1995a & b).

5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> autoreceptors in the terminal fields exert a negative influence on 5-HT release which after chronic antidepressant administration are believed to be desensitised (see Piñeyro & Blier, 1999). Studies in this thesis revealed a site specific down regulation of both 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors in the frontal cortex of SSRI treated rats but not those treated with tianeptine. This suggests that the adaptive process may in part be associated with a decrease in the number of inhibitory 5-HT terminal autoreceptors in this brain area. However these 5-HT receptor subtypes are also found on non-serotonergic nerve terminals and are also sensitive to antidepressant treatment (Bolaños-Jimenez *et al.*, 1994). To quantify the possible involvement of only serotonergic 5-HT<sub>1B/1D</sub> receptors, it will be important to combine chronic SSRI treatment studies with the methods outlined in this thesis for the preparation of pure, viable and functional serotonergic nerve terminals.

Like the 5-HT<sub>1B/1D</sub> receptors, there was also a site-specific decrease in the binding site density of the 5-HT<sub>7</sub> receptor in the frontal cortex after chronic SSRI treatment. Indeed a recent study has shown a neuroadaptive decrease of 5-HT<sub>7</sub> binding sites in the rat hypothalamus with SSRIs (Mullins *et al.*, 1999). It is therefore possible that the mechanism of action of SSRIs is mediated by 5-HT<sub>7</sub> receptors in these brain areas or alternatively that such adaptive processes could be linked to the side effect profiles of these drugs after treatment. As 5-HT<sub>7</sub> receptors are found in the DRN it is possible that they may also exert a negative influence on neuronal firing, just like 5-HT<sub>1A</sub> somatodendritic autoreceptors. However, until 5-HT<sub>7</sub> receptor antagonists and agonists become commercially available the reporting of such a role by electrophysiologists will be limited. The use of a 5-HT<sub>7</sub> receptor antagonist, such

as that reported by Forbes *et al.*, (1998), in combination with an SSRI may help to shorten the onset of action of antidepressant medications just as the 5-HT<sub>1A</sub> antagonist, pindolol, does in combination with fluoxetine (Perez *et al.*, 1997). The 5-HT<sub>7</sub> receptor may therefore be an important target for future antidepressant drug design.

It would appear that the antidepressant studies undertaken in this thesis have revealed that the SSRIs (fluoxetine and citalopram) share a common site-specific mechanism of action in decreasing the number of 5-HT<sub>1B/1D</sub> and 5-HT<sub>7</sub> receptors in the frontal cortex. It is therefore possible that this brain area plays a crucial role in mechanism of action of SSRIs. SSRIs inhibit SERT within minutes of dosing but do not appear, as in this thesis, to cause an adaptational change of SERT at the protein level after chronic treatment. SERT is also subject to various other types of regulatory influences (Qian *et al.*, 1997; Ramamoorthy *et al.*, 1998; Ramamoorthy & Blakely, 1999). To avoid a controversial scenario that has become apparent from receptor modulation studies (see Piñeyro & Blier, 1999), it will be important to ensure that a consistent approach is used for future antidepressant studies. For example it has become apparent that the administration of antidepressants in animals via a continuous delivery system, such as a mini-osmotic pump may be more consistent with studies in humans than repeated injections. In such studies a role of SERT in the mechanism of action of these antidepressants has been suggested as shown by clear adaptive changes in SERT density (Piñeyro *et al.*, 1994; Piñeyro *et al.*, 1995d; Benmansour *et al.*, 1999). This approach may also reveal other changes to receptor subtypes and second messenger systems which are important in the mechanism of action of antidepressant drugs, but which are not sensitive to adaptive changes from a spiked treatment regime as used in thesis. However, many novel strategies for the pharmacotherapy of depression have been detected in human and animal studies using the repeated injection method for administration of drugs (Maubach *et al.*, 1999). Future developments of specific PET and SPECT ligands may bypass these problems and allow the direct study of the mechanisms of action of antidepressant drugs in the living human brain.

