The effects of female sex steroids on central serotonergic neurotransmission.

by

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In memory of
my father.

I declare that the studies presented in this thesis are the result of my own independent investigation with the exception of the luteinising hormone and prolactin radioimmunoassays which were carried out by John Bennie and Sheena Carroll and the plasma oestradiol assay which was carried out in the Department of Clinical Biochemistry.

This work has not and is not currently being submitted for any other degree or professional qualification.

Elaine Shanks (Candidate)

Abstract

Changes in 5-hydroxytryptamine (5-HT) neurotransmission are implicated in several central nervous system disorders including depression, schizophrenia and Alzheimer's disease. Many studies have shown that there are differences in these diseases between men and women. The aim of this study was to examine the effects of the female sex steroids oestrogen and progesterone on specific aspects of serotonergic neurotransmission.

Acutely ovariectomised adult female Wistar rats were used to study the ability of oestrogen and progesterone to alter central 5-HT neurotransmission, with particular interest in changes in 5-HT_{2A} receptor and serotonin transporter (SERT) binding site density and the firing characteristics of 5-HT neurones in the dorsal raphe nucleus (DRN).

Changes in 5-HT_{2A} binding site density were evaluated using quantitative autoradiography on serial coronal sections using [^{3}H]MDL 100,907 and [^{3}H]ketanserin with non-specific binding being assessed using RP 62203. Binding site density was measured in the dorsal and median raphe nuclei, which contain serotonergic cell bodies, and regions of the forebrain which receive projections from these raphe nuclei e.g. the cortex and hypothalamus. The experiments examined the effects of oestrogen and progesterone on binding site density while also comparing the binding patterns of the two 5-HT_{2A} receptor ligands. Preliminary experiments were carried out using coronal sections containing cingulate cortex to obtain the optimal conditions for [^{3}H]MDL 100,907 binding experiments. Similar binding patterns were observed throughout the brain using [^{3}H]MDL 100,907 or [^{3}H]ketanserin although higher non-specific binding was observed with ketanserin. Specific binding levels differed between the two ligands with higher levels being observed in the cingulate cortex, nucleus accumbens and olfactory tubercle and lower levels in the amygdala and dorsal raphe using [^{3}H]MDL 100,907 compared to [^{3}H]ketanserin. Acute oestrogen treatment produced no significant differences in binding site density in any of the regions examined when [^{3}H]MDL 100,907 was used; however, an increase in binding was observed with [^{3}H]ketanserin in the cingulate and frontal cortex. Progesterone treatment alone or in combination with oestrogen produced no significant differences in binding site density in any of the regions examined with either [^{3}H]MDL 100,907 or [^{3}H]ketanserin. These results

suggest that progesterone has no effect alone but can attenuate the effect of oestrogen on ketanserin binding in specific brain regions.

Changes in SERT binding site density were examined using quantitative autoradiography on serial coronal sections using [^{3}H]paroxetine with non-specific binding being assessed using citalopram. Binding site density was measured in the dorsal and median raphe, in the locus coeruleus and in forebrain regions e.g. cortex. No significant differences in binding site density were found in any of the areas examined after oestrogen, progesterone or combination treatment suggesting that acute steroid treatment does not affect SERT binding site density in the regions examined.

In vivo extracellular recordings were carried out in the DRN of urethane anaesthetised rats which had been ovariectomised the previous day and acutely treated with oestrogen or vehicle. Spontaneously active 5-HT neurones in the DRN were classified into two types depending on their firing characteristics, these were regularly firing cells and doublet-firing cells. Oestrogen treatment produced a significant increase in the latency to antidromic stimulation of the medial forebrain bundle (MFB) and resulted in significantly fewer doublets being fired per minute by doublet-firing cells. Fifty nine percent of cells recorded from were subjected to subthreshold stimulation of the MFB. In 57% of these cells subthreshold stimulation produced inhibition of firing with the remainder showing either no effect or excitation. Significantly fewer cells in the oestrogen treated group showed a short duration of inhibition (<50 ms). The inhibition of firing was characterised by first administering the 5-HT$_{1A}$ antagonist WAY 100635 then repeating the subthreshold stimulation. In both oestrogen and control groups pre-treatment with WAY 100635 partially blocked the inhibition of firing suggesting the inhibition observed is mediated, at least in part, by 5-HT released from recurrent collaterals. These results show that acute treatment with oestrogen reduces the activity of doublet-firing cells and also that inhibition of firing produced by subthreshold stimulation involves the activation of 5-HT$_{1A}$ receptors.

In summary, this study shows that progesterone treatment has no effect on 5-HT$_{2A}$ receptor or SERT binding site density but attenuates the positive effect of oestrogen on ketanserin binding in discrete regions of female rat brain. This study also demonstrates that acute oestrogen treatment decreases the frequency of doublet-firing by 5-HT neurones in the DRN suggesting a possible mechanism for the modulation

of 5-HT release. Finally, this study shows that subthreshold stimulation of the MFB predominantly results in inhibition of firing and that this effect is partially due to activation of 5-HT_{1A} receptors.

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And finally, I give my thanks and love to my mum, my brother, Sarah, Hannah and Daniel. Thank you for being there, for listening and making me smile.

Contents

List of figures

List of tables

Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5 hydroxytryptophan
AC	adenylate cyclase
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole
BBB	blood brain barrier
BLA	basolateral amygdala
cAMP	cyclic 3',5'-adenosine monophosphate
CHO	Chinese hamster ovary
CLi	caudal linear nucleus of the raphe
CNS	central nervous system
CRF	corticotrophin-releasing factor
DAG	diacylglycerol
DBB	diagonal band of Broca
DEP	diethyl pyrocarbonate
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DR	dorsal raphe
DRD	dorsal region of dorsal raphe nucleus
DRN	dorsal raphe nucleus
DRV	ventral region of dorsal raphe nucleus
DRVL	ventrolateral region of dorsal raphe nucleus
DRY	dorsal raphe at the level of the locus coeruleus where it is Y-shaped in appearance
ECS	electroconvulsive shock treatment
EDTA	di-sodium ethylene diamine tetra acetic acid
ER	oestrogen receptor
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
i.p.	intraperitoneally
i.v.	intravenous
IgG	immunoglobulin G
IR	immunoreactivity
IP_3	inositol-1,4,5-trisphosphate
IUPHAR	International Union of Pharmacology

LC	locus coeruleus
LH	luteinising hormone
LHRH	luteinising hormone-releasing hormone
LSD	lysergic acid diethylamide
MAOI	monoamine oxidase inhibitor
MDMA	3,4-methylenedioxymethamphetamine
MFB	medial forebrain bundle
mlf	medial longitudinal fasciculus
MnR	median raphe
mPFC	medial prefrontal cortex
MPOA	medial preoptic area
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
OB	oestradiol benzoate
OVX	ovariectomised
PAG	periaqueductal gray
PBS	phosphate buffered saline
PCPA	parachlorophenylalanine
PET	positron emission tomography
PEV	periventricular region of the hypothalamus
PI	phosphoinositide
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PLC	phospholipase C
PMDD	premenstrual dysphoric disorder
PMN	polymorphonuclear
PR	progesterone receptor
PRL	prolactin
PSB	pontamine sky blue
PSTH	post stimulus time histogram
s.c.	subcutaneous
s.e.m.	standard error of the mean
SCN	suprachiasmatic nuclei
SERT	5-HT transporter
SNR	substantia nigra
SON	supraoptic nucleus

SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TH	tryptophan hydroxylase
UHP	ultra high pure
VMN	ventromedial nucleus of the hypothalamus
VNTR	variable number tandem repeat
VTA	ventral tegmental area
xscp	decussation of the superior cerebellar peduncle

Chapter 1

Introduction

5-hydroxytryptamine

5-hydroxytryptamine (5-HT, serotonin) is an endogenous indoleamine. It was first isolated from serum in 1948 by Rapport *et al* (1948) although it was another year until Rapport identified the active vasoconstrictive component as 5-hydroxytryptamine (Rapport 1949). 5-HT was first discovered in the brain by Twarog and Page (1953), by which time it was known to be present in three key locations - gut, brain and platelets.

5-HT is one of a number of neurotransmitters which is synthesised in the brain. The cell bodies of 5-HT neurones are found in the various raphe nuclei of the midbrain and brainstem from where they project to many areas of the brain (see diagram of ascending and descending projections from the raphe nuclei below adapted from Steinbusch and Nieuwenhuys 1983).

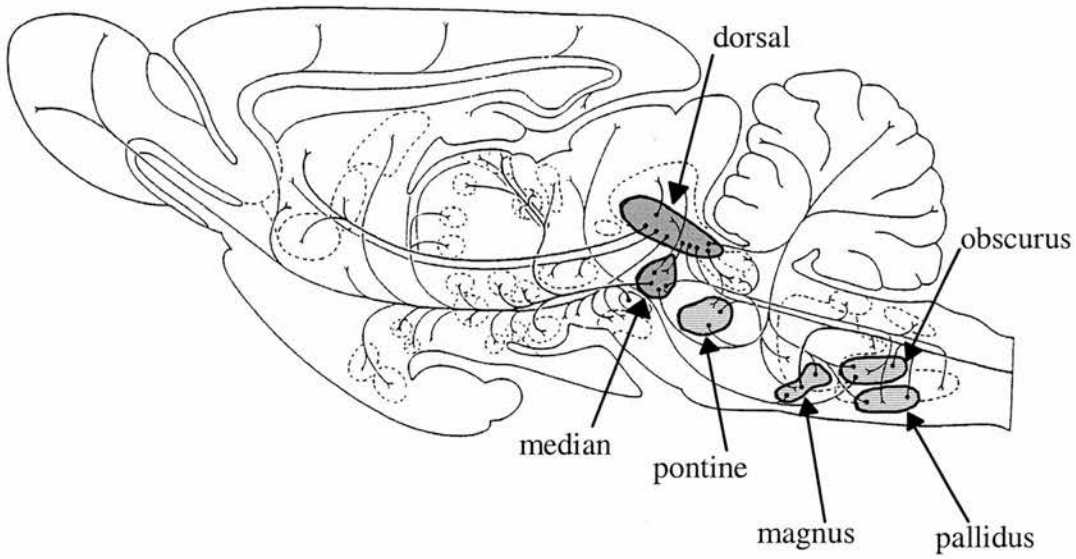


Figure 1.1: Ascending and descending projections from the raphe nuclei as illustrated on a sagittal diagram through the rat brain (from Steinbusch and Nieuwenhuys 1983). The dorsal and median raphe nuclei are shown in dark grey with the other raphe nuclei are shown in light grey and are, rostral to caudal, the pontine raphe nucleus, raphe magnus, raphe obscurus and raphe pallidus.

The starting compound for the synthesis of 5-HT is the essential amino acid tryptophan. This amino acid crosses the blood brain barrier via a carrier mediated transport system and is then taken up into the nerve terminals (Steinbusch 1984). The uptake of tryptophan into the nerve terminals is normally the rate limiting step in 5-HT synthesis (Hamon *et al* 1981). Once in the nerve terminals tryptophan is then converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase using oxygen and reduced pterin as substrates (Steinbusch 1984). Under normal conditions this reaction is not saturated, however, if excess tryptophan is present the synthesis of 5-HTP becomes the rate limiting step in 5-HT synthesis (Hamon *et al* 1981). The final step involves 5-HTP being converted to 5-HT by the enzyme aromatic-L-amino acid decarboxylase and this reaction is pyridoxal phosphate dependent (Steinbusch 1984). The synthesis of 5-HT can be blocked by drugs e.g. parachlorophenylalanine (PCPA) which acts by inhibiting tryptophan hydroxylase (Koe and Weissman 1966).

5-HT is released from the nerve terminal by Ca^{2+} dependent exocytosis. Upon release the neurotransmitter then diffuses across the synaptic cleft and interacts with receptors on the postsynaptic membrane. Neurotransmission relies on the rapid release and rapid inactivation of the transmitter. In the case of 5-HT it is inactivated by removal from the synaptic cleft by a specific transporter in the membrane of the presynaptic terminal (see figure 1.2). Recent reports suggest that the transporter may actually be situated just outside the synapse itself (Bunin and Wightman 1998, Zhou *et al* 1998).

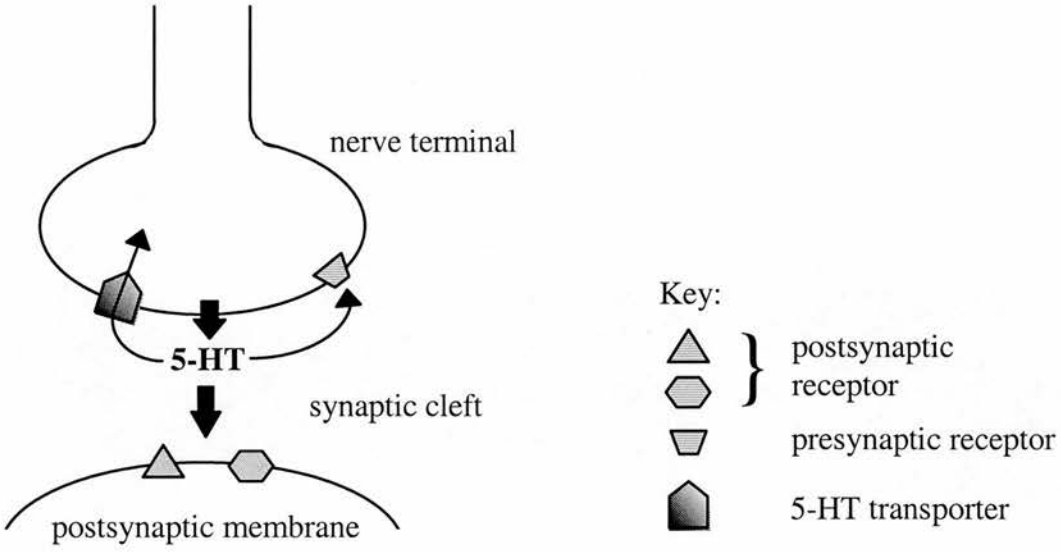


Figure 1.2: Schematic diagram illustrating the release of 5-HT from a nerve terminal and into the synaptic cleft. On release 5-HT can either act on 5-HT receptors which are located on the post- or presynaptic membrane. 5-HT is removed from the synaptic cleft by uptake by the 5-HT transporter.

The 5-HT transporter (SERT) is a high affinity uptake mechanism for 5-HT and is capable of removing 5-HT from extracellular concentrations of 10^{-7}M (Henn and Hamberger 1971). When 5-HT is taken back up into the nerve terminal it is either repackaged into vesicles for release or is metabolised to 5-hydroxyindoleacetic acid (5-HIAA). 5-HIAA is the major metabolite of 5-HT and is excreted in urine.

The presence of an uptake mechanism for 5-HT has been detected both peripherally in the membranes of platelets (Humphrey and Toh 1954, Hughes and Brodie 1959) and within the central nervous system (CNS) on neurones (Blackburn *et al* 1967, Palaic *et al* 1967) and on glial cells (Henn and Hamberger 1971). 5-HT uptake by the transporter involves Na^+-dependent co-transport where a sodium ion and a chloride ion are transported across the membrane at the same time as 5-HT (Keyes and Rudnick 1982, Kanner and Bendahan 1985, Reith *et al* 1989).

The 5-HT transporter was first cloned in 1991 (Blakely *et al* 1991, Hoffman *et al* 1991) and is reported to contain 12, or possibly 13, transmembrane spanning domains (Blakely *et al* 1991, Hoffman *et al* 1991). The transporter has been reported

to be encoded by a single 3.7 kb messenger ribonucleic acid (mRNA) in the rat (Blakely *et al* 1991) and is said to comprise of 629 amino acids (Hoffman 1994) or 653 amino acids (Hoffman *et al* 1991). In humans the 5-HT transporter has been cloned and has been localised to chromosome 17, specifically 17q11.1 - 17q12 (Ramamoorthy *et al* 1993). It has been reported that splice variants of the human transporter occur (Bradley and Blakely 1997) and that the transporter may also occur in two binding states (Staley *et al* 1998). In the rat brain, as in humans, the transporter is mainly localised in the dorsal and median raphe nuclei (Blakely *et al* 1991, Fujita *et al* 1993). The transporter is also found in the other raphe nuclei and, to a lesser extent, in the terminal regions of 5-HT pathways e.g. frontal cortex, hippocampus and neostriatum (Fujita *et al* 1993).

The transporter uptake mechanism can be blocked by drugs. These drugs can be used therapeutically as antidepressants and include tricyclic antidepressants (TCA's) e.g. imipramine (Blackburn 1967), and selective serotonin reuptake inhibitors (SSRI's) e.g. fluoxetine (Prozac) (Wong *et al* 1975). In addition to their therapeutic uses these compounds have been used experimentally to expand the understanding of serotonergic neurotransmission. For example, tritiated imipramine has been used to detect the 5-HT transporter and indicate the extent of serotonin projections in rat brain (Hensler *et al* 1994).

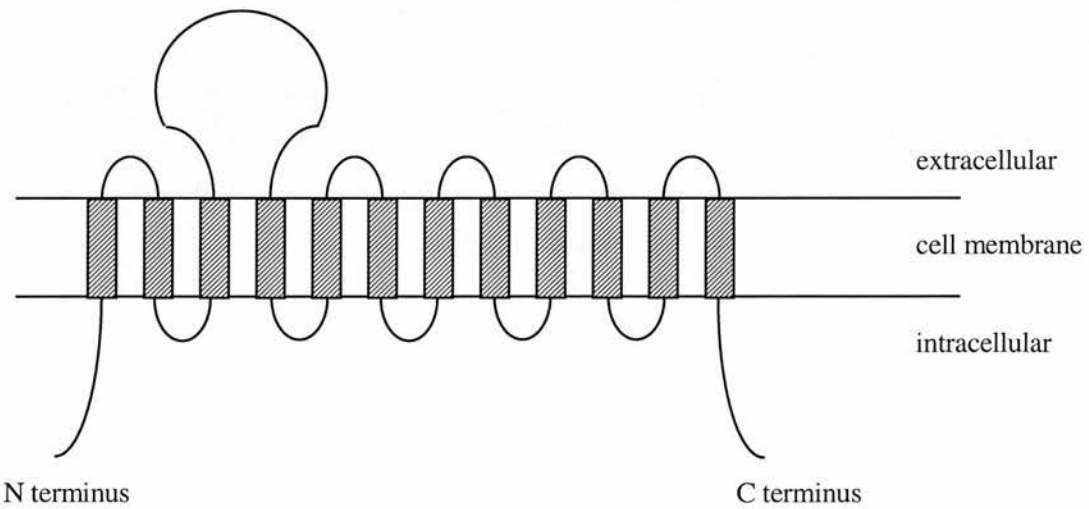


Figure 1.3: Schematic diagram of the structure of the 5-HT transporter. It has been reported that the N- and C-terminals of the transporter are intracellular and also that glycosolation sites have been detected on the large extracellular loop between transmembrane regions 3 and 4 (Hoffman *et al* 1991). It has been suggested that the

third transmembrane region contains residues which are involved in binding 5-HT (Chen *et al* 1997).

5-HT receptor nomenclature

Gaddum and Picarelli in 1957 were the first to propose that two types of tryptamine receptor existed in the intestine (Gaddum and Picarelli 1957). They named these two receptor types the M (morphine) receptor and the D (dibenzyline) receptor after the compounds which blocked each receptor. The D receptor was said to be present on the smooth muscle of the intestine while the M receptor was said to be present on nerve ganglia/fibres. In 1979 Peroutka and Snyder proposed that two different subtypes of the 5-HT receptor were present in the CNS, they designated them 5-HT_1 and 5-HT_2 (Peroutka and Snyder 1979). This definition was based on the observation that [^{3}H]5-HT and [^{3}H]spiroperidol labelled two distinct populations of 5-HT receptors in rat cerebral cortex while [^{3}H]lysergic acid diethylamide (LSD) was found to bind to both receptor populations but with different affinities.

By the mid-1980's more 5-HT receptors had been discovered and Bradley *et al* (1986) stated the need for a uniform terminology for classification of 5-HT receptors as the literature at that time had become confusing. In 1994 the International Union of Pharmacology (IUPHAR) (Hoyer *et al* 1994) reclassified 5-HT receptors. Three criteria were used to determine receptor subtypes. These were the operational (drug-related), transductional (receptor-coupling) and structural (primary amino acid sequence) characteristics of the receptor. Under this classification 5-HT receptors were divided into seven main subtypes. There were also some receptors which did not fulfil the criteria for entry into any of the seven subtypes, they have been termed orphan receptors as a result. Some of these orphan receptors have now been reclassified and fall within one of the seven main 5-HT receptor subtypes as a result of a function for the receptors being discovered (Eglen *et al* 1997).

Receptor subtype	Transduction mechanism	Location in the CNS
5-HT_{1A}	- AC	presynaptically on 5-HT cell bodies, postsynaptically in hippocampus, septum, cortex and spinal cord.
5-$HT_{1B/D}$	- AC	presynaptically on 5-HT nerve terminals.
5-HT_{1E}	- AC	caudate putamen, amygdala and frontal cortex.
5-HT_{1F}	- AC	hippocampus and cortex
5-HT_{2A}	+ PI	hypothalamus, cortex, nucleus accumbens, dorsal raphe.
5-HT_{2B}	+ PI	cerebellum, lateral septum, hypothalamus and amygdala.
5-HT_{2C}	+ PI	choroid plexus, cerebral cortex, hippocampus, substantia nigra and striatum
5-HT_3	ion channel	nucleus tractus solitarius, cortex, hippocampus and habenula.
5-HT_4	+ AC	hippocampus.
5-HT_{5A}	- AC	cortex, hippocampus and cerebellum.
5-HT_{5B}	- AC	habenula and hippocampus (CA1)
5-HT_6	+ AC	caudate, nucleus accumbens, piriform cortex, thalamus and hippocampus.
5-HT_7	+ AC	hippocampus and amygdala

Table 1.1: Summary table of 5-HT receptor subtypes detailing the transduction mechanism involved and listing examples of areas where the receptors have been detected in the CNS.

Abbreviations:
- AC :adenylate cyclase
- PI :phosphoinositide
- + AC :positively coupled to adenylate cyclase.
- - AC :negatively coupled to adenylate cyclase.
- + PI : positively coupled to PI hydrolysis.

With the exception of the 5-HT_3 receptor subtype, all the 5-HT receptors to date have seven transmembrane spanning domains and are coupled to G-proteins (Hoyer *et al* 1994, Martin and Humphrey 1994) (see figure 1.4).

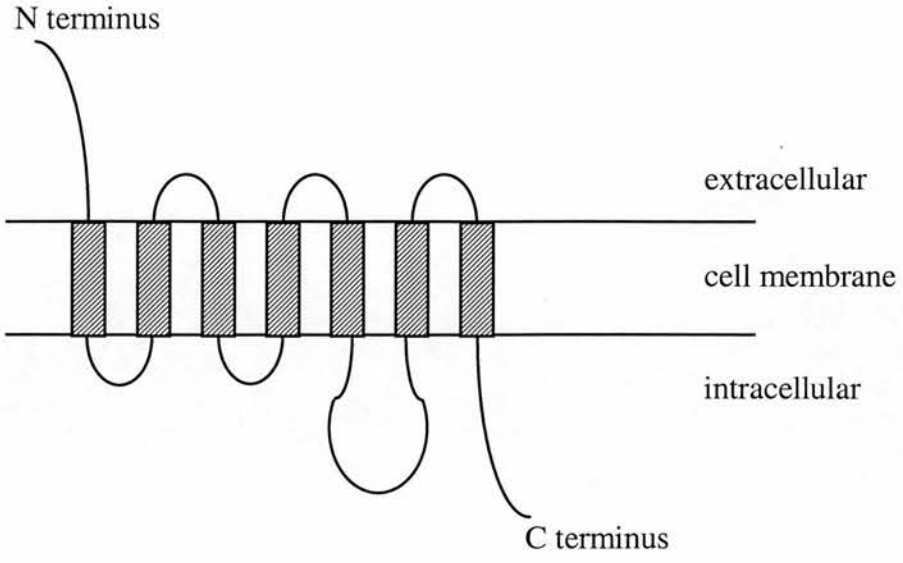


Figure 1.4: Illustration of a seven transmembrane receptor.

The 5-HT_1 class of receptors has been further subdivided into 5 subtypes, these are 5-HT_{1A}, $_{1B}$, $_{1D}$, $_{1E}$ and $_{1F}$. All five of the 5-HT_1 receptor subtypes are negatively coupled to the enzyme adenylate cyclase via a G-protein and the genes encoding these receptors have been reported to have no introns (Martin and Humphrey 1994). The 5-HT_{1A} receptors are found both pre- and post-synaptically (Verge *et al* 1985). Presynaptically they are found on the serotonergic cell bodies where they act as autoreceptors and their activation results in a decrease in the activity of the neurone (Verge *et al* 1985, Sprouse and Aghajanian 1987). The properties of the 5-HT_{1A} receptor are discussed in more detail on page 14. The 5-HT_{1B} and $_{1D}$ subtypes appear to display a similar pharmacology although they differ in amino acid sequence (Oksenberg *et al* 1992). The nomenclature for both these receptor subtypes has recently been revised (Hartig *et al* 1996) such that human 5-$HT_{1D\alpha}$ are reclassified as h5-HT_{1D} and human 5-$HT_{1D\beta}$ as h5-HT_{1B}, rat 5-HT_{1B} and $_{1D}$ receptors now appear with a "r" prefix i.e. r5-HT_{1D}. 5-$HT_{1B/1D}$ receptors are located on the presynaptic nerve terminal where they act as terminal autoreceptors causing a decrease in 5-HT release (Middlemiss 1985).

5-HT_2 receptors are classified into 3 subtypes based on the 1994 IUPHAR classification (Humphrey *et al* 1993, Hoyer *et al* 1994), these are 5-HT_{2A}, $_{2B}$, and $_{2C}$. 5-HT_2 receptors are positively coupled to the enzyme phospholipase C (PLC) and increase the conversion of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-

1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Conn and Sanders-Bush 1986). Both IP_3 and DAG act to produce an increase in the level of intracellular calcium, $[Ca^{2+}]_i$. All three 5-HT_2 receptor subtypes are found in the brain. The 5-HT_{2A} receptor was previously referred to as the 5-HT_2 receptor or the classical D receptor (as classified by Gaddum and Picarelli in 1957) and is discussed in more detail on page 15. Immunoreactivity for the 5-HT_{2B} receptor has been detected on Purkinje cells in the cerebellum, in the medial amygdala, lateral septum and in the dorsal hypothalamic nucleus (Duxon *et al* 1997). The mRNA for 5-HT_{2C} receptors is found in the choroid plexus, hippocampus, striatum and prefrontal cortex (Meibach *et al* 1980, Pazos *et al* 1984). Also, different isoforms and conformations of the 5-HT_{2C} receptor have been reported to occur (Burns *et al* 1997 and Berg *et al* 1998 respectively).

The 5-HT_3 receptor is a receptor activated ion channel which allows the passage of cations e.g. Na^+ and each subunit contains only 4 transmembrane regions. At present no subtypes of this receptor have been detected although a report has suggested their existence (Bonhaus *et al* 1993). Evidence for species differences in 5-HT_3 receptors comes from studies examining drug effects on the vagus nerve. Phenylbiguanide was shown to produce depolarisation in rat vagus nerve but not in that of guinea-pig (Ireland and Tyres 1987, Butler *et al* 1990). These studies also examined the properties of several antagonists, including MDL 72222, showing that antagonists were more potent in the guinea-pig vagus nerve compared to the rat (Ireland and Tyres 1987, Butler *et al* 1990). Splice variants of the 5-HT_3 receptor have also been reported both in the rat (Micquel *et al* 1995) and in the mouse (Hope *et al* 1993). Whether these splice variants occur in particular regions of the brain has yet to be determined but this, in conjunction with the possibility of subtypes of the receptor, may allow differential regional effects of 5-HT via the 5-HT_3 receptor making the response of this receptor more versatile.

5-HT_4 receptors are positively coupled to adenylate cyclase (Fagni *et al* 1992) although they have also been shown to decrease potassium ion conductance in the hippocampus (Andrade and Chaput 1991) via activation of the enzyme protein kinase A (PKA) (Fagni *et al* 1992, Torres *et al* 1995). In common with other 5-HT receptor subtypes splice variants of 5-HT_4 receptors have been shown to exist (Gerald *et al* 1995). In rats the receptors have been found in the striatum, substantia nigra and olfactory tubercle (Grossman *et al* 1993) while 5-HT_4 receptor mRNA has been

detected in the olfactory tubercle, dentate gyrus and hippocampus (Ullmer *et al* 1996).

5-HT_5 receptors occur as two subtypes, 5-HT_{5A} and 5-HT_{5B}, with activation of these receptors producing inhibition of adenylate cyclase (Francken *et al* 1998). The gene for the 5-HT_{5A} receptor consists of two exons separated by a large intron (Rees *et al* 1994) and in humans the mRNA is expressed in the cerebral cortex, hippocampus and cerebellum (Pasqualetti *et al* 1998). In the rat 5-HT_{5B} receptor mRNA is expressed in the habenula and CA1 region of the hippocampus (Matthes *et al* 1993).

The gene for the 5-HT_6 receptor contains two introns and functional receptors have been shown to be positively coupled to adenylate cyclase (Kohen *et al* 1996). The mRNA for the 5-HT_6 receptor has been reported to be expressed in the nucleus accumbens, olfactory tubercle, striatum and hippocampus (Yoshioka *et al* 1998). The receptors themselves have been detected in the caudate, nucleus accumbens, olfactory tubercle, piriform cortex, thalamus, hippocampus (CA1) and cerebellum although no receptors have been detected in the raphe nuclei (Kohen *et al* 1996, Gerard *et al* 1997).

The 5-HT_7 receptor has been found to exist in multiple isoforms in both rat and human (Heidmann *et al* 1997). Like the 5-HT_4 and 5-HT_6 subtypes, 5-HT_7 receptors are positively coupled to adenylate cyclase (Tsou *et al* 1994). The mRNA for this receptor is located in various regions of the limbic system including the pyramidal cells in the hippocampus and in the amygdala (Ruat *et al* 1993). A summary table of the information on 5-HT receptor subtypes is given in table 1.1.

The development of molecular biological techniques in recent years has facilitated the cloning of both the serotonin transporter and serotonin receptors. The functional characterisation of 5-HT receptors and SERT has been achieved with the help of several compounds including 8-OH DPAT (5-HT_{1A} agonist), WAY 100,635 (5-HT_{1A} antagonist), ketanserin and MDL 100,907 (5-HT_{2A} antagonists), MDL 72222 (5-HT_3 antagonist), GR113808 and SB 207266 (5-HT_4 antagonists) (Gale *et al* 1994, Wardle *et al* 1996 respectively) and the SSRI's e.g. fluoxetine and paroxetine. The recent discovery of selective antagonists for both the 5-HT_6 (Sleight *et al* 1998, Bromidge *et al* 1999) and the 5-HT_7 (Forbes *et al* 1998, Kikuchi *et al* 1999) receptors will hopefully lead to the discovery of the function of these receptors.

The dorsal and median raphe nuclei

These two raphe nuclei are responsible for the majority of the innervation of the forebrain (refer to the projection diagram, figure 1.1, from Steinbusch and Nieuwenhuys 1983). Other raphe nuclei exist (see figure 1.1) however these nuclei send few projections to the forebrain and predominantly innervate the cerebellum, brainstem and the spinal cord (Steinbusch and Nieuwenhuys 1983). Both the dorsal and the median raphe nuclei innervate specific brain regions however, these areas can overlap hence some brain regions receive projections from one nucleus and some regions receive projections from both. The dorsal raphe (DR) has been reported to solely innervate the nucleus accumbens, striatum and amygdala whilst the median raphe (MnR) is reported to solely innervate the dorsal hippocampus, olfactory bulb and dentate gyrus (Azmitia and Segal 1978, Kosofsky and Molliver 1987). Other regions such as the thalamus, suprachiasmatic nuclei (SCN) and hypothalamus receive innervation from both nuclei while the cortex is predominantly innervated by the dorsal raphe (Azmitia and Segal 1978, Kosofsky and Molliver 1987). The innervation of a specific structure may be derived not only from a particular raphe nucleus but from a specific region within that nucleus. For example, the nucleus accumbens is innervated by 5-HT neurones arising throughout the DR whereas the prefrontal cortex is innervated only by 5-HT neurones which have their cell bodies along the midline of the DR (Van Bockstaele *et al* 1993).

Projection areas are not the only difference between the dorsal and median raphe nuclei and it has been reported that the axons originating from these nuclei differ. Axons from 5-HT neurones in the MnR have large spherical varicosities while axons from DR neurones are reported to be fine and have small polymorphic varicosities (Kosofsky and Molliver 1987). The observation that the morphology of the axons differs in the two nuclei has been used to explain why DR projections are more susceptible to the effects of some compounds. For example, in rats where 5-HT synthesis has been blocked by PCPA the 5-HT_{1A} agonist 8-OH DPAT was used to study 5-HT release (Blier *et al* 1990). This study showed that a dose of 8-OH DPAT which decreased 5-HT release in the frontal cortex (DR projection area) had no effect on 5-HT release in the dentate gyrus (MnR projection area) (Blier *et al* 1990) suggesting the DR was more responsive to 8-OH DPAT under these conditions. Another example of this phenomenon is the effect of the γ-aminobutyric acid $(GABA)_A$ antagonist bicuculline, infusion of this compound into the DR increases

5-HT levels in both the DR and nucleus accumbens while infusion into the MnR produces no effect (Tao *et al* 1996). Although several compounds have different effects on the DR and MnR in many instances drugs produce the same effect in either nuclei. Several drugs have been shown to inhibit DR and MnR firing by their effects on 5-HT levels and/or actions on 5-HT_{1A} receptors. Acute administration of 3,4-methylenedioxymethamphetamine (MDMA) has been shown to inhibit firing in both the DR and MnR with similar potency and to increase 5-HT release in the terminal regions of these nuclei (frontal cortex and hippocampus respectively) by equal amounts (Gartside *et al* 1997). Other examples are paroxetine and 8-OH DPAT which inhibit the firing of 5-HT neurones in both the DR and MnR giving similar ED_{50} values in either nuclei (Hajos *et al* 1995a).

Dorsal and median raphe neurones have a well characterised firing pattern. These neurones fire spontaneously with a slow, regular discharge rate of 0.2-3 spikes s^{-1} (Wang and Aghajanian 1977). This firing pattern is observed both *in vivo* and *in vitro* although *in vitro* 5-HT neurones must be driven, usually by phenylephrine, as the tonic noradrenergic input to the raphe from the locus coeruleus has been removed (Baraban *et al* 1978, Baraban and Aghajanian 1981). More recently a second firing pattern has been described. It has been observed in both the DR and MnR and relates to "burst-firing" neurones. These neurones were reported to fire with the same action potential pattern described above but occasionally a doublet or triplet was observed where only a single spike was expected (Hajos *et al* 1995b). These burst-firing neurones are reported to be serotonergic as their firing is inhibited by paroxetine and 8-OH DPAT in a manner similar to that of traditional 5-HT neurones (Hajos *et al* 1995b and 1996). These cells are found throughout the DR, they account for approximately 30% of 5-HT neurones recorded from and they are believed to be a discrete subpopulation of 5-HT neurones since cells do not alternate between being regularly firing and "burst-firing" (Hajos *et al* 1995b and 1996).

The firing frequency of 5-HT neurones in the DR and MnR is regulated by somatodendritic 5-HT_{1A} receptors and activation of these receptors decreases neuronal firing (Sprouse and Aghajanian 1987). Several *in vivo* studies have indicated the presence of other 5-HT receptors on the cell bodies of 5-HT neurones. These studies would suggest that although 5-HT_{1A} receptors are the main autoregulatory receptor other receptors play a role in the regulation of 5-HT neuronal firing. One example is 5-HT_{2A} receptors which have been detected on 5-HT neurones in the DR (Davies *et al* 1988). This study showed that iontophoretic

application of 5-HT in the DR increased firing rate and this increase was blocked by ketanserin. Other receptors found presynaptically which affect 5-HT release include 5-$HT_{1B/D}$ receptors and the ion channel 5-HT_3 receptor (Davidson and Stamford 1995, Bagdy *et al* 1998 respectively). Stimulation of 5-HT_3 receptors in the dorsal raphe has been shown to facilitate somatodendritic 5-HT release (Bagdy *et al* 1998).