In studies for this thesis, tianeptine had no effect on any of the components of the 5-HT system investigated. Despite the commonly believed notion that this drug

may actually enhance 5-HT uptake (Mennini *et al.*, 1987; Fattaccini *et al.*, 1990) this drug has been shown to influence other neurotransmitter systems. For example, without producing marked changes in the 5-HT system, short-term administration of tianeptine increases NA content and decreases NA turnover in specific nuclei related with mood such as the preoptic area, DRN and sensory cortex (Frankfurt *et al.*, 1994b). Acute and prolonged tianeptine treatment may also increase extracellular DA concentrations in striatum and nucleus accumbens in a 5-HT-independent manner (Invernizzi *et al.*, 1992b). Therefore despite no observed effect on the areas of the 5-HT system studied, the mechanism of action of tianeptine might be associated with adaptive changes of other neurotransmitter systems. It is also possible that despite the relatively specific nature of SSRIs, indirect changes in other aminergic systems may also additionally account for their mechanism and delayed onset of action. For example  $\alpha_2$  receptors exist on serotonergic neurons exerting an inhibitory action on 5-HT release (Maura *et al.*, 1982) and the stimulation of various 5-HT receptors can influence DA release (see Table 1.5 in the Introduction of this thesis).

### **Repeated MDMA Administration Studies**

MDMA produces long lasting changes in various 5-HT parameters in the brain including: a reduction in 5-HT and 5-HIAA content, a decline in tryptophan hydroxylase activity, and a decrease in the number of SERT binding sites accompanying the degeneration of serotonergic terminals (see Sprague *et al.*, 1998). The mechanisms by which 5-HT release occurs are not fully understood at present, but it is believed to involve a carrier-mediated (Rudnick & Wall, 1992) and  $\text{Ca}^{2+}$  dependent process (Crespi *et al.*, 1997). Repeated MDMA administration caused a significant reduction in the number of [ $^3\text{H}$ ]citalopram binding sites after a 2 week recovery period in both rat terminal field brain areas and the rat adrenal medulla. Following a 13-week recovery period a significant reduction in SERT density in the brain was still apparent, but not in the rat adrenal medulla. Though it is not known whether the initial effect seen in the adrenal gland is a toxic event, as in the brain, it is possible that changes in SERT density in the adrenal medulla may be responsible for the previously reported changes in aldosterone secretion following MDMA administration (Burns *et al.*, 1996). The recovery of adrenal SERT number

after a longer recovery period may reflect differences in the rate of SERT synthesis and trafficking in these two tissues.

The density and affinity of 5-HT<sub>1A</sub> receptors remained unchanged after MDMA administration following both 2-week and 13-week recovery periods. Previous reports have suggested an upregulation of this 5-HT receptor subtype in the frontal cortex following a 1-week recovery period after MDMA administration (Aguirre *et al.*, 1995, 1997 & 1998). It is therefore possible that 5-HT<sub>1A</sub> receptors are involved in the immediate but not longer term consequences of MDMA usage. The density of 5-HT<sub>7</sub> receptors was increased in both the frontal and caudal cortex after repeated MDMA administration following a 2-week recovery period. This may suggest that 5-HT<sub>7</sub> receptors are involved in the later consequences of MDMA usage. It may also suggest a postsynaptic localisation of this 5-HT receptor subtype in this brain area. Such an inference has previously for been made for 5-HT<sub>7</sub> receptors in the hypothalamus following an upregulation of binding sites after destruction of 5-HT terminals using 5,7-DHT (Clemett *et al.*, 1999). The results suggest a possible role for 5-HT<sub>1A</sub>, 5-HT<sub>7</sub> receptors and the adrenal gland in the psychopathobiological changes elicited in humans at various stages after MDMA usage. As these parameters have been described to be involved in depression, the studies further add concern that MDMA usage in humans may lead to neuropsychiatric conditions in later life.