The above studies show that the firing rate of 5-HT neurones can be regulated by the action of 5-HT on autoreceptors within the DR. Two reports have also suggested that the firing of a 5-HT neurone could be regulated by an adjacent 5-HT neurone. The first study demonstrated that 5-HT raphe neurones were connected to one another through dendrodendritic synaptic junctions (Felten and Harrigan 1980). The second study reported a high degree of dye coupling between DR neurones as dye injected into one cell body was found to spread into the cell body of one or two adjacent cells (Stezhka and Lovick 1995). These findings would suggest that linked cells could produce a similar firing pattern and that the effects of drugs or neurotransmitters upon one cell could be transmitted to the other(s).

The regulation of raphe neuronal firing is not confined to 5-HT acting on autoreceptors. Other neurotransmitters, for example noradrenaline, GABA, glutamate and opioids have also been reported to have effects on raphe neuronal firing. As mentioned above, in raphe slices 5-HT cell firing must be driven and commonly this is achieved by including phenylephrine in the solution which bathes the slice. The phenylephrine is required because the tonic noradrenergic input to the raphe from the locus coeruleus has been removed (Baraban *et al* 1978, Baraban and Aghajanian 1981). However, some of the inputs to raphe neurones are still present within raphe slices, one set of these inputs comes from GABA interneurones. GABA interneurones have been reported to occur within the DR (Gallager 1978, Nanopoulos *et al* 1982, Wang *et al* 1992) and GABAergic terminals have been shown to exist in close proximity to both the dendrites and the cell bodies of 5-HT neurones (Wang *et al* 1992). The effect produced by GABA on 5-HT raphe neurones is dependant on which GABA receptor is activated. Infusion of the $GABA_A$ agonist muscimol into the DR produced a decrease in the extracellular level of 5-HT in this nucleus whereas application of the $GABA_B$ agonist bicuculline produced an increase in extracellular levels of 5-HT in the DR (Tao *et al* 1996). Increased extracellular levels of 5-HT have also been observed in the DR and in the nucleus accumbens after kainate was infused into the DR (Tao *et al* 1997). Kainate receptor antagonists do not affect basal 5-HT release suggesting release is not under tonic control by glutamate (Tao *et*

al 1997). This study also showed that activation of α-amino-3-hydroxy-5-methyl-isoxazole (AMPA) receptors had little effect on 5-HT release suggesting that the response observed is dependant on the subtype of glutamate receptor involved. A third set of modulatory inputs which are preserved in raphe slices comes from opioid neurones. Opioid receptors and axon terminals have been demonstrated to exist in the DR and met-enkephalin-like immunoreactive nerve terminals have been observed in contact with 5-HT cell bodies (Wang *et al* 1991, 1998). Like GABA and glutamate, opioids produce complex actions on firing of 5-HT neurones within the raphe nuclei. In the case of opioids this variability in effect is due to the fact that opioid neurones synapse directly onto 5-HT neurones but also act on both glutamate and GABA interneurones which independently synapse onto DR neurones (Jolas and Aghajanian 1996).

5-HT_{1A} receptors

The importance of 5-HT_{1A} receptors in the DR is primarily related to their somatodendritic location and the fact that activation of these receptors reduces raphe neuronal firing (Verge *et al* 1985, Sprouse and Aghajanian 1987). However, it should not be forgotten that 5-HT_{1A} receptors also occur postsynaptically (Hoyer *et al* 1986). The mRNA for 5-HT_{1A} receptors has been detected both in presynaptic locations i.e. the raphe nuclei and in postsynaptic regions e.g. in the hippocampus (dentate gyrus and layers CA1-3), septum, olfactory bulb and the entorhinal cortex (Wright *et al* 1995). The activity of ligands at the post- and presynaptic receptors differ as demonstrated by the observation that compounds which act as antagonists postsynaptically can act as partial agonists at the presynaptic receptor e.g. BMY 7378 (Hjorth *et al* 1995). A possible explanation for the discrepancy in ligand activity is the difference in receptor reserve between the two sites (reviewed by Hoyer and Boddeke 1993). At postsynaptic sites e.g. the hippocampus it has been shown that no receptor reserve for 5-HT_{1A} receptors exists (Yocca *et al* 1992). However, in the dorsal raphe it is estimated that the receptor reserve for 5-HT_{1A} receptors is greater than 50% (Cox *et al* 1993) and possibly as high as 80% (Meller *et al* 1990). This means that maximal inhibition of DR firing can be achieved by an agonist without all available receptors being occupied and that low doses of antagonist have little effect in reversing agonist inhibition.

At a cellular level, 5-HT_{1A} receptor immunoreactivity has been detected on the dendritic processes and cell bodies of 5-HT neurones in the dorsal raphe where it is unevenly distributed on the plasma membrane (Kia *et al* 1996). In the brain as a whole 5-HT_{1A} receptor immunoreactivity is found on the dendritic branches, shafts and spines in the dorsal raphe and locus coeruleus and it has also been detected on the axon hillock of the pyramidal cells in the cerebral cortex and on the cell body and processes of astrocytes (Azmitia *et al* 1996).

Characterisation of 5-HT_{1A} responses has been facilitated by the 5-HT_{1A} agonist 8-OH DPAT and the antagonist WAY 100,635. 8-OH DPAT preferentially binds to receptors bound to G-proteins i.e. active receptors (Hall *et al* 1985) and this is illustrated by the fact that the B_{max} for WAY 100,635 is 50-60% higher than that for 8-OH DPAT (Fletcher *et al* 1996). WAY 100,635 is selective for 5-HT_{1A} receptors with over 100 fold selectivity over all other receptors tested including other 5-HT_1 receptor subtypes, 5-HT_{2A}, 5-HT_{2C}, α_2-adrenoceptors, dopamine (D_1, D_2 and D_4), $GABA_A$ and $GABA_B$, histamine (H_1, H_2 and H_3) and N-methyl-D-aspartate (NMDA) receptors (Fletcher *et al* 1996). In the hippocampus antagonism by WAY 100,635 is partly non-competitive however in the dorsal raphe the antagonism is competitive (Corradetti *et al* 1996). This is a further example of the differences in ligand activity at post- and presynaptic 5-HT_{1A} receptors.

5-HT_{2A} receptors

The 5-HT_2 receptor family has undergone reclassification by IUPHAR (Hoyer *et al* 1994) and is now said to contain three subtypes, namely 5-HT_{2A}, $_{2B}$, and $_{2C}$. The 5-HT_{2A} receptor was previously referred to as the 5-HT_2 receptor, the 5-HT_{2C} receptor was previously classed as the 5-HT_{1C} receptor and the 5-HT_{2B} receptor was previously known as 5-HT_{2F} receptor due to its classical discovery in the stomach fundus.

The interest in the function of 5-HT_{2A} receptors within the CNS is a result of them being located in regions which have specific roles. For example, they are found in the cortex (Pazos *et al* 1985) which is the region of the brain which is associated with the emotional balance of the individual (for review see Price 1999). They are also found in the hypothalamus which is involved in the regulation of many endocrine hormones (Pazos *et al* 1987).

5-HT_{2A} receptors are found to occur in many brain regions and also in the periphery (Bradley *et al* 1986). Within the brain they are found in specific laminae of the neocortex, I and IV in the rat (Pazos *et al* 1985, Hoyer *et al* 1986, Pazos *et al* 1987), and III and V in humans (Pazos *et al* 1985, Hoyer *et al* 1986, Pazos *et al* 1987). 5-HT_{2A} receptors are also found in other brain regions e.g. the limbic system (Hoyer *et al* 1986, Pazos *et al* 1987), the basal ganglia (Pazos *et al* 1987) and the hypothalamus (Meibach *et al* 1980, Pazos *et al* 1987, Sumner and Fink 1992). 5-HT_{2A} receptors are G-protein linked receptors and it is the third intracellular loop of these receptors which confers coupling efficiency (Oksenberg *et al* 1995). The transmembrane regions 2 and 7 of these receptors appear to be in close contact with each other and are reported to interact in the activation of the receptor by an agonist (Sealfon *et al* 1995).

It has been suggested that there may be species differences in 5-HT_{2A} receptors since receptor heterogeneity has been observed *in vitro* (Mylecharne *et al* 1997). Further evidence for species differences comes from antagonist studies which show that some antagonists have higher affinity for rat 5-HT_{2A} receptors e.g. mesulergine whereas some have higher affinity in monkeys, pigs and humans e.g. LY 86057 (Johnson *et al* 1994 and 1995). The latter study (Johnson *et al* 1995) also showed ketanserin had equal affinity at rat and human receptors. It is possible that species differences could be explained by the fact that two affinity states of the 5-HT_{2A} receptor have been demonstrated (Gurguis *et al* 1998). It should also be noted that the regulation of 5-HT_{2A} receptors is unusual since chronic administration of agonists (Buckholtz *et al* 1988) or of antagonists (May *et al* 1986, Eison *et al* 1989) results in receptor downregulation, normally chronic treatment with an antagonist results in receptor supersensitivity. Downregulation of 5-HT_{2A} receptors by agonist activation has been shown to involve a decrease both in receptor number and receptor RNA as determined by Northern Blot analysis (Saucier and Albert 1997).

Antagonism of 5-HT_{2A} receptors has been shown to block the 5-HT induced inhibition of dopamine release (Muramatsu *et al* 1988a), acetylcholine release (Muramatsu *et al* 1988b) and adrenaline release (Fenuik *et al* 1981). Activation of 5-HT_{2A} receptors by 5-HT or a 5-HT_{2A} agonist (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, DOI) was reported to inhibit glutamate release (Maura *et al* 1988). Neurotransmitters are not the only compounds whose release is modulated by 5-HT_{2A} receptor activation, hormones too are affected. For example, the oestrogen-induced

surge of luteinising hormone-releasing hormone (LHRH) is blocked by administration of 5-HT_{2A} antagonists (Fink *et al* 1996). It is obvious that receptors which are involved in controlling the release of so many compounds will have an important role to play in normal brain function. This role of 5-HT_{2A} receptors encompasses a wide range of functions from gonadotrophin release to learning (Meneses and Hong 1997) and slow-wave sleep, which can increased by administering 5-HT_{2A} receptor antagonists in both humans and rats (Idzikowski *et al* 1986, Dugovic and Wauquier 1987 respectively).

However, 5-HT_{2A} receptors are also implicated in several disorders in the CNS. For example, increases in 5-HT_{2A} receptor binding sites have been observed in anxiety (Iversen 1984) and depression (Meltzer 1990, Yates *et al* 1990). Increases in 5-HT_{2A} receptor binding sites have also been reported in the frontal cortex of suicide victims (Mann *et al* 1990) although no change was observed in an earlier study (Cheetham *et al* 1988). In the periphery increases in 5-HT_{2A} receptor binding sites have been detected on platelets from suicidal patients and this has been suggested as a test for suicidal behaviour (Pandey *et al* 1995). Not all CNS disorders involve increases in 5-HT_{2A} receptors, a decrease is seen in the brains of patients with Alzheimer's disease (Reynolds *et al* 1984).

Depression

Depression is defined in DSM-IV (American Psychiatric Association 1994) as a depressed mood or a loss of interest or pleasure which lasts for at least two weeks and is present for most of the day and nearly every day. Other symptoms are often experienced and include weight loss/gain, sleep disturbance, feelings of worthlessness, difficulty thinking and concentrating, fatigue and suicidal thoughts. Depression is diagnosed only if several of these symptoms are present and if behaviour of the individual is significantly different from normal.

Depression is a complex CNS disorder as it involves the interaction of more than one transmitter system and this makes it difficult to determine the precise cause of depression, if there is indeed any one cause, and as a result depression is difficult to treat. The drugs currently used in the treatment of depression all involve a lag time between initial administration and clinical improvement, this can cause anxiety in the patient as they believe their depression shows no signs of alleviating.

The monoamine theory of depression implicates a deficiency in noradrenaline and 5-HT release and increases in β-adrenoceptor and 5-HT_2 receptor binding sites (review by Garver and Davis 1979). Studies on brain and platelets have shown that there is a decrease in 5-HT levels in depression (Risch and Nemeroff 1992). Increased levels of 5-HT_{2A} receptor binding have been detected in the cortex of drug-free depressive patients although the increase was not seen in depressed patients treated with antidepressants (Yates *et al* 1990). This observation is supported by the finding that chronic antidepressant treatment decreases 5-HT_{2A} receptor density (Peroutka and Snyder 1980, Wamsley *et al* 1987). This decrease in 5-HT_{2A} receptor density is thought to be linked to desensitisation and downregulation of the receptor rather than an absolute decrease in receptor numbers (Wamsley *et al* 1987, Roth and Ciaranello 1991). Although antidepressant treatment decreases 5-HT_{2A} receptor density the opposite effect is seen with electroconvulsive shock treatment (ECS) which actually produces an increase in receptor density (Green *et al* 1983). The contradicting effects on 5-HT_{2A} receptor density produced by these two treatments for depression may be linked to the unusual regulation of 5-HT_{2A} receptors discussed earlier. 5-HT_{1A} receptors may also be involved in depression since 5-HT_{1A} agonists have antidepressant effects (Kennett *et al* 1987). It has been suggested that the antidepressant actions of both 5-HT_{1A} agonists and other antidepressant drugs are mediated through the desensitisation of the somatodendritic 5-HT_{1A} receptor (reviewed by Blier and de Montigny 1994).

It is not just in the receptors themselves that changes have been found in patients with depression, but also in the 5-HT transporter. One study reported that the number of transporter binding sites was decreased in depression (Watanabe *et al* 1993). Also, it has shown that susceptibility to depression is linked to the possession of different alleles of a variable number tandem repeat (VNTR) sequence in intron 2 of the transporter gene (Ogilvie *et al* 1996) however this finding was not confirmed in a more recent study (Guitierrez *et al* 1998).

Many of the antidepressant drugs, including lithium which is used to treat biphasic depression, modify serotonergic transmission. Chronic administration of lithium has been shown to desensitise 5-HT_{1A} autoreceptors (Wang and Friedman 1988) although the mechanism involved appears to be different from that observed after chronic SSRI's (Price *et al* 1990). Chronic antidepressant drugs have been shown to decrease 5-HT_{2A} receptor number (Lesch *et al* 1992). The effect of chronic antidepressant

treatment on the 5-HT transporter is less clear. Levels of SERT mRNA have been reported to increase after chronic treatment with both TCA's and monoamine oxidase inhibitors (MAOI's) (Lopez *et al* 1994). However, chronic treatment with other antidepressants, including fluoxetine and desipramine produced decreases in the expression of SERT mRNA (Lesch *et al* 1992, Kuroda *et al* 1994).

There has always been one major problem with the treatment of depression and this is the lag time between commencing treatment and a therapeutic effect being observed. This lag time is usually in the order of 2 to 3 weeks and is likely to be due to a complex interaction because it has been shown that drugs which inhibit the transporter do so within minutes (Wong *et al* 1975). Since uptake inhibition occurs rapidly this is unlikely to be responsible for the long lag time in therapeutic effect and there are many theories as to what causes this phenomenon.

One theory is that a new balance between neurotransmitters, receptors and second messengers must be reached (reviewed by Blier and de Montigny 1994). For example, chronic SSRI's cause downregulation of 5-HT_1 receptors (Dumbrille-Ross and Tang 1983, Wamsley *et al* 1987). These 5-HT_1 receptors are likely to include the somatodendritic 5-HT_{1A} receptors which regulate the activity of the neurone. Downregulation of the 5-HT_{1A} receptors will decrease the inhibitory effect of these receptors on raphe neurone firing and hence cause an increase in 5-HT released from the neurone (Welner *et al* 1989). The downregulation or desensitisation of these autoreceptors may be a delayed effect (Finley 1994) and this would explain the lag time before a therapeutic effect is observed. A second theory is that TCA's may increase transporter gene transcription and produce an increase in 5-HT turnover (Lopez *et al* 1994).

Animal models of depression do exist such as the learned helplessness (Seligman and Beagley 1975) and the chlorimipramine-induced model of depression in rats where a decrease in neuronal firing in the DR has been observed (Yarari *et al* 1993). In humans changes in brain function can only be determined under certain circumstances e.g. brain scans (magnetic resonance imaging [MRI] and positron emission tomography [PET]) or from *post-mortem* tissue, neither of which is ideal.

Depression in women

It has been shown that there is a gender difference in depression with twice as many women being diagnosed with depression as men (Parry 1995). This observation may relate to differences in the symptoms experienced by men and women with depression. A recent report suggested somatic depression (depression with sleep disorders, fatigue and appetite disturbance) was twice as common in women than men but that pure depression (depression without these three other symptoms) was found to occur equally in men and women (Silverstein 1999).

Although women are susceptible to depression throughout their lives there are certain times which appear to have a higher risk associated with them, these occur premenstrually, postpartum and postmenopausally. The chances of suffering from depression at any one of these times is also dependant on a previous history of depression. Women who have already suffered from a bout of depression are far more likely to suffer from another episode postpartum or postmenopausally (Haynes and Parry 1998). The peak incidence of depression around the menopause is highest during the perimenopause which is the time between regular menstrual cycling and cessation of menses for at least a year i.e. the menopause (Haynes and Parry 1998). Of the three types of depression premenstrual depression is the most difficult to categorise since the symptoms range widely from woman to woman with only approximately 5% of women meeting the criteria for premenstrual dysphoric disorder (PMDD) (Haynes and Parry 1998). The number of women who actually complain of premenstrual depression is much greater; however as the DSM-IV classification for PMDD requires a significant impairment in normal functioning (American Psychiatric Association 1994) many women do not meet the criteria to be classified as depressed.

Though many women suffer from these specific types of depression no absolute cause has been defined. The most obvious cause of depression in women would be the decrease in levels of oestrogen and progesterone particularly premenstrually and postpartum. Although premenstrual depression appears to occur prior to menstruation studies have been unable to link premenstrual depression with decreased oestrogen levels (Studd and Zamblera 1994). Studies have also failed to detect any specific abnormalities in either the levels of oestrogen and progesterone or in the regulation of these hormones in women who suffer from premenstrual depression (Rubinow *et al* 1988). A recent theory has suggested premenstrual

depression is related not to the absolute levels of oestrogen and progesterone but to the sensitivity of the individual to these hormones (Schmidt *et al* 1998). This theory suggests that these individuals form a subpopulation which is more susceptible to depression.

Unfortunately, since an absolute cause has not been defined for these forms of depression there is no clear cut treatment for the sufferers. One treatment approach relies on the knowledge that depressed mood has been shown to correlate with lower blood 5-HT levels (Verkes *et al* 1998) and hence suggests conventional antidepressants may be effective. Indeed premenstrual depression is more effectively treated using SSRI's than with hormone replacement therapy but these drugs are still only effective in 65-70% of cases (Haynes and Parry 1998). However, it has been shown that oestrogen replacement therapy can be efficacious as a supplementary therapy alongside conventional antidepressants (Chouinard *et al* 1987). Several studies have shown that oestrogen treatment is effective in treating postpartum depression (Gregoire *et al* 1996, Sichel *et al* 1995) and also premenstrual depression (Studd and Zamblera 1994). Oestrogen replacement therapy alone has also been shown to be useful in treating women who do not respond to other antidepressants (Klaiber *et al* 1979).

In summary, women appear to be more susceptible to depression than men and particularly so premenstrually, postpartum and postmenopausally. This increased risk of depression has not been found to correlate with decreased levels of sex steroids. However, oestrogen replacement can be used therapeutically to treat depression alone or in conjunction with antidepressants and as a result it is important to study the effects of the sex steroids on neuronal function.

Oestrogen

Oestrogens are a group of female sex hormones, produced in the granulosa cells of the ovarian follicle, whose role is to promote ovulation (Brown 1994). Oestrogens are synthesised from cholesterol in a multi-step reaction pathway and as a result they are steroidal in structure. There are three main oestrogens in humans, oestradiol, oestrone and oestriol. They differ in the number of hydroxyl groups on the steroid ring and this affects their potency (Jozan *et al* 1981). Due to their steroidal structure oestrogens can penetrate the blood brain barrier (BBB) easily. Oestrogens are also

found in the brains of males where they are a metabolic by-product of testosterone metabolism by aromatase (Celotti *et al* 1991).

The levels of oestrogens in the circulation vary within the oestrous cycle, see schematic diagram of the rat oestrous cycle below (from Fink 1977). This variation in the plasma levels of sex steroids can produce changes within the brain which correlate with the stage of the oestrous cycle. One such brain region is the hippocampus where cyclical changes in spine density in the CA1 region have been observed (Woolley and McEwen 1994). The spine density is maximal during pro-oestrus when oestrogen levels are at their peak and lowest 24 hours later (oestrus) when oestrogen levels are at their lowest. A possible mechanism for this effect is by a decrease in GABA transmission in hippocampal neurones (Murphy *et al* 1998) or by NMDA receptor activation (Woolley and McEwen 1994).

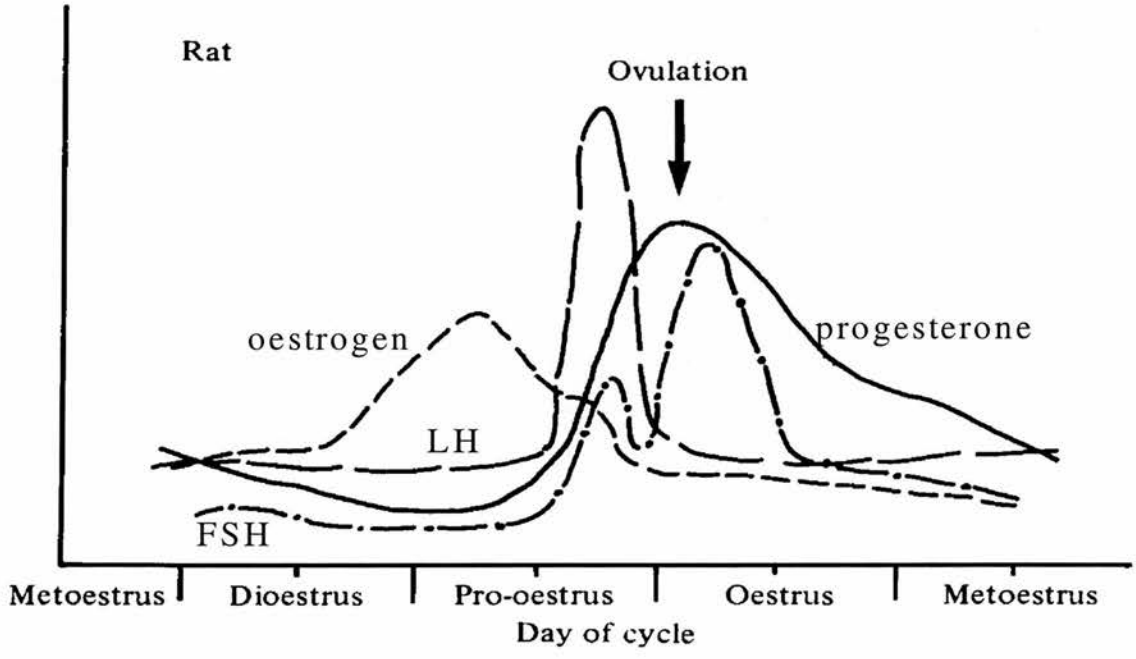


Figure 1.5: Diagram illustrating plasma levels of luteinising hormone (LH), follicle stimulating hormone (FSH), oestrogen and progesterone throughout the oestrous cycle of a rat (adapted from Fink 1977).

Oestrogen receptors are believed to be located within the nucleus of the cell. Two types of oestrogen receptors are known to exist, these are the classical oestrogen receptor (ERα) and the recently discovered ERβ (Kuiper *et al* 1996). In rat brain ERα is found in the medial preoptic area (MPOA), median eminence and the ventromedial nucleus of the hypothalamus (VMN) (see figure 1.6 from Brown 1994). The cells in the preoptic area, median eminence and the pituitary gland are involved in the feedback regulation for the release of LHRH and pituitary gonadotropic hormones (Brown 1994).

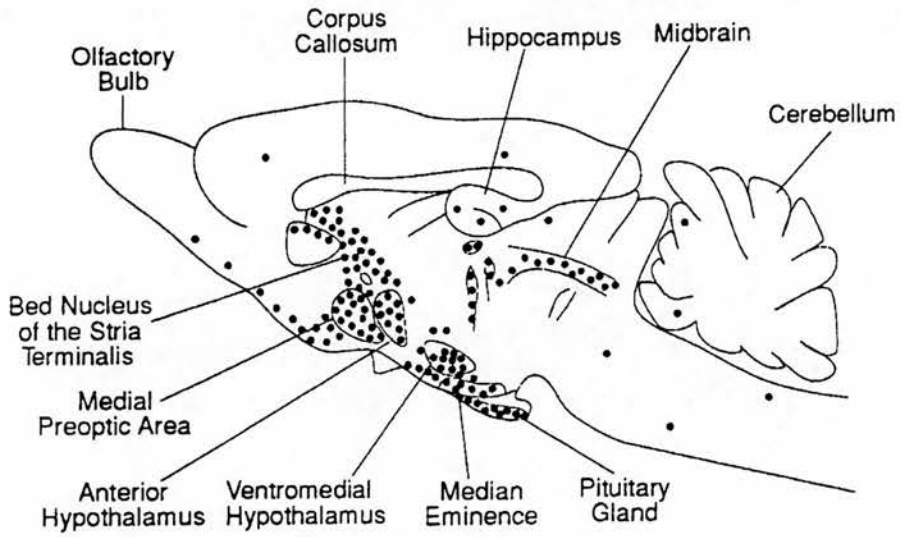


Figure 1.6: Diagram of a sagittal section through a rat brain illustrating the location of classical oestrogen receptors (ERα) (from Brown 1994).

The major action of oestrogens is to stimulate protein synthesis (Biegon 1990) although oestrogens also have non-genomic actions. The genomic actions, e.g. protein synthesis, involve a time delay of hours before their effects are observed. For example, oestrogen stimulates corticotrophin-releasing factor (CRF) gene transcription *in vitro* and *in vivo* in rats (Paulmyer-Lacroix *et al* 1996). Oestrogen has also been shown to increase low voltage-activated calcium current density in a cell line, a process which was found to be dependent on protein synthesis (Ritchie *et al* 1993). The genomic effects of oestrogen are known to be mediated by the two intracellular oestrogen receptors ERα and ERβ (Kuiper *et al* 1996). It has been shown that the distribution of the two oestrogen receptors differs throughout the rat

brain. ERα mRNA has been detected mainly in the hypothalamic nuclei (including the arcuate nucleus, VMN and MPOA) but also in the olfactory tubercle, amygdala, periaqueductal central gray (PAG) and nucleus tractus solitarius (Shughrue *et al* 1997a). In rats ERβ mRNA has been detected in the cortex, hippocampus, thalamus, MPOA, cerebellum, supraoptic nucleus (SON) and DR (Shughrue *et al* 1997a) and in the spinal cord (Kuiper *et al* 1997). Immunoreactivity for ERβ has been detected in the lateral septum, SON, medial amygdala, MPOA, arcuate nucleus and hippocampus (Li *et al* 1997). It is apparent from the data above that some areas contain both receptors e.g. MPOA whereas some contain only ERα e.g. VMN or ERβ e.g. cerebellum (see table 1.2). The DR has been reported to contain only ERβ mRNA (Shughrue *et al* 1997a) however Alves *et al* (1998) detected only ERα and not ERβ immunoreactivity within the DR. It is also unclear which cells in the DR contain the oestrogen receptors since the ERα immunoreactivity detected by Alves *et al* (1998) does not appear to be localised to 5-HT neurones and may be located on GABA interneurones.

location in rat brain	ERα		ERβ	
cortex			mRNA	
olfactory tubercle	mRNA			
lateral septum				IR
supraoptic nucleus			mRNA	
thalamus			mRNA	
Hypothalamus				
arcuate nucleus	mRNA	receptor		IR
VMN	mRNA	receptor		
MPOA	mRNA	receptor	mRNA	IR
anterior pituitary		receptor		
hippocampus			mRNA	IR
amygdala	mRNA			IR
PAG	mRNA			
dorsal raphe		IR	mRNA	
cerebellum			mRNA	

Table 1.2: Summary of brain regions in the rat where oestrogen receptors ERα and ERβ have been detected.

Abbreviations: IR = immunoreactivity, receptor = receptor binding

Some effects of steroids occur much more rapidly, i.e. within minutes. These rapid effects are unlikely to be due to protein synthesis however they could be produced by a non-genomic mechanism. Non-genomic actions of oestrogen can be result from oestrogen binding to receptors on the cell membrane (Bression *et al* 1986, Schumacher 1990) or altering the release of neurotransmitters (McEwen 1991). Oestrogens may also cause changes in ion permeability (Bression *et al* 1986), for example an increase in K^+ permeability leading to hyperpolarisation of medial amygdala neurones (Nabekura *et al* 1986).

Oestrogen has been shown to affect the electrical activity of cells in various brain regions *in vivo*. In the medial nucleus of the amygdala oestrogen has been shown to increase the spontaneous electrical activity of neurones (Schiess *et al* 1988). A similar effect was observed in striato-nigral neurones where oestrogen treatment produced an increase in the spontaneous firing of these neurones in both male and female rats (Tansey *et al* 1983). However, oestrogen was found to produce no change in firing rate in various regions of the hypothalamus including MPOA, VMN and arcuate nucleus (Bueno and Pfaff 1976). In these hypothalamic regions oestrogen was found to increase the number of spontaneously active cells per track in the VMN and arcuate nucleus but decrease the number of spontaneously active cells in other regions including the MPOA (Bueno and Pfaff 1976). The differential effects of oestrogen in the MPOA compared to other hypothalamic regions may play a role in hormone release. Several studies *in vitro* have shown rapid effects of oestrogen suggesting a non-genomic action. In neostriatal neurones, oestradiol was shown to reduce calcium currents (Mermelstein *et al* 1996). In the hippocampus, oestradiol has been reported to depolarise pyramidal neurones (Wong and Moss 1991) and to potentiate kainate induced currents in CA1 neurones (Gu and Moss 1996). Although oestrogen has been shown to affect kainate currents in CA1 neurones neither acute (4/5 days) nor chronic (2/3 weeks) treatment with oestrogen produced any change in 5-HT_{1A} mediated outward currents in these neurones (Dijcks *et al* 1994). However, oestradiol treatment has been shown to increase potassium currents in a human colonic epithelial cell line (T84) (Condliffe *et al* 1998).

Progesterone

The second female sex steroid is progesterone and, like oestrogen, the levels of this hormone fluctuate throughout the oestrous cycle as shown in figure 1.5. It is possible that progesterone may also have a role to play in the gender differences found in the CNS disorders described above. Like oestrogen it too is synthesised in the ovary from cholesterol, is steroidal in structure and hence readily crosses the BBB. Progesterone has also been reported to be synthesised within the peripheral nervous system in Schwann cells (Koenig *et al* 1995).

Progesterone receptors (PRs) occur in the brain in several regions including the pituitary, hypothalamus, PAG and cerebral cortex (Rainbow *et al* 1982, Parsons *et al* 1982). They are also found in the peripheral nervous system in Schwann cells (Jung-Testas *et al* 1996). The expression of progesterone receptors can be induced by oestrogen in some but not all of these brain regions. For example, in the hypothalamus (MPOA, arcuate nucleus and SCN) PRs are oestrogen-inducible whereas in the cortex they are not (Rainbow *et al* 1982, Parsons *et al* 1982). The mRNA for progesterone receptors is found in the cortex, hippocampus (pyramidal layers of CA1 and CA3), diagonal band of Broca (DBB), SON, arcuate nucleus, VMN and MPOA (Hagihara *et al* 1992). The mRNA for PRs has only been detected in neurones not in glial cells or fibres (Hagihara *et al* 1992). Immunocytochemical detection of PRs in the DR has revealed the presence of more labelled cells in females than in males (Alves *et al* 1998). The PRs in the DR are affected by oestrogen in a region specific manner. In the lateral wings of the DR progesterone receptor immunoreactivity has been shown not to be induced by oestrogen whereas PR immunoreactivity was increased in the other regions of the DR (Alves *et al* 1998). Different isoforms of the PR have been detected and it would appear that these isoforms are short and long forms of the receptor (Bethea and Widmann 1998). The transduction mechanism of the human PR isoforms has been examined and it is believed that the different isoforms signal through different transduction pathways (Wen *et al* 1994).

Progesterone has been classed as a neurosteroid since it is synthesised from cholesterol within both the central and peripheral nervous systems (Baulieu and Schumacher 1997). Neurosteroids can modulate neurotransmission by acting directly on the neuronal membrane and a membrane binding site has been demonstrated for progesterone within the CNS (Tischkau *et al* 1993). Non-genomic effects of

progesterone have been reported, for example in the ventral tegmental area (VTA) application of progesterone results in an increase in sexual behaviour (DeBold and Frye 1984). Progesterone has also been shown to inhibit the current produced by nicotinic receptor activation within seconds (Bertrand *et al* 1991). This initial inhibition was found to be reversible and after several hours an irreversible suppression of the current was observed, the timing suggests this second effect of progesterone may be genomic (Bertrand *et al* 1991). Nicotinic receptors are not the only receptors modulated by progesterone, $GABA_A$ receptors are also affected with progesterone producing increases in [^{3}H]muscimol binding in the caudate and cortex (Maggi and Perez 1984). It is thought that the anxiolytic effects of progesterone are mediated through modulation of the $GABA_A$ receptor (Bitran *et al* 1995).

Progesterone also produces electrophysiological effects in the brain and has been shown to increase orthodromic latency of neurones in the MPOA on stimulation of the median eminence-arcuate nucleus (Watanabe 1992), an effect which may result, in part, from progesterone's effects on ion channels. The effects of progesterone are not solely mediated through ion channels, some are mediated by second messenger systems. For example, progesterone has been shown to produce an increase in adenylate cyclase activity and cyclic 3',5'-adenosine monophosphate (cAMP) levels in the hypothalamus and cortex two and 24 hours after progesterone treatment (Collado *et al* 1985).

Progesterone has been shown to promote formation of new myelin sheaths (Baulieu *et al* 1996) and to promote the expression of myelin basic protein in cultured oligodendrocytes (Baulieu and Schumacher 1997). These actions of progesterone suggest that it may have a role to play in nerve regeneration. In the brain progesterone also has other positive roles to play, one such role is in protection against ischaemia after middle cerebral artery occlusion (Jiang *et al* 1996).

Oestrogen and progesterone

Since the levels of oestrogen and progesterone rise then fall during the oestrous cycle (figure 1.5) it is likely that the effects of these steroids will interact, these interactions have been observed in several studies. Oestrogen has been reported to produce an increase in levels of both GABA and glutamate in the hypothalamus compared to levels in ovariectomised (OVX) rats, the addition of progesterone reduced the levels

of these transmitters to that in OVX rats (Luine *et al* 1997). Serotonergic neurotransmission is also affected by these two steroids, examples of this are discussed below.

Oestrogen, Progesterone and 5-HT

Studies have examined several parameters of serotonergic neurotransmission throughout the oestrous cycle and between males and females. In the forebrain 5-HT turnover (the ratio of 5-HIAA/5-HT) has been shown to differ between male and female rats, with females showing an increased turnover compared to males (Rosecrans 1970). This observation may be explained by the increased tryptophan hydroxylase activity, hence 5-HT synthesis, in females compared to males (Carlsson and Carlsson 1988). However, in humans it appears that men have a higher rate of 5-HT synthesis than women (Nishizawa *et al* 1997).