The selective neurotoxicity of MDMA to serotonergic terminals is presently not understood, though many drugs which have a direct action on the serotonergic and dopaminergic systems or induce a hypothermic response are capable of preventing such damage (see Sprague *et al.*, 1998). The immunosuppressant drug FK506 prevented MDMA induced depletion of SERT in the brain but not in the adrenal gland. Though FK506 is known to produce a hypothermic response in reducing focal cerebral ischaemic damage (Bochelen *et al.*, 1999), it is believed that other mechanisms may also account for this reduction in neurotoxicity (Yagita *et al.*, 1996). Thus, glutamate neurotoxicity, which has been suggested to be involved in the mechanism of action of MDMA (Sprague *et al.*, 1998), is also inhibited by FK506 (Kikuchi *et al.*, 1998). Glutamate binding sites in the rat brain are pharmacologically different to those in the rat adrenal medulla (Yoneda & Ogita, 1986), which may

explain why FK506 can prevent MDMA induced depletion of SERT in the brain but not in the adrenal medulla. Another possible mechanism of MDMA neurotoxicity includes the formation of free radicals (Colado *et al.*, 1997b). This was confirmed using the free radical scavenger FR122175 that was more effective than FK506 in preventing MDMA induced depletion in the rat brain. Both drugs were also equally effective in preventing the increase in 5-HT<sub>7</sub> receptor number as a consequence of MDMA administration. FK506 has also been shown to decrease *in vitro* oxidative phosphorylation of mitochondria from rat forebrain at complex III of the mitochondrial electron transport chain, which is particularly involved with the formation of reactive oxygen species (Zianni *et al.*, 1998). Therefore, whereas FR122175 scavenges free radicals, the neuroprotective action of FK506 may in part be due to direct inhibition of free radical formation.

### **Concluding Remarks**

Neurochemical and electrophysiological experiments have indicated a role for 5-HT in a variety of pathological conditions including depression. The 5-HT transporter is the primary target for many antidepressants and drugs of abuse, such as ecstasy (MDMA). However the mechanism of action of these drugs is poorly understood. The studies undertaken in this thesis suggest an involvement of a variety of pre- and post-synaptic components of the serotonergic system in the mechanism and delayed onset of action of antidepressant drugs. The studies also suggest a role for such components in the psychopathological changes elicited by MDMA in man. Such findings may help to improve antidepressant drug design and to increase public awareness that the use of apparently “safe” recreational drugs may have detrimental consequences.

## APPENDICES



## APPENDIX I: Abbreviations.

The abbreviations used in this thesis are in accordance with the guidelines set out in the British Journal of Pharmacology instructions to authors. Those not defined in the above publication are listed below. Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, H<sup>+</sup> and Cl<sup>-</sup> refer to the ionic species of calcium, potassium, sodium, hydrogen and chlorine respectively

### Miscellaneous

5,7-DHT	5,7-dihydrotryptamine
6-OH DA	6-hydroxydopamine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine; serotonin
5-HTP	5-hydroxytryptophan
ABC	avidin biotin complex
ABTS	2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid)
ACH	acetylcholine
ACTH	adrenocorticotrophin hormone
AKP	alkaline phosphatase
ATP	adenosine 5' triphosphate
BDNF	brain derived neurotrophic factor
B <sub>max</sub>	density of radiolabelled binding sites
BNF	British National Formulary
BSA	bovine serum albumin
R <sub>f</sub>	'relative to the front' – the distance a protein moves relative to the dye front
cAMP	adenosine 3' 5' cyclic monophosphate
CCK	cholecystoskinin
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
COOH-tail	carboxyl tail
CRH	corticotrophin releasing hormone
d.H <sub>2</sub> O	distilled water

DA	dopamine
CSF	cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DAG	diacylglycerol
DAT	dopamine transporter
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
ECL	enhanced chemiluminescence
ECT	electroconvulsive shock therapy
EDTA	(ethylenedinitrilo) tetraacetic acid
ELISA	enzyme linked immunosorbent assay
G protein	guanyl nucleotide binding protein
GPCRs	G protein coupled receptors
GABA	gamma aminobutyric acid
GAT	gamma aminobutyric acid transporter
GDP	guanosine 5' diphosphate
GFAP	glial fibrillary acidic protein
GTP	guanosine 5' triphosphate
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HPA	hypothalamus-pituitary-adrenal
HRP	horseradish peroxidase
i.p.	intraperitoneal
IC <sub>50</sub>	concentration of competing ligand which displaces 50% of the specific binding of the radioligand
IgG	immunoglobulin G
IP	immunoprecipitation
IP <sub>3</sub>	inositol phosphates
IUPHAR	International Union of Pharmacology
k <sub>+1</sub>	association rate constant
k <sub>-1</sub>	dissociation rate constant
K <sub>D</sub>	equilibrium dissociation constant
kDa	kilodalton

$K_i$	inhibitor constant
KLH	keyhole limpet haemocyanin
$k_{obs}$	observed constant
L-DOPA	l-dihydroxyphenylalanine
LSD	lysergic acid diethylamide
LTP	long term potentiation
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MDMA	3,4-methylenedioxymethamphetamine; ecstasy
MPP <sup>+</sup>	N-methyl-4-phenylpyridinium
MPTP	N-methyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
N-terminal	amino terminal
NA	noradrenaline
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaSSA	noradrenergic and specific serotonergic antidepressant
NET	noradrenaline transporter
NGS	normal goat serum
nH	Hill slope or coefficient
NP-40	nonylphenoxy polyethoxy ethanol
nsb	non specific binding
O.D.	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethyleneamine
PET	positron emission tomography
PKA	protein kinase A
PKC	protein kinase C
$pK_D$	negative log of the equilibrium dissociation constant
$pK_i$	negative log of the inhibitor constant
PNGASE F	peptide -N-glycosidase F

pNpp	p-nitrophenyl phosphate
PRP	platelet rich plasma
PVDF	polyvinylidene difluoride.
r	correlation coefficient
rpm	revolutions per minute
s.c.	subcutaneous
SCN	suprachiasmatic nucleus
s.e. mean	standard error of the mean
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERT	5-HT transporter
SNRI	serotonin-noradrenaline reuptake inhibitor
SPECT	single photon emission tomography
SSRI	serotonin specific reuptake inhibitor
TCA	tricyclic antidepressant
TEMED	N, N, N', N'-Tetramethylethylenediamine
TMD	transmembrane domain
TPH	tryptophan hydroxylase
tween-20	polyoxyethylenesorbitan
VMAT	vesicular monoamine transporter
VNTR	variable number tandem repeat

## Compounds

The full chemical names of those drugs experimentally used in this thesis are given below. The full chemical names of those compounds which are referred to in the text but which were not used experimentally, may be found in their accompanying reference and/or those references cited within.

5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytryptamine; serotonin
5-MeOT	5-methoxytryptamine
8-OH DPAT	8-hydroxy-2-dipropylaminotetralin