In females changes have been seen in 5-HT levels and 5-HT turnover in the brain during the oestrous cycle. 5-HT levels in the hypothalamus decrease on the afternoon of pro-oestrus when oestrogen levels are at their peak (see figure 1.5) (Biegon *et al* 1982, Biegon *et al* 1983). This change in 5-HT levels may be related to the fact that tryptophan is displaced from its plasma albumin binding sites by oestrogen both *in vivo* and *in vitro* (Aylward 1973). The uptake of 5-HT is also affected and a significant increase in 5-HT uptake has been shown to occur in the hypothalamus on the day of pro-oestrus (Biegon *et al* 1980). Regional changes in 5-HT receptors have also been reported during the oestrous cycle. There is a significant decrease in B_{max} of 5-HT_1 receptors on pro-oestrus and oestrus compared to dioestrus when the levels of sex steroids are lowest (Biegon *et al* 1980). This effect on 5-HT_1 receptors (subtype not determined) was observed in the preoptic area of the hypothalamus but not in the cortex or hippocampus (Biegon *et al* 1980) hence may relate to hormone regulation. The response to 8-OH DPAT (5-HT_{1A} agonist) changes during the oestrous cycle with least hyperphagia on pro-oestrus and maximal hyperphagia on dioestrus (Uphouse *et al* 1991). Also, in females more 5-HT was released in the hypothalamus after administration of 8-OH DPAT on pro-oestrus than on dioestrus (Maswood *et al* 1995). Changes in 5-HT_{2A} receptors have also been reported over the oestrous cycle. In regions of the forebrain 5-HT_{2A} receptor binding site density is higher during pro-oestrus than during dioestrus (Sumner and Fink 1997). The

regions of higher 5-HT_{2A} receptor binding were the frontal and cingulate cortex, nucleus accumbens and olfactory tubercle.

Many other studies have examined the effects of steroid replacement on various aspects of 5-HT neurotransmission. Most of these have examined the effects of oestrogen alone while others have studied the effects progesterone in oestrogen-primed rats, few studies have examined the effects of progesterone alone. The results of these studies will be discussed in respect of release/metabolites, uptake and finally receptors.

It is possible that the synthesis of 5-HT is under steroidal control since the expression of tryptophan hydroxylase (TH) mRNA is regulated by gonadal steroids. In macaques oestrogen treatment (28 days) produced an increase in expression of TH mRNA compared to spayed controls and this increase was augmented further by the addition of progesterone (Pecins-Thompson *et al* 1996). In ovariectomised rats an increase in 5-HT content was observed in the DR and substantia nigra (SNR) 24 hours after the rats had been treated with oestrogen (Di Paolo *et al* 1983). In the same study no changes were observed in 5-HT content in either the hypothalamus, DR or SNR after chronic (28 days) oestrogen treatment. In oestrogen-primed rats (oestrogen for 4 days) decreases in the 5-HIAA/5-HT ratio have been shown in the VMN and the periventricular region of the hypothalamus (PEV) 4 hours after progesterone treatment (Gereau 4th *et al* 1993) demonstrating there is a decrease in 5-HT turnover. In the presence of pargyline (a monoamine oxidase inhibitor) a decrease in 5-HT accumulation was observed in both the hypothalamus (VMN and PEV) and also in the PAG of these oestrogen and progesterone treated rats (Gereau 4th *et al* 1993). In contrast, no changes in the 5-HIAA/5-HT ratio were shown after oestrogen treatment (12 and 24 hours or 4 weeks; Di Paolo *et al* 1983) (2 days; Lu *et al* 1998) or after the addition of progesterone in oestrogen-primed rats (4 hours; Lu *et al* 1998). However, the study by Lu *et al* (1998) did report that oestrogen, in the presence or absence of progesterone, produced a decrease in 5-HT concentrations in the MPOA and VMN though not in the lateral septum (Lu *et al* 1998). The results from this study contrast with those shown by Di Paolo *et al* (1983) which reported no change in 5-HT levels in these regions. Lu *et al* (1998) also demonstrated that oestrogen, in the presence or absence of progesterone, produced a decrease in the density of 5-HT immunoreactive fibres in these hypothalamic regions but again no change was found in the lateral septum.

Studies into the effects of gonadal steroids on the 5-HT uptake have also been carried out in both rats and macaques. Studies have examined both uptake binding sites and mRNA expression. In the rat, oestrogen has been shown to increase binding sites using both imipramine (Rehavi *et al* 1987) and paroxetine (McQueen *et al* 1997 and 1998) as radioligands. Rehavi *et al* (1987) demonstrated that chronic oestrogen treatment (12 days) produced increases in binding sites and 5-HT uptake in the hypothalamus and frontal cortex. Acute administration of oestrogen (32 hours) produced different effects with no change in transporter binding sites in the cortex but increases in the VMN, basolateral amygdala (BLA) and a decrease in the PAG (McQueen *et al* 1997 and 1998). This difference may be the result of the duration of oestrogen treatment or the binding assay methodology since the study by Rehavi *et al* (1987) was carried out using tissue homogenates while that of McQueen *et al* (1997 and 1998) was carried out using quantitative autoradiography. A study examining paroxetine binding in the cortex and hippocampus concurs with that of McQueen *et al* (1997 and 1998) in that no change was found in the cortex after oestrogen treatment (7 days; Mendelson *et al* 1993). The study by Mendelson *et al* (1993) also showed a decrease in several regions of the hippocampus after oestrogen treatment however these results were pooled from both male and female rats. Examination of the effects of oestrogen on 5-HT transporter mRNA expression has been carried out in both macaques and rats. In rats no increase was observed in the grain density of labelled cells in the DR but there was an increase of approximately 50% in the number of labelled cells (McQueen *et al* 1997). Increases in transporter mRNA of this magnitude have also been reported for rats chronically treated with oestrogen (3 weeks; Thomas *et al* 1997). In macaques the effects of both oestrogen and progesterone have been studied. This study showed a decrease in the expression of transporter mRNA in the DR after both oestrogen (28 days) and oestrogen plus progesterone (oestrogen for 28 days with progesterone given for the last 14 days) treatments (Pecins-Thompson *et al* 1998). The difference between the rat and macaque study may be a result of an acute versus chronic (respectively) treatment regime. It is interesting to note that both these studies show differences in the dorsal raphe and not the median raphe, this may relate to differences between these two nuclei, some of which were highlighted earlier.

Changes in the turnover, concentration and uptake of 5-HT as a result of oestrogen treatment are likely to have a knock-on effect on receptors and several studies have examined the effects of gonadal steroids on the expression and binding site density of 5-HT receptors. Initially effects were studied in the oestrogen concentrating nuclei of

the hypothalamus. Several regions, including the arcuate nucleus/median eminence, anterior hypothalamus and MPOA reportedly show increased binding of 5-HT after oestrogen treatment (48 hours; Biegon *et al* 1982). This study also examined changes in 5-HT_1 receptors (subtype not determined as non-specific ligand used) in brain regions which are reported not to concentrate oestrogen (Pfaff and Keiner 1973) e.g. the frontal cortex, caudate, medial septum, ventral thalamus, SNR, DR and cerebellum and found no change in binding in any of the regions examined. Using the same steroid treatment protocol Biegon and McEwen (1982) demonstrated that oestrogen resulted in an increased B_{max} of 5-HT_1 receptors in the MPOA and hypothalamus but not the cortex. This suggests the increased binding observed by Biegon *et al* (1982) was the result of an increase in receptor number, the time course involved in these experiments (48 hours) would allow for a genomic action of oestrogen (Sumner and Fink 1993, McQueen *et al* 1997). The study by Biegon and McEwen (1982) also showed an increased inhibition of 5-HT binding with increasing oestrogen doses, the maximal inhibition achieved was approximately 70% and this effect could also be attributed to a decrease in 5-HT_1 B_{max}. *In vivo* oestradiol treatment produces a biphasic effect on 5-HT_1 receptor density in the female rat. Initially there is an acute decrease in density (Biegon *et al* 1982) which is followed 48 hours later by a selective increase in density in brain regions known to contain oestrogen receptors (Biegon *et al* 1982).

In the hippocampus oestrogen treatment increases the efficacy of 5-HT_{1A} receptors to inhibit cAMP formation in isolated membrane fractions (Clarke and Maayani 1990). Changes in 5-HT_{1A} receptors have also been studied in animals treated with oestrogen and progesterone. These steroids slightly attenuated the effects of 8-OH DPAT in inhibiting lordosis (Uphouse *et al* 1994) and the degree of attenuation appears to be dependent on the dose of oestrogen (Jackson and Uphouse 1998). Another study has suggested neither oestrogen nor oestrogen + progesterone have any effect on 5-HT_{1A} receptor density (Frankfurt *et al* 1994). However, this latter study did suggest that the two steroids in combination increased the density of 5-HT_{1B} receptors in the VMN when compared to oestrogen alone. Steroid administration in the experiment by Frankfurt *et al* (1994) was, like that in Uphouse *et al* (1994), only for two days except the rats had been treated the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) at the time of OVX to remove presynaptic receptors. The differing results between this study (Frankfurt *et al* 1994) and the two previous studies by Biegon (Biegon *et al* 1982, Biegon and McEwen 1982) illustrate the effect produced by denervation of the raphe nuclei. The effect of chronic steroid treatment with

oestrogen, progesterone or a combination of both steroids on 5-HT_1 receptors has been examined in the cortex. Decreases in B_{max} were observed with oestrogen (2 weeks), progesterone (2 weeks) and the steroid combination but affinity was not affected (Biegon *et al* 1983). The results from this experiment agree with the previous findings on 5-HT_1 receptors after acute (48 hour) steroid treatment (Biegon *et al* 1982, Biegon and McEwen 1982). Studies have not only examined the binding sites for 5-HT_1 receptors but their mRNA expression after steroid treatment. Studies examining mRNA have looked at both acute (2, 24 and 30 hours) and chronic (3 weeks) oestrogen treatment. Two hours after oestrogen 5-HT_{1A} receptor mRNA levels were decreased in the medial amygdala, piriform cortex and after 24 hours were still decreased in these two areas and also in the perirhinal cortex (Osterlund and Hurd 1998). No effect was found on 5-HT_{1A} receptor mRNA in the hippocampus after oestrogen treatment in this or another acute study (30 hours; Sumner and Fink 1993). The study by Sumner and Fink (1993) found no changes in 5-HT_{1A} receptor mRNA in any region examined, which included frontal and cingulate cortex, caudate, MPOA, arcuate nucleus, VMN and DR. Given chronically oestrogen was found only to increase 5-HT_{1A} receptor mRNA in the DR with increases in mRNA in the hypothalamus failing to reach statistical significance (Thomas *et al* 1997). In summary, oestrogen and progesterone decrease the B_{max} of 5-HT_{1A} receptors after both acute and chronic steroid treatment. Acutely these steroids appear to decrease mRNA expression in discrete brain regions while chronic treatment increases 5-HT_{1A} mRNA expression in the DR.

The effects of oestrogen upon 5-$HT_{2A/2C}$ receptors have also been studied. Acute oestrogen (30 hours) has been shown to produce no changes in 5-HT_{2C} mRNA (termed 5-HT_{1C} in the study) in any of the regions examined (Sumner and Fink 1993). However, this study found differences in the expression of 5-HT_{2A} receptor mRNA in discrete brain regions. In the DR oestrogen produced an increase in the percentage of labelled cells and in the amount of mRNA in cells. In the medial septum and DBB the amount of 5-HT_{2A} receptor mRNA decreased and in the MPOA there was a decrease in the percentage of labelled cells. Other studies have examined the effects of steroids on 5-HT_{2A} receptor binding site density. Biegon *et al* (1983) reported an increase in B_{max} of 5-HT_2 receptors in the cortex after oestrogen (2 weeks) although the combination of oestrogen plus progesterone (both given for 2 weeks) produced a return to control B_{max} values. Further studies in rats with other radioligands (ketanserin and RP 62203) have shown increases in 5-HT_{2A} receptor binding in a variety of forebrain regions and in the DR after acute oestrogen

treatment (30 hours; Sumner and Fink 1995, Fink and Sumner 1996). The forebrain regions where increases were observed were the cingulate and frontal cortex, piriform cortex, olfactory tubercle and nucleus accumbens, other forebrain regions showed no change. After chronic treatment with oestrogen (2 weeks) similar changes in 5-HT_{2A} receptors were observed in the frontal cortex as had been shown with acute steroid treatment (Cyr *et al* 1998). In this study the increase in 5-HT_{2A} receptor binding with chronic oestrogen was shown to be due to an increase in the B_{max} of 5-HT_{2A} receptors with no change in affinity. The expression of mRNA was also examined and an increase, relative to vehicle, was observed in the cortex after two weeks in Fisher rats (Cyr *et al* 1998). This increase was strain dependant since no increases were seen in either 5-HT_{2A} binding site density or mRNA in Sprague-Dawley rats after two weeks of oestrogen but increases in both were observed in this strain after 3 months of steroid treatment (Cyr *et al* 1998). The effect of oestrogen on 5-HT_{2A} receptors in the cortex has been linked with depression because the areas where 5-HT_{2A} receptor binding is increased are those involved in mood and mental state (reviewed by Price 1999).

Non-genomic actions of oestrogen could also explain the changes in receptor and transporter binding sites. A possible mechanism is by causing an increase in the microviscosity of the membrane leading to an increase in the exposure of receptors, an increase in membrane viscosity after oestrogen treatment has been observed previously (Biegon *et al* 1982). This explanation could be true for the changes in the B_{max} of the 5-HT_1 receptors on pro-oestrus and oestrus. As for the transporter, the increase in 5-HT uptake on pro-oestrus could be explained by decreased internalisation of the transporter or by a change in the affinity of the transporter for 5-HT possibly due to phosphorylation. There are still many unanswered questions regarding the interactions of the sex steroids and 5-HT and how these affect women both during their reproductive life and after it. The latter is becoming more important as the percentage of our population which is postmenopausal increases.

The aims of this project

The aim of this project was two-fold. The first aim was to extend the work conducted within the department and in the literature on the 5-HT_{2A} receptor and 5-HT transporter in female rats by examining the effects of acute progesterone treatment on serotonergic neurotransmission in the presence or absence of oestrogen. For the 5-HT_{2A} receptor ligand binding experiments it was desirable to use a more specific 5-HT_{2A} antagonist than ketanserin since these were now available. At the time of this study tritiated RP 62203 was no longer available and had been replaced with MDL 100,907. Part of the project therefore involved evaluating MDL 100,907 (since renamed M100907) as a ligand for quantitative autoradiography. The second aim of this project was to examine the firing characteristics of putative 5-HT neurones in the dorsal raphe nucleus of female rats and to see if these properties changed after acute treatment with oestrogen. Standard firing characteristics such as firing rate and the proportion of cells which fired doublets were recorded. In addition to this all cells recorded from were antidromically activated from the medial forebrain bundle and antidromic latencies recorded. Part of this study involved stimulating the recurrent inhibitory pathway (Wang and Aghajanian 1978) and characterising whether 5-HT_{1A} receptors were involved in this response. The binding experiments were conducted in the MRC Brain Metabolism Unit and the electrophysiology experiments were conducted in Laboratory of Neuroendocrinology, Physiology Department.

Chapter 2

Methods

2.1 BIOCHEMICALS

COMPOUND	SOURCE
Receptor agonists/antagonists:	
Ketanserin tartrate Prazosin HCl WAY 100,635: (N-tert-butyl-3-(4-[2-methoxyphenyl]piperazin-1-yl)-2-phenylpropanamide)	purchased from Research Biochemicals Incorporated, One Stratmore Road, Natick, MA 01760. USA.
RP 62203: (2-[3-(4-(fluorophenyl)-piperazinyl)propyl]naphto[1,8-cd]isothiazole-1,1-dioxide)	gifted from Rhone-Poulenc Rorer, Centre de Recherche, de Vitry-Alfortville, Vitry sur Seine, France.
8-OH DPAT: (8-hydroxy-2-(di-*n*-propylamino)tetralin) NAN-190: (1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine)	purchased from Sigma-Aldrich Co. Ltd., Dorset, BH12 4QH.
Citalopram	gifted by H Lundbeck, Copenhagen, Denmark.
Antibodies:	
anti-rLH-S-10 and anti-rPRL-S-9	were raised in rabbits against luteinising hormone (LH) and prolactin (PRL) respectively by the National Hormone and Pituitary Program of NIDDK, Baltimore, Maryland, USA.
rLH-RP-2, rLH-I-9, rPRL-RP-3 and rPRL-I-6 are purified hormones. rLH-I-9 and rPRL-I-6 are highly purified (>90% hormone). rLH-RP-2 and rPRL-RP-3 are less pure hormone preparations (10-40% hormone).	obtained from the National Hormone and Pituitary Program of NIDDK, Baltimore, Maryland, USA.
Donkey anti-rabbit immunoglobulin G (IgG) non-immune rabbit serum	obtained from the Scottish antibody production unit, Lanarkshire, Scotland, ML8 5ES.

Steroids:	
Oestradiol benzoate (Benztrone) Progesterone (Gestrone)	purchased from Paines & Bryne Ltd., West Byfleet, Surrey, UK.
Radiochemicals:	
[^{3}H]ketanserin [^{3}H]paroxetine	purchased from NEN Life Science Products, Boston, MA, USA.
[^{3}H]MDL 100,907: (R-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol) ^{125}I (IMS30)	purchased from Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA.
^{3}H microscales (2.68-1236 Bq/mg tissue equivalent) ^{3}H Hyperfilm	purchased from Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA.
Miscellaneous drugs:	
Carprofen (Zenecarp) - analgesic	purchased from C-Vet, Veterinary Products, Division of Grampian Pharmaceuticals Ltd, UK.
Halothane (Fluothane)	purchased from ICI, Macclesfield, Cheshire, UK.
Heparin	purchased from CP Pharmaceuticals Ltd, Wrexham, UK.
Sodium pentobarbitone (Sagatal)	purchased from Rhone Merieux, Spring Green Centre, Harlow, Essex, UK.
Urethane	purchased from Sigma-Aldrich Co. Ltd., Dorset, BH12 4QH.
Chemicals and dyes:	
Boric acid di-sodium hydrogen EDTA: (di-sodium ethylene diamine tetra acetic acid) sodium bicarbonate sodium di-hydrogen sodium metabisulphite	purchased from Merck Ltd, Dorset, UK. BH15 1TD.

Chemicals and dyes (continued):	
Bovine albumin chloramine T: (N-chloro-p-toluenesulfonamide sodium salt) N,N- dimethylformamide diethyl pyrocarbonate gelatine ovalbumin poly-L-lysine (564,000 polymer) sodium azide	purchased from Sigma-Aldrich Co. Ltd., Dorset, BH12 4QH.
Chromic potassium sulphate cresyl fast violet neutral red pontamine sky blue potassium dichromate	purchased from BDH, Poole, Dorset, UK.
Acetone, paraformaldehyde, potassium chloride, sucrose, sodium chloride, sodium hydroxide Tris: (tris(hydroxymethyl)methylamine) xylene	purchased from Fisons, Loughborough, UK.
Methanol	purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland.
Miscellaneous:	
Arachis oil	purchased from Mackenzie & Co.
Hypam fixer Phenisol	purchased from Ilford Ltd., Mobberley, Knutsford, Cheshire, UK.
Sephadex G50 columns	purchased from Pharmacia LKB Biotechnology, Milton Keynes, MK5 8PH.
Slides - 76 x 26 mm, thickness 1.0/1.2 mm, ground edges	purchased from Raymond A Lamb, 6 Sunbeam Road, London, NW10 6JL.
Whatman No.1 filters (27 cm diameter)	purchased from Whatman Ltd, England.

Stock solutions:

A 1 mg/ml stock solution of prazosin HCl was produced by dissolving 10 mg prazosin in 1ml of methanol, the resulting solution was dissolved in 9 mls of ultra high pure (UHP) water, aliquoted and stored at -40°C until required.

Ketanserin tartrate was dissolved in UHP water to produce a 1 mg/ml solution which was aliquoted and stored at -40°C until required.

Citalopram was dissolved in 0.05M Tris buffer pH 7.7 (see 2.3.4) to produce a 4 mM solution which was stored at 4°C for up to 6 months.

RP 62203 was dissolved in dimethylformamide to produce a 10^{-2}M solution. This solution was diluted with UHP water to a concentration of 10^{-4}M and this was aliquoted and stored at -40°C until use.

Poly-L-lysine coated slides:

The solutions used were made up as follows:

Chromic acid was produced by dissolving 100 g potassium dichromate in 850 ml distilled water then adding 100 ml concentrated sulphuric acid.

DEP water was produced by adding 320 µl diethyl pyrocarbonate (DEP) to 500 ml UHP water. The resulting solution was shaken and allowed to settle (4-24 hours) before being autoclaved.

The gelatine subbing solution was made by dissolving 15 mg sodium azide + 0.75 g gelatine in 30 ml DEP water. This was then made up to 500 ml with DEP water and the resulting solution was filtered through a Whatman No.1 filter.

The poly-L-lysine solutions was produced by dissolving 100 mg poly-L-lysine in 500 ml DEP water.

The process began with soaking slides in chromic acid overnight. The next day, slides were rinsed in tap water to remove the acid, were placed in racks and left in water overnight. The following day, slides were rinsed twice in DEP water for 15 minutes, soaked in absolute ethanol for 15 minutes and then placed in an oven at 55°C overnight. The next day, slides were subbed in gelatine solution for 5 minutes and returned to the oven at 55°C overnight. On the final day, slides were rinsed twice in poly-L-lysine solution for 10 seconds. The slides were then returned to the oven set at 55°C to dry for 4-24 hours before being boxed. Coated slides had a shelf life of 3-6 months.

2.2 ANIMALS

Female COB Wistar rats were obtained from Charles River UK Ltd or were derived from a colony bred within the department from stock originally obtained from Charles River. The female rats were between 6-10 weeks old and weighed between 160 and 300 g. They were maintained under controlled lighting (lights on from 05.00 to 19.00h) and temperature (22 ± 1°C) with free access to food pellets (SDS RM1C, Special Diet Services, Witham, Essex, UK) and tap water.

Rats were assessed for a minimum of two 4-day oestrous cycles (as determined by vaginal lavage). The cytological characteristics of the vaginal smears from a female rat with a 4-day oestrous cycle were as follows;

Metoestrus: large number of polymorphonuclear (PMN) leucocytes and epithelial cells with possibly some cornified epithelial cells.

Dioestrus: either a mixture of PMN leucocytes, epithelial cells and cornified epithelial cells or all PMN leucocytes.

Pro-oestrus: mainly epithelial cells with some cornified epithelial cells.

Oestrus: predominantly cornified epithelial cells

2.3 BINDING EXPERIMENTS

2.3.1 General: steroid treatment and tissue preparation

In order to control the levels of sex steroids, female rats were bilaterally ovariectomised and then treated with sex steroids or vehicle. Bilateral ovariectomies were carried out under halothane anaesthesia (2% halothane, 2 litres min^{-1} oxygen) between 09.00-10.00h on the morning of dioestrus, after animals had shown at least two consecutive 4-day oestrous cycles. The animal was placed on its side and a patch of hair (approximately 2 cm x 2 cm) was shaved with the centre of the area being located 3 cm rostral to the tip of the pelvis. A small incision (approximately 1 cm long) was made in the skin in the centre of this area. A small hole was then made in the muscle layer below allowing the ovary to be removed from the abdomen. The

uterine horn below the ovary was ligated and the ovary was then excised. The uterine horn was replaced into the abdomen and the incision in the muscle wall was sutured. The incision in the skin was then sutured together and the whole process was repeated on the other side in order to remove the second ovary. Immediately after ovariectomy, rats were injected subcutaneously (s.c.) with either oestradiol benzoate (OB) (30 μg in 0.2 ml arachis oil) or vehicle (0.2 ml arachis oil) and an analgesic (Carprofen, 5 mg kg^{-1} s.c.). The next day, animals were injected s.c. at 12.00h with either progesterone (2.5mg in 0.2 ml arachis oil) or vehicle. Between 16.30h and 17.30h, the time of the LH surge, animals were anaesthetised with sodium pentobarbitone (30 mg kg^{-1} intraperitoneally [i.p.]), decapitated, and their brains were removed and flash frozen for 5 minutes in isopentane cooled to -35°C by dry ice/ethanol. Trunk blood was collected from the animals for radioimmunoassay of plasma luteinising hormone (LH) concentration and prolactin (PRL) concentration (see 2.5 and 2.6). The results from these assays are shown in chapter 4 figure 4.5. The uterine horns were dissected out and weighed since oestrogen treatment has been shown to increase uterine weights (Aiyer and Fink 1974). The results from the examination of the uterine horns is shown in chapter 4 figure 4.5. Brains were stored at -70°C until sectioning in a cryostat (Bright 5030 rotary retracting microtome, Bright Instrument Co. Ltd) at -16°C. Serial 15 μm coronal sections were thaw mounted on acid cleaned, gelatine/poly-L-lysine subbed slides and stored at -70°C until required. The sections were taken at the following levels (Paxinos and Watson 1986): area 1 (nucleus accumbens) bregma +1.60 to +0.70 mm; area 2 (lateral septum) bregma +0.48 to -0.26 mm; area 3 (preoptic area) bregma -0.30 to -0.80 mm; area 4 (hypothalamus and amygdala) bregma -2.12 to -2.80 mm; area 5 (midbrain raphe) -7.30 to -7.80 mm; area 6 (locus coeruleus) bregma -9.30 to -9.80 mm.

2.3.2 Quantitative autoradiography using [^{3}H]ketanserin binding to detect 5-HT_2 receptor binding sites.

[method based on Pazos *et al* 1985]

Ketanserin was the first selective 5-HT_2 receptor antagonist. However, ketanserin only shows 5 fold higher affinity for 5-HT_2 receptors over α_1-adrenoceptors and H_1-histamine receptors (Leysen *et al* 1978). In the cortex there is little binding to H_1-histamine receptors (Leysen *et al* 1982). Therefore, in order to use [^{3}H]ketanserin to detect 5-HT_2 receptors, only the α_1-adrenoceptor binding component of ketanserin requires to be blocked. This is commonly achieved by using prazosin (Pazos *et al*

1985), which binds to α_1-adrenoceptors (Ireland *et al* 1997) and to α_{2B} and α_{2C}-adrenoceptors (Marjamaki *et al* 1993).

Sections from areas 1 to 5 were used and slides from one animal per treatment group (n=6 per treatment group) were processed together. Slides were allowed to thaw at room temperature for 30 minutes. The slides were placed in racks and the sections were fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 5 minutes. Slides were then washed twice in PBS at room temperature for 5 minutes followed by pre-incubation in 0.17M Tris HCl pH 7.7 at room temperature for 15 minutes. The edges of each slide were dried and the slide was then placed in an incubation box. Each section was overlaid with 50µl of incubation buffer which contained the tritiated ligand dissolved in 0.17M Tris HCl pH 7.7. The incubation buffer for total binding contained 2 nM [^{3}H]ketanserin (specific activity 2.46 TBq mmol^{-1}) and 0.1 mM prazosin to block binding to the α_1-adrenoceptor (Pazos *et al* 1985). The incubation buffer for non-specific binding contained 2 nM [^{3}H]ketanserin, 0.1 mM prazosin and 1 µM RP 62203. Alternate slides were used to determine non-specific binding. The sponges in the base of the incubation boxes had been moistened with 0.17M Tris HCl pH 7.7 to prevent the slides from drying out. Slides were incubated at room temperature in the dark for two hours as ketanserin and RP 62203 are light sensitive. At the end of the incubation period, slides were removed from the incubation boxes and the excess buffer was drained off. Slides were placed in racks and washed twice in ice-cold 0.17M Tris HCl pH 7.7 for 10 minutes followed by a 10 second dip in ice-cold UHP water. Slides were drained and dried in a vacuum desiccator for 2-3 hours. The following day, slides were again dried in a vacuum desiccator for 2-3 hours, then mounted in x-ray cassette boxes and apposed to ^{3}H Hyperfilm. Each cassette also contained a set of tritium standards (^{3}H microscales range 2.68-1236 Bq/mg tissue equivalent). The tritium microscale standards had been calibrated by Amersham from tritiated standards made from brain paste of telencephalon (Davenport *et al* 1988). Cassettes were wrapped in black bags and stored at 4°C for 5 weeks before developing the autoradiographs.

2.3.3 Quantitative autoradiography using [^{3}H]MDL 100,907 binding to detect 5-HT$_{2A}$ receptor binding sites.

[method development is discussed in chapter 3]

Since the discovery of ketanserin as a selective ligand at the 5-HT$_2$ receptor (Leysen *et al* 1978), a ligand more selective for the 5-HT$_{2A}$ receptor has been needed. A recent addition to the selective 5-HT$_{2A}$ antagonists is MDL 100,907. This ligand is reportedly at least 100 times more selective for 5-HT$_{2A}$ receptors than 5-HT$_{2C}$ and α_1-adrenoceptors (Kehne *et al* 1996). The selectivity of MDL 100,907 for 5-HT$_{2A}$ receptors has been confirmed in rat cortical homogenates (Johnson *et al* 1996).

The development of the methodology for the [^{3}H]MDL 100,907 binding experiments is explained in detail in chapter 3. The method used for [^{3}H]MDL 100,907 binding was similar to that used for [^{3}H]ketanserin binding. The differences between the two protocols are highlighted below.

For these binding experiments sections from areas 1 to 5 were used and the Tris buffer was 0.17M Tris HCl pH 7.4. Slides were racked and washed twice in PBS at room temperature for 5 minutes. There was no fixation step prior to the PBS washes. The PBS washes were followed by pre-incubation in Tris buffer at room temperature for 15 minutes. The incubation buffer for total binding contained 0.75 nM [^{3}H]MDL 100,907 (specific activity 3.0 TBq mmol^{-1}) and for non-specific binding 0.75 nM [^{3}H]MDL 100,907 and 1 μM RP 62203. Slides were incubated at room temperature in the dark for one hour as RP 62203 is light sensitive. Slides were placed in cassettes containing a set of tritium standards (^{3}H microscales range 2.68-1236 Bq/mg tissue equivalent) and stored at 4°C for 4 weeks before developing the autoradiographs.

2.3.4 Quantitative autoradiography using [^{3}H]paroxetine binding to detect 5-HT transporter binding sites.

[based on the method of De Souza and Kuyatt 1987 as described by Battaglia *et al* 1991]

Sections from areas 1, 2 and 4 to 6 were used and slides from one animal per treatment group (n=6 per treatment group) were processed together. Slides were allowed to thaw at room temperature for 30 minutes then slides were placed in racks and incubated in troughs at room temperature for 3 hours. Troughs for total binding

contained approximately 250 pM [^{3}H]paroxetine (mean 252.4 pM, range 247.6-259 pM, specific activity 632.7 GBq mM^{-1}) in 0.05M Tris buffer containing 120 mM NaCl and 5 mM KCl (pH 7.7). Troughs for non-specific binding also contained 4 μM citalopram. Alternate slides were used to determine non-specific binding. After incubation, slides were washed twice for 30 minutes in 0.05M Tris buffer pH 7.7 at room temperature followed by a 10 second dip in ice-cold UHP water. Slides were drained and dried in a vacuum desiccator for 2-3 hours. The following day, slides were again dried in a vacuum desiccator for 2-3 hours, then mounted in x-ray cassette boxes and apposed to ^{3}H Hyperfilm. Each cassette also contained a set of tritium standards (^{3}H microscales 2.68-1236 Bq/mg tissue equivalent). Cassettes were wrapped in black bags and stored at -40°C for 8 weeks before developing the autoradiographs.

2.3.5 Autoradiograph development and analysis

Films were processed one at a time. Films were removed from cassettes and placed in developer (20% aqueous solution of Phenisol in distilled water) at 20°C for 4 minutes. The films were then rinsed in distilled water and placed in fixer (20% aqueous solution of Hypam fixer in distilled water) at room temperature for 10 minutes. Films were rinsed in running tap water for 30 minutes then air dried. Neuroanatomical regions were identified using the atlas of Paxinos and Watson (1986). For each brain, optical density measurements were made from 6-8 sections (total binding) and 3-4 sections (non-specific binding) using a Seescan image analysis system (Seescan plc, Poseidon House, Castle Park, Cambridge, UK). The optical density was measured over an area between 0.05-1 mm^2 depending on the size of the structure examined. The [^{3}H]microscale standards (10.88-1236 Bq/mg tissue equivalent) were used to produce a standard curve allowing optical density readings for each region to be expressed as Bq/mg. At this stage, specific binding was determined by subtracting non-specific binding from total binding. Specific binding values were then converted to fmol/mg tissue using the specific activity of the tritiated ligand.

2.4 *IN VIVO* ELECTROPHYSIOLOGY

2.4.1 Animals and surgery

Adult, female COB Wistar rats (aged 6-9 weeks and weighing 160-270 g, mean 215 g) were bilaterally ovariectomised under general anaesthesia (as detailed in 2.3.1) on the morning of dioestrus between 09.00-10.00h and immediately injected s.c. with either 30 μg oestradiol benzoate (OB-treated group, n=34: Paines and Bryne, West Byfleet, Surrey, UK) in 0.2 ml arachis oil or 0.2 ml arachis oil alone (oil control group, n=40). All animals also received an analgesic (Carprofen, 5 mg kg^{-1} s.c.). The next day, animals were deeply anaesthetised with urethane (1.1 g kg^{-1} i.p. in distilled water) and the right jugular vein was cannulated to allow drug administration and blood sampling. A 2 mm wide craniotomy was carried out on the right hemisphere of the skull adjacent to the midline (-4.5 to -7.7 mm A-P; Paxinos and Watson 1986). The rat was mounted in a stereotaxic frame (Narishige, Tokyo, Japan) with the incisor bar set ~4 mm below the interaural line and the skull levelled between lambda and bregma. The experiments and subsequent data analysis were carried out blind.

2.4.2 Recording Techniques

Recording Electrodes

A bipolar stimulating electrode (Clark Electromedical Instruments, Pangbourne, UK) was placed in the medial forebrain bundle (MFB) (-4.8 mm from bregma, 0.9 mm lateral to the midline of the skull, 8.5 mm ventral to the dural surface [Paxinos and Watson 1986]). Single barrelled glass microelectrodes were made from borosilicate glass capillaries with an inner filament (120F-10, Clark Electromedical Instruments) drawn out using a vertical electrode puller (Narishige). Electrodes were broken back to obtain an external tip diameter of approximately 2 μm and were filled with a solution of 0.9% NaCl with 2% pontamine sky blue (PSB). The microelectrode (resistance 3-10 MΩ) was implanted above the DR at an angle of 15° to the vertical (-7.5 mm from bregma, 1.5 mm lateral to the midline of the skull, 5.0 mm ventral to the dural surface [Paxinos and Watson 1986]) after which it was lowered into the DR by means of a micromanipulator to a maximum depth of 6 mm below the dural surface.

Extracellular Recordings of DR Neurones

Dorsal raphe cells were identified as putative 5-HT neurones on the basis of their firing characteristics as described by Aghajanian *et al* (1978) and by antidromic stimulation of the MFB (Hajos and Sharp 1995). When an antidromically-activated cell was encountered, the time, depth and latency of the antidromic spike, and the threshold voltage required to initiate the antidromic spike were recorded. Baseline firing frequency was recorded for 10 minutes after which time, in most experiments, a sub-threshold stimulus was applied to the MFB for 10 minutes to examine the effects of activating the recurrent inhibitory pathway (Wang and Aghajanian 1978). In order to further examine the mechanism involved in producing the inhibition of firing observed after sub-threshold stimulation, the stimulation was repeated after a 10 minute interval. Initially, a second stimulation was applied to 5 cells from each treatment group to examine any changes in the properties of the inhibition. Subsequently, in a small number of cells in which inhibition was observed, stimulation was repeated after a 10 minute interval with a 5-HT_{1A} antagonist administered 100 seconds prior to the second stimulation. The purpose of this experiment was to determine whether 5-HT_{1A} receptors were involved in the inhibition produced by sub-threshold stimulation of the MFB. The 5-HT_{1A} partial agonist NAN-190 (Hodgkiss *et al* 1992) (5 μg kg^{-1} intravenously [i.v.] in 0.1 ml saline) was tested on 3 cells in the oil control group and 5 cells in the OB-treated group. The 5-HT_{1A} antagonist WAY 100,635 (Fletcher *et al* 1996), (1 mg kg^{-1} i.v. in 0.1 ml saline) was tested on 6 cells in the oil control group and 4 cells in the OB-treated group.