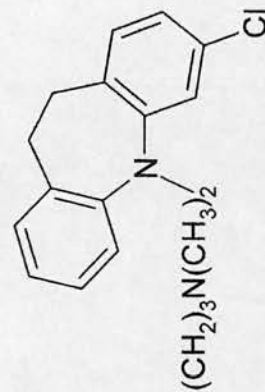
BRL15572	3-[4-(3-chlorophenyl)piperazin-1-yl]-1,1-diphenyl-2-propanol
CGS12066B	7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]-quinoxaline
citalopram	1-[3-(dimethylamino) propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile
clozapine	8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine
CP93129	1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5H-pyrrolo[3,2-b]pyridin-5-one
cyanopindolol	4-[3-[ <sup>t</sup> Butylamido]-2-hydroxypropoxy]-1H-indole-2-carbonitrile
DHE	dihydroergotamine; 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)ergotaman-3',6',18-trione
DMI	desmethylinipramine; 10,11-dihydro-5-(3-methylaminopropyl)-5H-dibenz(b,f)azepine
DP-5-CT	dipropyl-5-carboxamidotryptamine
ergotamine	12'-hydroxy-2'-methyl-5'α-(phenylmethyl)ergotaman-3',6',18-trione
FK506	macrolide tectrolimus; 15,19-Epoxy-3H-pyrido(2,1-c)(1,4)oxaazacyclotricosine-1,7,20,21(4H,23H)-tetrone, 5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-3-(2-(4-hydroxy-3-methoxycyclohexyl)-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(2-propenyl)-, (3S-(3R*(E(1S*,3S*,4S*)),4S*,5R*,8S*,9E,12R*,14R*,15S*,16R*,18S*,19S*,26aR*))-
fluoxetine	(±)-N-methyl-γ-[4-(trifluoromethyl)phenoxy]-benzenepropanamine
FR122175	development compound-no name supplied
GR125,743	N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide

GR127,935	N-[4-methoxy-3-(4-methyl-1-piperzinyloxy)phenyl]2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide
GR46611	3-[3-(2-dimethylamino-ethyl)-1H-indol-6-yl]-N-(4-methoxybenzyl)acrylamide
GR85548	N-methyl-2-[3-(1-methylpiperidin-4-yl)-1H-indol-5-yl] 4'-(methylenedioxyphenoxy) [methyl] piperidine
ketanserin	3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1H,3H]-quinazolinedione
LY694,247	2-[5-[3-(4-methylsulphonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1H-indole-3-yl]ethylamine
mesulergine	N'-[(8a)-1,6-dimethylergolin-8-yl]-N,N-dimethylsulfamide
metergoline	[(8β)-1,6-dimethylergolin-8-yl]-methyl]carbamic acid phenylmethyl ester
methiothepin	1-[10,11-dihydro-8-(methylthio)dibenzo[b,f]thiepin-10-yl]-4-methylpiperazine
methysergide	[8β(S)]-9,10-didehydro-N-[1-(hydromethyl)propyl]-1,6-dimethylergoline-8-carboxamide
mianserin	1,2,3,4,10,14b-hexahydro-2-methyldibenzo[c,f]pyrazino[1,2-a]azepine
nisoxetine	(±)-γ-(2-methoxyphenoxy)-N-methylbenzenepropanamine
nomifensine	1,2,3,4-tetrahydro-2-methyl-4-phenyl-8-isoquinolinamine
PAPP	1-(2-[4-aminophenyl]ethyl)-4-(3-trifluoromethylphenyl)piperazine
pargyline	N-methyl-N-2-propynylbenzylamine
paroxetine	(-)-(3S,4R)-4-(p-Fluorophenyl)-3-((3,4-(methylenedioxy)phenoxy)methyl)piperidine
pentobarbitone	5-ethyl-5-(1-methylbutyl)-2,4,6(1H,3H,5H)-pyrimidinetrione sodium
pimozide	1-[1-[4,4-bis(4-fluorophenyl)butyl]4-piperidinyl]-1,3-dihydro-2H-benzimidazol-2-one
pindolol	1-(1H-indol-4-yloxy)-3-[(1-methylethyl)amino]-2-propanol