Amplification and Filtering

The output signals over 300 Hz were filtered and amplified 100 fold by the amplifier (model 1800, A-M Systems). These signals were then relayed from a spike processor (Digitimer D.130, Digitimer, England) to a digital to analogue converter (CED 1401, Cambridge Electronic Design) and displayed on-line using Spike2 software (Cambridge Electronic Design).

Data Display and Storage

Digitised data were captured and simultaneously viewed using Spike2 software on the PC. Digitised records were stored on the hard disk of the PC for off-line analysis using Spike2 software.

2.4.3 Recording and stimulating electrode localisation

At the end of each experiment, the position of the recording electrode tip was marked by iontophoretic ejection of PSB dye and the position of the stimulating electrode was marked by an electrolytic lesion. Rats were then perfused with 30 ml heparinised saline (50 units ml^{-1}) followed by 100 ml 4% buffered paraformaldehyde solution. The brain was removed and stored in 4% buffered paraformaldehyde at 4°C for at least 48 hours. After this time it was transferred to a solution containing 2% paraformaldehyde and 15% sucrose at 4°C for at least 12 hours before being sectioned. The brains were either sectioned on a freezing microtome (Bright Instrument Co. Ltd, Huntingdon, England) or were flash frozen and cut at -16°C using a cryostat (Bright 5030 rotary retracting microtome, Bright Instrument Co. Ltd). Twenty micron coronal sections were mounted on gelatine/chrome alum coated slides and examined under a microscope to localise the electrode position. Sections from early experiments were counterstained with 0.15% cresyl fast violet. Sections from later experiments were counterstained with 4% neutral red, as this provided a better contrast with the PSB. Only those neurones confirmed to be within the DR were included in this study.

2.4.4 Data Analysis

Criteria for Cell Type Classification

Cells were initially classified as either spontaneously active or silent, the latter being described as a cell which fired fewer than one action potential in two minutes. As described by Hajos *et al* (1995b), the spontaneously active cells in both groups could be separated into two subtypes on the basis of their action potential firing pattern. To determine whether the cells fired doublets, the control period (120 s to 600 s) was analysed using the burst analysis programme in Spike2. The instantaneous frequency between doublets was found to be approximately 200 Hz. This information along with information in Hajos *et al* (1995b), was used to set the parameters in the burst

analysis programme. A cell was considered to be doublet firing if >5% of spikes were contained in doublets.

Firing Rate

The firing rate was calculated by importing the Spike2 files into Excel (version 5.0, Microsoft, USA) and counting the number of action potentials within the control period (120 to 600 seconds). In this way, both the basal firing rate, where only the first action potential of a doublet was counted, and the total firing rate of cells could be calculated.

Subthreshold Stimulation of the MFB

Using the histogram analysis programme in Spike2, a post-stimulus time histogram (PSTH) was constructed. Subthreshold stimulation of the MFB either had no effect on the firing of the cell or produced inhibition or stimulation of firing. Both the latency and duration of these effects were measured. The onset of inhibition was defined as a total cessation of firing for at least 10 ms. The offset of inhibition was defined as the first bin equal to or above the prestimulus baseline level. Stimulation was defined as an increase of 25% or more in the bin height compared to baseline levels.

2.4.5 Verification of steroid treatment

The uterine horns were dissected out and weighed after the animal had been perfused. Oestrogen treatment is known to increase uterine weights (Aiyer and Fink 1974) and this was confirmed as animals in the OB-treated group had significantly higher uterine weights than oil controls (444.6 ± 34.3 mg vs. 270.8 ± 16.4 mg; mean ± s.e.m. n=13 in both groups; $P<0.005$ Wilcoxon Rank Sum). In order to check oestradiol levels, a blood sample was taken from the jugular vein prior to perfusion, centrifuged at 13000 revs for 5 minutes (MSE Micro Centaur). The plasma was then removed, kept on ice (<1 hour) and stored at -40°C to be assayed at a later date. Plasma levels of oestradiol were determined by fluorescence polarisation immunoassay (Abbott Diagnostics, Maidenhead, Berkshire, UK) by the Department of Clinical Biochemistry, Royal Infirmary, Edinburgh. Animals in the OB-treated group had significantly higher plasma oestradiol levels than oil controls (178.5 ± 33.8 pg ml^{-1} vs. 62.3 ± 28.9 pg ml^{-1}, n=13 per treatment; $P<0.001$ Wilcoxon Rank Sum).

2.5 RAT LH RADIOIMMUNOASSAY

The rat LH and PRL radioimmunoassays detailed in 2.5 and 2.6 were carried out by John Bennie and Sheena Carroll, Radioimmunoassay Team, MRC Brain Metabolism Unit.

This assay was carried out on plasma samples obtained from the animals used in the binding experiments. The samples of plasma were stored at -40°C prior to being assayed. All preparations of standards, iodinated antigen, antiserum, donkey anti-rabbit IgG double antibody and knowns were carried out on ice, as was the setting up of the assay and all subsequent additions to the assay.

2.5.1 Iodination of the antigen

The antigen, rLH-I-9 (>90% pure LH), was dissolved in PBS to give a concentration of 2.5 μg in 10 μl. The dissolved antigen was aliquoted (10 μl) and stored at -40°C until use. For each iodination, one 10 μl aliquot was defrosted and 10 μl 0.5M sodium phosphate pH 7.5 followed by 5 μl ^{125}I (18.5 MBq) and 10 μl chloramine T (5 mg in 2 ml PBS) was added. This solution was mixed gently for 60 seconds then 10 μl sodium metabisulphite (5 mg in 2 ml PBS) was added. This was mixed very gently and transferred to a 1 x 15 cm glass column containing Sephadex G50 (fine) in PBS. The column had been previously coated with 1.5 ml ovalbumin (5% in PBS) to prevent the iodinated antigen binding to the column. The eppendorf was washed with 1 ml PBS and transferred to the column. The column was eluted with PBS and twenty four 1 ml fractions were collected. Tubes 4-12 contained 0.5 ml 5% ovalbumin, hence only 0.5 ml fractions were taken from the column. All the tubes were counted in a desensitised gamma counter (EG&G Wallac, 20 Vincent Avenue, Crownhill Business Centre, Crownhill, Milton Keynes, UK). The iodinated antigen from the post peak tube of the iodination purification procedure was used.

2.5.2 Assay procedure

The standards were produced by dissolving rLH-RP-2 (10-40% pure LH) in PBS/1% bovine albumin. Standard values ranged from 0.1 ng ml^{-1} to 5.0 ng ml^{-1}. Aliquots of standards (200 μl) were stored at -40°C until required. For the assay, three aliquots

of each standard were defrosted and 200 µl of PBS/1% bovine albumin added. Zero standards of 400 µl PBS/1% bovine albumin were prepared in triplicate. To each standard tube, 200 µl of antiserum was added (the antiserum [anti-rLH-S-10 raised in rabbits] had been stored at -40°C and had been diluted to $^{1}/_{450}$ using PBS/0.5% non-immune rabbit serum). For the assay, the antiserum was further diluted to $^{1}/_{72000}$ using PBS/0.05M EDTA/0.5% non-immune rabbit serum. Non-specific binding tubes were prepared in triplicate and contained 400 µl PBS/1% bovine albumin and 200 µl PBS/0.05M EDTA/0.5% non-immune rabbit serum at the $^{1}/_{450}$ dilution. To assess drift throughout the assay, two sets of known LH concentrations were included. These knowns had a concentration of approximately 2 ng ml^{-1} and 7 ng ml^{-1}; 50 µl aliquots of these samples were stored at -40°C until required. For the assay, two aliquots of each known were defrosted and 350 µl PBS/1% bovine albumin followed by 200 µl of antiserum was added (as described above). The unknowns were prepared from rat plasma samples. 25-100 µl samples were taken, the volume was dependant on the expected result. Unknowns were prepared in duplicate and were made up to 40 µl with PBS/1% bovine albumin, to which 200 µl of antiserum was added (as described above). All tubes (standards, non-specific, zero, knowns and unknowns) were then mixed carefully to prevent splashing and incubated overnight at 4°C.

The next day, the iodinated antigen (see 2.5.1 for details of preparation) was diluted with PBS/1% bovine albumin to give approximately 10000 cpm in 200 µl. To each tube, 200 µl of the diluted antigen was added; 200 µl was also added to 3 empty tubes to give total counts. All tubes were mixed carefully and incubated at 4°C. On day 4 of the assay, the double antibody (donkey anti-rabbit IgG) was diluted to $^{1}/_{20}$ with PBS/1% bovine albumin and 200 µl was added to each tube (except the total count tubes). The tubes were mixed again and incubated at 4°C. The next day, all the tubes (except the total counts) were centrifuged at 1500-2000g for 30 minutes. The supernatant was removed from these tubes and discarded. All tubes were counted in an automatic gamma counter.

2.6 RAT PROLACTIN RADIOIMMUNOASSAY

This assay was carried out on plasma samples obtained from the animals used in the binding experiments. The samples of plasma were stored at -40°C prior to being assayed. All preparations of standards, iodinated antigen, antiserum, donkey anti-

rabbit IgG double antibody and knowns were carried out on ice, as was the setting up of the assay and all subsequent additions to the assay.

2.6.1 Iodination of the antigen

The antigen, rPRL-I-6 (>90% pure PRL) was dissolved in 0.01M sodium bicarbonate to give a concentration of 5.0 μg in 10 μl. The dissolved antigen was aliquoted (20 μl and stored at -40°C until use. For each iodination one 20 μl aliquot was defrosted, to which 10 μl 0.5M sodium phosphate pH 7.5, followed by 5 μl ^{125}I (18.5 MBq) and 10 μl chloramine T (5 mg in 2 ml PBS) was added. This solution was mixed gently for 60 seconds then 10 μl sodium metabisulphite (5 mg in 2 ml PBS) was added. This was mixed very gently then transferred to a 1 x 25 cm glass column containing Sephadex G50 (fine) in PBS. The column had been previously coated with 1.5 ml ovalbumin (5% in PBS) to prevent the iodinated antigen binding to the column. The eppendorf was washed with 1 ml 0.01M borate buffer and this was transferred to the column. The column was eluted with 0.01M borate buffer and twenty four 1 ml fractions were collected. Tubes 4-12 contained 0.5 ml 5% ovalbumin, hence only 0.5 ml fractions were taken from the column. All the tubes were counted in a desensitised gamma counter. The iodinated antigen from the post peak tube of the iodination purification procedure was used.

2.6.2 Assay procedure

The standards were produced by dissolving rPRL-RP-3 (10-40% pure PRL) in PBS/1% bovine albumin. Standard values ranged from 0.125 ng ml^{-1} to 16.0 ng ml^{-1}. Aliquots of standards (200 μl) were stored at -40°C until required. For the assay, three aliquots of each standard were defrosted and 200 μl of PBS/1% bovine albumin added. Zero standards of 400 μl PBS/1% bovine albumin were prepared in triplicate. To each standard tube, 200 μl of antiserum was added (the antiserum [anti-rPRL-S-9 raised in rabbits] had been stored at -40°C and had been diluted to $^{1}/_{10}$ using PBS/0.5% non-immune rabbit serum). For the assay, the antiserum was further diluted to $^{1}/_{5000}$ using PBS/0.05M EDTA/0.5% non-immune rabbit serum. Non-specific binding tubes were prepared in triplicate and contained 400 μl PBS/1% bovine albumin and 200 μl PBS/0.05M EDTA/0.5% non-immune rabbit serum. To assess drift throughout the assay, two sets of known prolactin concentrations were included. These knowns had a concentration of approximately 5 ng ml^{-1} and 30 ng ml^{-1}; 20 μl aliquots of these samples were stored at -40°C until required. For the

assay, two aliquots of each known were defrosted and 0.38 ml PBS/1% bovine albumin, followed by 200 µl of antiserum, was added (as described above). The unknowns were prepared from rat plasma samples. Samples of between 2-20 µl were taken, the volume was dependant on the expected result. Unknowns were prepared in duplicate and were made up to 400 µl with PBS/1% bovine albumin to which 200 µl of antiserum was added (as described above). All tubes (standards, non-specific, zero, knowns and unknowns) were then mixed carefully to prevent splashing and incubated overnight at 4°C.

The next day, the iodinated antigen (see 2.6.1 for details of preparation) was diluted with PBS/1% bovine albumin to give approximately 10000 cpm in 200 µl. To each tube, 200 µl of the diluted antigen was added; 200 µl was also added to 3 empty tubes to give total counts. All tubes were mixed carefully and incubated at 4°C. On day 4 of the assay, the double antibody (donkey anti-rabbit IgG) was diluted to $^{1}/_{20}$ with PBS/1% bovine albumin and 200 µl was added to each tube (except the total count tubes). The tubes were mixed again and incubated at 4°C. The next day, the tubes (except the total counts) were centrifuged at 1500-2000g for 30 minutes. The supernatant was removed from these tubes and discarded. All tubes were counted in an automatic gamma counter.

2.7 STATISTICAL ANALYSIS

All data are presented as means ± s.e.m. unless otherwise stated. Comparisons between two treatment groups were made using non-parametric statistics (Wilcoxon Rank Sum test) using GB-STAT software (Dynamic Microsystems Ltd, Silver Spring, USA). Comparisons for multiple treatment groups (e.g. plasma LH and PRL levels, uterine weights and binding studies) were carried out using one-way analysis of variance followed by a *post-hoc* Tukey test. Fisher's exact test was used in chapter 5 to examine the frequency of short duration inhibitions between the control and treated groups.

Chapter 3

Changes in 5-HT_{2A} receptor density in female rats acutely treated with steroids as determined by quantitative autoradiography using [^{3}H]ketanserin and [^{3}H]MDL 100,907.

3.1 INTRODUCTION

It is now accepted that there are three subtypes of 5-HT_2 receptor. These are the 5-HT_{2A} receptor (formerly the 5-HT_2 receptor), the 5-HT_{2B} receptor (formerly known as the 5-HT_{2F} receptor) and the 5-HT_{2C} receptor (formerly known as the 5-HT_{1C} receptor) (Hoyer *et al* 1994, Hoyer and Martin 1997). The first ligand used to detect 5-HT_2 receptors was [^{3}H]spiperone in 1978 (Leysen *et al* 1978) however spiperone is an antagonist at the D_2 dopamine receptor and also possesses 5-HT_1 binding affinity (Urwyler and Coward 1987, Peroutka and Snyder 1981 respectively). The first selective 5-HT_2 receptor antagonist was ketanserin (Leysen *et al* 1982). Ketanserin was shown to be selective for the 5-HT_2 receptor subtype over 5-HT_1 receptors, however ketanserin only has a 5 fold higher affinity for 5-HT_2 receptors over α_1-adrenoceptors and H_1-histamine receptors (Leysen *et al* 1982). In the cortex there is little binding to H_1-histamine receptors (Leysen *et al* 1982) but in order to use [^{3}H]ketanserin to detect 5-HT_2 receptors the α_1-adrenoceptor binding component requires blocking with an α_1-antagonist e.g. prazosin (Pazos *et al* 1985). Ketanserin does show some selectivity between 5-HT_2 receptor subtypes with 25 fold selectivity for 5-HT_{2A} receptors over 5-HT_{2C} (Choudhary *et al* 1992). Despite the inherent problems regarding its lack of specificity ketanserin has proved extremely useful in discovering the location and function of 5-HT_{2A} receptors.

In 1992 the discovery of a more selective ligand for 5-HT_{2A} receptors was reported. RP 62203 is, like ketanserin, an antagonist at this receptor and was shown to have potent effects both *in vivo* and *in vitro* (Doble *et al* 1992). It was reported to have 10 fold selectivity over α_1-adrenoceptors, 100 fold over H_1-histamine receptors and 1000 fold over dopamine receptors (Doble *et al* 1992). It was also shown to bind to the same areas of the brain as ketanserin (Fajolles *et al* 1992). However, RP 62203 has since been shown to bind to 5-HT_{2C} receptors (Malgouris *et al* 1993) and to dopamine D_4 receptors in Chinese hamster ovary (CHO) cells (Heuillet *et al* 1996).

A recent addition to the selective 5-HT_{2A} antagonists is MDL 100,907. This ligand is reportedly at least 100 times more selective for 5-HT_{2A} receptors than 5-HT_{2C} and α_1-adrenoceptors and has more than 600 fold selectivity over D_4 receptors (Kehne *et al* 1996). The selectivity of MDL 100,907 for 5-HT_{2A} receptors has been confirmed in rat cortical homogenates (Johnson *et al* 1996) and more recently using autoradiography with rat brain sections (Lopez-Gimenez *et al* 1997). *In vitro* studies have also assessed the potential of MDL 100,907 as a 5-HT_{2A} antagonist both in cell

lines (Garcia and Kim 1997) and using *in vitro* electrophysiology (Marek and Aghajanian 1994). MDL 100,907 has been used *in vivo* to detect 5-HT_{2A} receptors in both behavioural studies (Gleason and Shannon 1997) and using the [^{11}C] form as a ligand for positron emission tomography (Lundkvist *et al* 1996). The evaluation of [^{3}H]MDL 100,907 as a ligand to detect 5-HT_{2A} receptors is described within this chapter and is based on the methodology described by Johnson *et al* (1996) as the data from Lopez-Gimenez *et al* (1997) was unpublished at the time these experiments commenced.