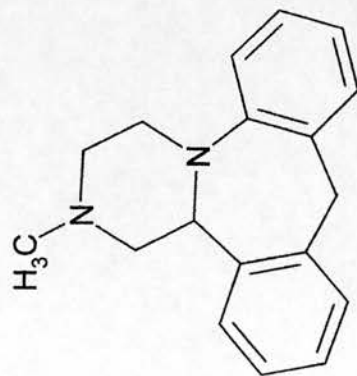
ritanserin	6-[2-[4-[ <i>bis</i> (4-fluorophenyl)methylene]-1-ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one
SB216641	N-[3-(2-dimethylamino)ethoxy-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl)-4-carboxamide
sumatriptan	3-[2-(dimethylamino)ethyl]-N-methyl-indole-5-methanesulfonamide
tianeptine	[3-chloro-6-methyl-5,5-dioxo-6,11-dihydro-(c,f)-dibenzo-(1,2-thiazepine)-11-yl)amino]-7-heptanoic acid
WAY100635	(N-[2-[4-(2-methoxyphenyl)piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexanecarboxamide



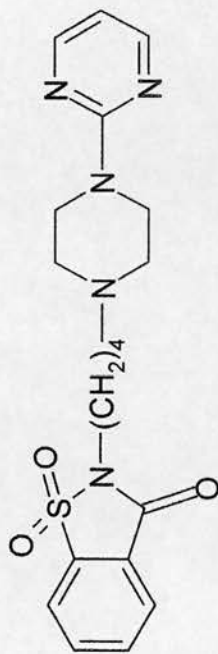
**moclobemide (monoamine oxidase inhibitor)**



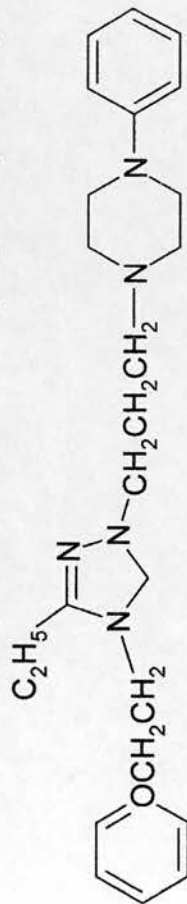
**clomipramine (tricyclic antidepressant)**



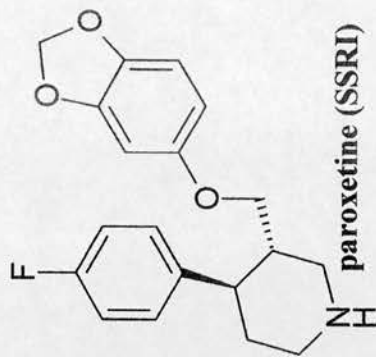
**mianserin (atypical antidepressant)**



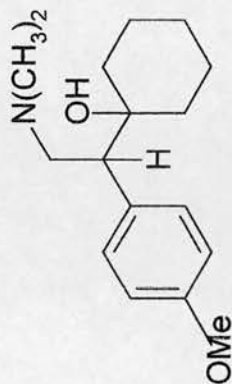
**ipsapirone (5-HT<sub>1A</sub> partial agonist)**



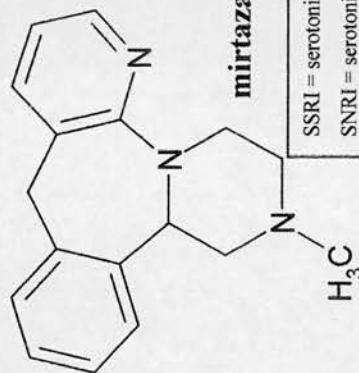
**nefazodone (serotonin uptake & 5-HT<sub>2</sub> antagonist)**



**paroxetine (SSRI)**



**venlafaxine (SNRI)**



**mirtazapine (NaSSA)**

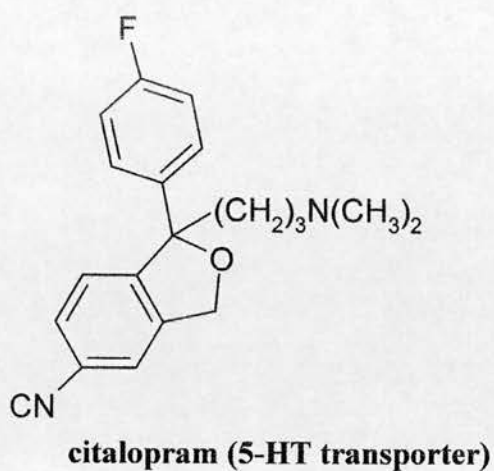
SSRI = serotonin reuptake inhibitor

SNRI = serotonin/noradrenaline reuptake inhibitor

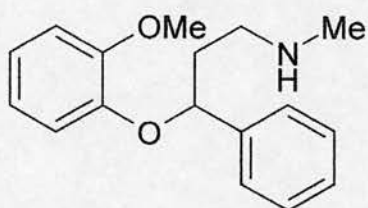
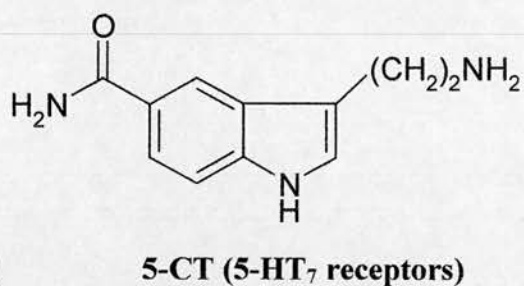
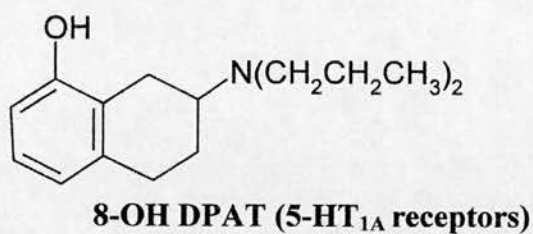
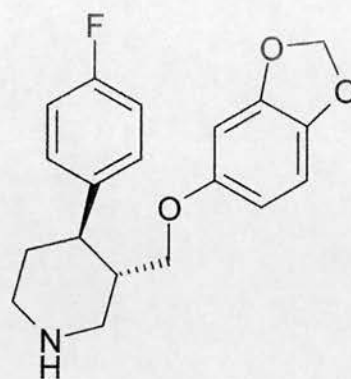
NaSSA = noradrenergic and specific serotonergic antidepressant

## APPENDIX II: Example Structures of Different Classes of Antidepressants



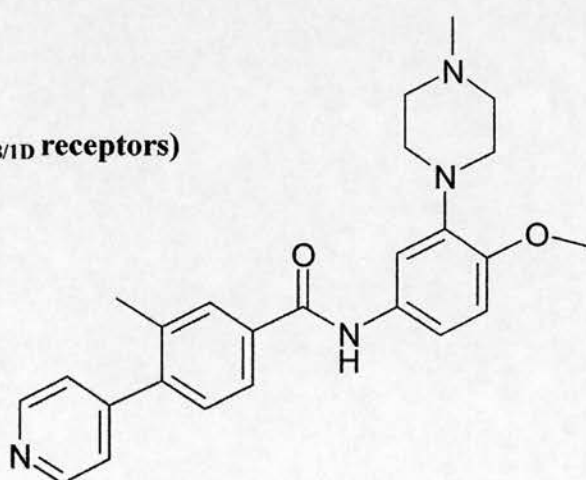


**paroxetine (5-HT transporter)**

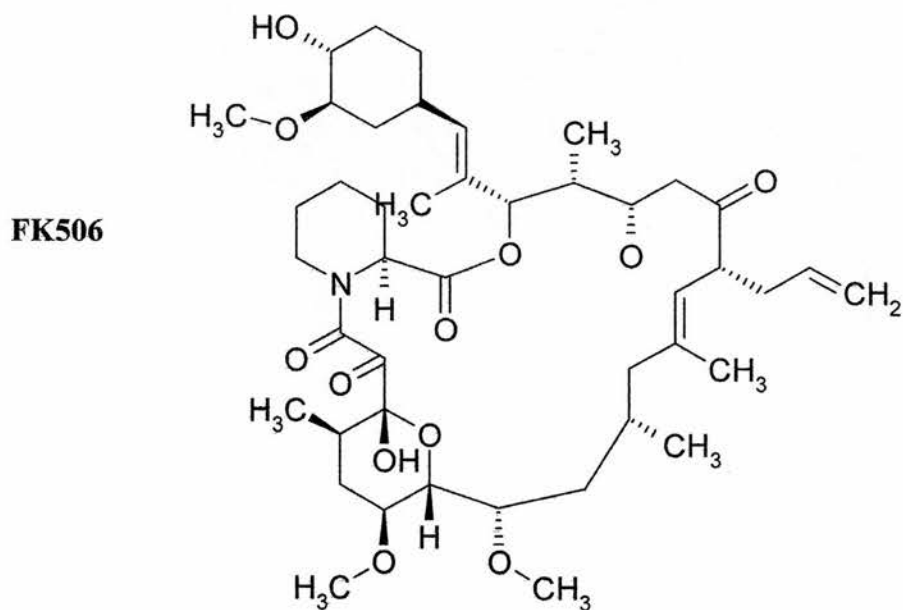
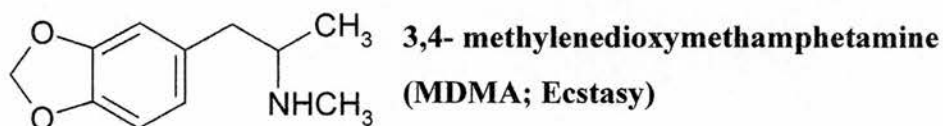
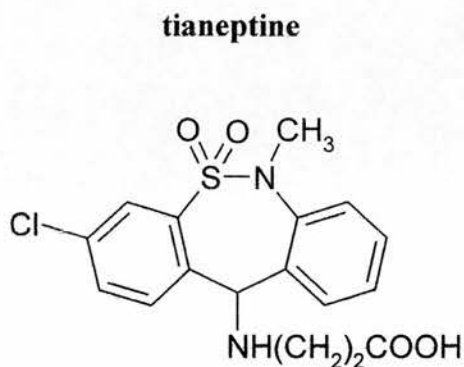
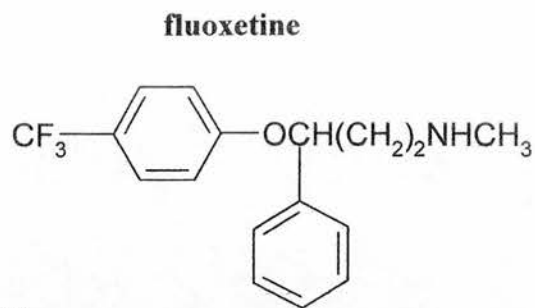
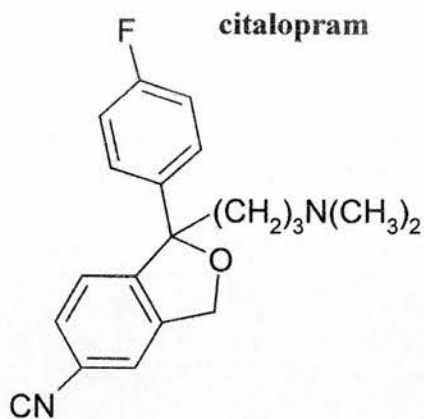


**nioxetine (noradrenaline transporter)**

**GR125,743 (5-HT<sub>1B/1D</sub> receptors)**



**APPENDIX III: Structures of Unlabelled Form of Radioligands  
Used in This Thesis**



**APPENDIX IV: Structures of Drugs Used In Animal Treatments**

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