The rationale behind examining 5-HT_{2A} receptors is that changes in these receptors have been implicated in several CNS disorders. For example in the cortex 5-HT_{2A} receptor binding has been observed to increase in depression (Yates *et al* 1990) but decrease in Alzheimer's disease (Reynolds *et al* 1984, Hoyer *et al* 1986). Also, many of the antipsychotic drugs used in the treatment of schizophrenia have affinity for the 5-HT_{2A} receptor (Lieberman *et al* 1998). Differences have been observed between men and women with these conditions. For example, in schizophrenia differences can be observed in the peak age of onset which occurs between 15-20 years old in men and between 20-30 years old in women (McGlashan and Bardenstein 1990). Differences are also observed in depression with twice as many women as men suffering from depression (Parry 1995). Several studies have examined changes in 5-HT_{2A} receptors and the effects of sex steroids upon them. Using [^{3}H]ketanserin it has been reported that the binding site density of 5-HT_{2A} receptors in cycling female rats oscillates during the oestrous cycle (Sumner and Fink 1997). This study showed binding site density in the forebrain was higher in pro-oestrous females that dioestrous females and that binding site density in males was similar to dioestrous rats in the frontal, cingulate and piriform cortex but similar to that of pro-oestrous rats in the hypothalamic regions examined. In ovariectomised rats, acute oestrogen treatment (30 hours) has been shown to increase 5-HT_{2A} receptor mRNA in the DR (Sumner and Fink 1993). Acute oestrogen treatment has also been shown to increase 5-HT_{2A} receptor binding in discrete regions of the forebrain such as the cingulate cortex and nucleus accumbens using either [^{3}H]ketanserin or [^{3}H]RP 62203 (Sumner and Fink 1995, Fink and Sumner 1996 respectively). Chronic treatment with oestrogen has also been reported to increase 5-HT_{2A} receptors in the cortex using tissue homogenates (Biegon *et al* 1983) or quantitative autoradiography (Cyr *et al* 1998). In the study by Biegon *et al* (1983) rats were ovariectomised (OVX) then treated with oestrogen for two weeks. Cyr *et al* (1998) also treated rats with oestrogen for two weeks but did so either immediately after OVX or ten weeks after

OVX. The ligands used in these chronic oestrogen experiments to detect 5-HT_{2A} receptors were [^{3}H]spiroperidol (Biegon *et al* 1983) and [^{3}H]ketanserin (Cyr *et al* 1998). The effect of progesterone on 5-HT_{2A} receptor binding is less well studied. In rats chronically treated with progesterone for two weeks after OVX a slight increase in 5-HT_2 receptor (since reclassified as 5-HT_{2A} receptors) binding was found although when oestrogen and progesterone were given together for two weeks the oestrogen dependent increase in 5-HT_2 binding was attenuated (Biegon *et al* 1983).

The aim of these experiments was three fold. Firstly, they were to determine the optimal experimental conditions for using [^{3}H]MDL 100,907 as an autoradiography ligand to detect 5-HT_{2A} receptors in female rat brain. Secondly, the experiments were designed to compare the binding patterns of two 5-HT_{2A} ligands, namely [^{3}H]ketanserin and [^{3}H]MDL 100,907. For this part of the study experiments would be carried out on adjacent sections from the same rat brains. This second aim would determine whether MDL 100,907 was an effective substitute for ketanserin to detect 5-HT_{2A} receptors using quantitative autoradiography. Thirdly, the experiments were to determine the effect of acute oestrogen, progesterone and the combination of oestrogen and progesterone on 5-HT_{2A} binding in discrete regions of female rat brain using quantitative autoradiography

3.2 METHODS

The methodology used to determine the optimal conditions for using [^{3}H]MDL 100,907 as an autoradiography ligand to detect 5-HT_{2A} receptors in female rat brain was based on Johnson *et al* (1996) and is described in 3.2.1 below. Slides with sections from two rats were processed together in each of the four experiments.

The methodology used to examine the effects of acute treatment with oestrogen and progesterone on 5-HT_{2A} receptor binding site density using either [^{3}H]ketanserin or [^{3}H]MDL 100,907 is described in chapter 2 sections 2.3.2, 2.3.3 and 2.3.5.

3.2.1 [^{3}H]MDL 100,907: methodology development experiments.

The brains from six cycling adult female COB Wistar rats were obtained at the time of the LH surge on pro-oestrus as previously described in chapter 2. Serial 15 μm

coronal sections containing frontal and cingulate cortex were taken between bregma +1.60 and +1.00 mm (Paxinos and Watson 1986).

A series of experiments were carried out to determine the optimal conditions for quantitative autoradiography using [^{3}H]MDL 100,907. These experiments examined ligand concentration, exposure time, fixation, incubation time, incubation temperature and post-incubation wash times. The protocol used is illustrated in figure 3.1 and table 3.1 and is similar to that described in chapter 2. In experiment 1 the ligand concentrations used ranged from 0.25 nM to 2 nM in 0.25 nM steps and the exposure times used were 2, 4 and 6 weeks. These conditions were chosen as Johnson *et al* (1996) reported a K_D for [^{3}H]MDL 100,907 of 0.56 nM and higher concentrations (up to 2 nM) were included to confirm [^{3}H]MDL 100,907 bound to a saturable site. Exposure times were determined by examining the exposure times required for other 5-HT_{2A} ligands. The exposure time when using [^{3}H]RP 62203 has been reported as 10 days (Malgouris *et al* 1993) or 3 weeks (Sumner and Fink 1997) and using [^{3}H]ketanserin exposure time was reported as 5 weeks (Sumner and Fink 1995). Half the slides in experiment 2 were fixed in 1% paraformaldehyde in PBS at room temperature for 5 minutes prior to the PBS step. Experiment 3 produced four treatment groups using the combination of two incubation times (1 and 2 hours) and two temperatures (room temperature and 37°C). Slides in experiment 4 were processed as normal until the post-incubation washes when half the slides were washed in ice cold Tris buffer pH 7.7 for 2x 5 minutes and the other half were washed for 2x 10 minutes. In experiment 4 tissue sections were then removed from the slides using glass fibre filter papers (Whatman GF/C filters, Whatman International Ltd, Maidstone, UK.). The filter paper was placed in scintillation vials and the amount of radioactivity present was assessed by counting dpm.

3.3 RESULTS

3.3.1 [^{3}H]MDL 100,907 methodology development experiments.

The levels of specific binding in the frontal and cingulate cortex at each exposure time (2, 4 and 6 weeks) is shown in figure 3.2. The values of specific binding are shown in fmol/mg tissue and are an average of the results obtained from the two brains which were processed together. A larger variation was seen in results after 2 weeks exposure compared to 4 and 6 weeks. This was most obvious at low ligand

concentrations and is probably due to the images of these sections not being fully developed on the film. The error bars at four and six weeks exposure are of comparable size hence a four week exposure was adopted as the standard condition. Values for total and non-specific binding in both the cingulate and frontal cortex after 4 weeks exposure are shown in figure 3.3. Non-specific binding in both areas was found to be approximately 5% of the total binding which is consistent with previous reports (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997). Figure 3.2 also illustrates that the binding sites for [^{3}H]MDL 100,907 saturate at a concentration around 1 to 1.5 nM. In order to detect both increases and decreases in binding site density a concentration below the level of saturation would be required hence the concentration of 0.75 nM was chosen as the standard condition. Analysis over a limited range of ligand concentrations (0.25-2 nM) gave Hill coefficients of 0.81 and 0.94 for the cingulate and frontal cortex respectively providing no evidence of heterogeneity of binding sites.

The effect of fixation of the tissue in 1% paraformaldehyde prior to incubation with [^{3}H]MDL 100,907 is illustrated in figure 3.4a. Fixation produced a slight increase in specific binding in both the cingulate and frontal cortex but this increase was not significant. The effect of different incubation times and temperatures on specific binding in the cingulate and frontal cortex is illustrated in figure 3.4b. A reduction in specific binding was observed in slides incubated at 37°C for two hours and deterioration of the tissue itself was visibly apparent. Incubating sections at room temperature produced no significant increase in binding compared to incubating at 37°C however tissue integrity was preserved. No significant increase in binding was produced by incubating sections for two hours at room temperature compared to one hour hence one hour was adopted as the standard condition in order to preserve the tissue.

The results of the post-incubation wash times are shown in figure 3.4c. There was no significant difference in total binding counts or non-specific counts between the two washing protocols. However, there was a slight decrease in the ratio of non-specific to total binding between the short and long wash times (14.8 ± 0.8% vs. 12.7 ± 0.8% respectively; n=4) hence the longer wash time was chosen as it improved the signal to noise ratio for the assay.

In summary, the optimal experimental conditions for [^{3}H]MDL 100,907 binding were defined as the incubation of unfixed sections for one hour at room temperature with

0.75 nM [^{3}H]MDL 100,907. These sections were then washed for 2x 10 minutes, dried and exposed for 4 weeks at 4°C to ^{3}H-Hyperfilm before developing and analysis.

3.3.2 Acute treatment with oestrogen and progesterone.

Distribution of binding: comparison between [^{3}H]ketanserin and [^{3}H]MDL 100,907.

The binding pattern of [^{3}H]ketanserin was similar to that described for both male rats (Pazos *et al* 1985) and female rats (Sumner and Fink 1995). High densities of binding were observed in the cingulate and frontal cortex and low densities were observed in the BLA, VMN and the DR. Non-specific binding for ketanserin was approximately 30% in the cortex and agreed with previously reported values (Leysen *et al* 1982). An example of non-specific and total binding is illustrated in figure 3.5.

The pattern of binding produced by [^{3}H]MDL 100,907 in female rat brain was similar to that previously described in sagittal sections from male rats (Lopez-Gimenez *et al* 1997). In general the pattern of binding was also similar to that described for ketanserin binding in both male rats (Pazos *et al* 1985) and female rats (Sumner and Fink 1995). Using [^{3}H]MDL 100,907 non-specific binding was approximately 5% of total binding and is illustrated in figure 3.5.

Although the general binding pattern of these two ligands was similar differences were observed when comparing specific binding site densities in control animals (table 3.2). In the cingulate cortex, nucleus accumbens and olfactory tubercle specific binding site density was significantly higher using [^{3}H]MDL 100,907 with a trend towards increased specific binding in both the frontal and piriform cortex ($p=0.08$; Wilcoxon Rank Sum). In the MPOA, BLA and DR the reverse was found with [^{3}H]ketanserin showing a significantly higher specific binding site density. In the other regions examined e.g. claustrum, DBB and VMN no differences in specific binding site density were observed between these two ligands. Examples of total binding patterns for both ligands are illustrated in the cortex (figure 3.5), in the BLA and VMN (figure 3.6) and the DR (figure 3.7).

Effects of steroid treatment on 5-HT_{2A} receptor binding site density.

Using [^{3}H]ketanserin an increase in 5-HT_{2A} receptor binding site density was observed in the frontal and cingulate cortex in oestrogen treated rats compared to controls ($p<0.05$; Wilcoxon Rank Sum), however no changes were observed in either progesterone or oestrogen and progesterone treated rats compared to controls (figure 3.8 and table 3.3). The attenuation of the oestrogen increase in binding by co-administration of progesterone described here agrees with that found in rats chronically treated with these two steroids (Biegon *et al* 1983). In other regions examined e.g. nucleus accumbens and piriform cortex no changes in binding site density were observed using [^{3}H]ketanserin in either oestrogen, progesterone or oestrogen + progesterone treated rats (figure 3.9 and table 3.3). Significance between the multiple treatment groups was assessed using one-way analysis of variance followed by a *post-hoc* Tukey test.

No differences were found in binding site density in the cortex of oestrogen, progesterone or oestrogen + progesterone treated rats when [^{3}H]MDL 100,907 was used as the ligand (figures 3.8 and 3.9, table 4.1). As detailed above, significance between the multiple treatment groups was assessed using one-way analysis of variance followed by a *post-hoc* Tukey test. In the MPOA a trend towards an increase in binding site density was observed after oestrogen treatment compared to control but this was not significant ($p=0.08$; Wilcoxon Rank sum).

In summary, the results demonstrate that the increase in 5-HT_{2A} receptor binding site density in the cingulate and frontal cortex produced by acute oestrogen, as detected using [^{3}H]ketanserin, was attenuated by administering progesterone and that progesterone alone had no effect on binding site density. The results also show that although ketanserin and MDL 100,907 are reported to bind to 5-HT_{2A} receptors they do not produce the same binding pattern throughout the brain. In general, higher specific binding site density is observed in the cortex using [^{3}H]MDL 100,907 but higher specific binding site density is seen in the dorsal raphe, MPOA and BLA using [^{3}H]ketanserin.

3.4 DISCUSSION

3.4.1. [^{3}H]MDL 100,907 methodology development.

These experiments indicate that [^{3}H]MDL 100,907 binds to a single saturable binding site in the cingulate and frontal cortex of female rat brain. These experiments also show that there is a low level of non-specific binding with this ligand which is consistent with previous reports (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997). No significant difference in binding site density was observed between unfixed and fixed tissue or between incubation times of 1 and 2 hours. Incubating sections at 37°C did not significantly decrease binding site density but had a deleterious effect on the tissue sections themselves.

The method derived above is similar to that of Lopez-Gimenez *et al* (1997) with respect to buffer used, incubation time and a post-incubation wash time of 2x 10 minutes. However, there are also differences between the protocols in that Lopez-Gimenez *et al* (1997) used 0.4 nM of tritiated ligand, 30 minutes pre-incubation and a shorter exposure time of 15 days. These differences may, in part, be accounted for by the fact that the sections used by Lopez-Gimenez *et al* (1997) were fixed in 4% paraformaldehyde prior to incubation (Lopez-Gimenez personal communication) and this may have affected the binding parameters of [^{3}H]MDL 100,907. However, it should be noted that no significant difference in binding site density was found when 1% paraformaldehyde was used to fix sections prior to incubation in this study (figure 3.4). The other major difference between this study and that of Lopez-Gimenez *et al* (1997) was the plane of sectioning, their study used sagittal sections while the results above were from binding in coronal sections. The regions analysed in this study, cingulate and frontal cortex, are more easily examined using coronal sections.

The findings from this study agree with previous reports (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997 and 1998) which show a low level of non-specific binding (<5% of total binding) with [^{3}H]MDL 100,907 (figure 3.3). This study illustrates that in the cortex specific binding for this ligand is high in comparison to that of other 5-HT_{2A} ligands such as ketanserin and RP 62203 which are reported to exhibit specific binding of approximately 70% (Leysen *et al* 1982) and 80% (Malgouris *et al* 1993) of total binding respectively. In the cortex the superficial layer of lamina V has been shown to contain the highest density of 5-HT_{2A} receptors (Lopez-Gimenez

et al 1997) and this was also demonstrated using [^{3}H]MDL 100,907 in this study. Previous studies have suggested that [^{3}H]MDL 100,907 binds to a single site (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997 and 1998) and this was confirmed in the present study. [^{3}H]MDL 100,907 has been evaluated as an *in vivo* ligand to detect 5-HT_{2A} receptors in monkeys (Lundkvist *et al* 1996) and in humans (Grunder *et al* 1997). In both species [^{3}H]MDL 100,907 was shown to bind rapidly, within 60 minutes, to 5-HT_{2A} receptors in the cortex. It has also been used *in vitro* to study 5-HT_{2A} receptors using quantitative autoradiography in both monkeys and humans (Lopez-Gimenez *et al* 1998). The specificity of this compound for the 5-HT_{2A} receptor subtype has led to the suggestion that MDL 100,907 could act as an atypical antipsychotic drug (Kehne *et al* 1996).

3.4.2. Effect of oestrogen and progesterone on 5-HT_{2A} receptor binding site density.

Although [^{3}H]ketanserin and [^{3}H]MDL 100,907 produced similar binding patterns in the regions examined in this study significant differences were found in the absolute values for specific binding site density in control animals between the two ligands. In the cortex the use of [^{3}H]MDL 100,907 revealed, with respect to [^{3}H]ketanserin, a higher specific binding site density in the cingulate cortex, nucleus accumbens and olfactory tubercle with a trend towards an increase in both the frontal and piriform cortex ($p=0.08$). This observed increase in binding site density may be a reflection of the slightly higher B_{max} observed for MDL 100,907 compared with that of ketanserin (Johnson *et al* 1996). In other regions of the forebrain e.g. MPOA and BLA, and in the dorsal raphe the reverse was found with significantly higher specific binding using [^{3}H]ketanserin. The level of specific binding in the dorsal raphe was over 3 times higher with ketanserin than MDL 100,907. This large increase in specific binding may have resulted from ketanserin binding to tetrabenzamine sites which are known to occur in this region (Leysen *et al* 1987). In the other regions examined which included the median raphe, VMN and medial septum no differences were found in the specific binding site density using [^{3}H]MDL 100,907 or [^{3}H]ketanserin. The data presented here demonstrate that MDL 100,907 and ketanserin do not produce the same binding pattern in female rat brain. However, the majority of studies which have compared the effects of ketanserin and MDL 100,907 suggested that the two compounds produce similar results *in vitro* and *in vivo*. In cell culture it has been reported that ketanserin and MDL 100,907 both decrease DOI stimulated release of arachidonic acid and docosahexaenoate (Garcia and Kim 1997). Both

compounds also produced similar effects on GABA release when examined by *in vitro* electrophysiology (Cozzi and Nichols 1996). *In vivo* the two 5-HT_{2A} antagonists have been shown to produce the same effect in behavioural studies examining head twitches (Schreiber *et al* 1995, Willins and Meltzer 1997) and in studies examining hyperlocomotion (Gleason and Shannon 1997). However, some studies have indicated that the two 5-HT_{2A} antagonists do not always produce the same effect. In a behavioural model of learning the cognitive effects of both DOI (agonist) and ketanserin (antagonist) were abolished by MDL 100,907 (Meneses *et al* 1997). Using another *in vivo* model, this time to examine schizophrenia using MK-801 treated NMRI mice, it was found that the effects of MDL 100,907 were counteracted by ritanserin (Martin *et al* 1997). Further evidence showing the different effect of MDL 100,907 is its inability to block the release of luteinising hormone-releasing hormone in female rats, while other classical 5-HT_{2A} receptor antagonists, including ketanserin, do (Fink 1997, Fink 1999 and personal communication).

Using [^{3}H]ketanserin to detect 5-HT_{2A} receptors previous reports have demonstrated that acute oestrogen treatment produces increases in 5-HT_{2A} receptor binding sites in both female (Sumner and Fink 1995) and male (Sumner and Fink 1998) rats. Similar increases have also been reported in the cortex of female rats chronically treated with oestrogen whether assessed by tissue homogenate binding experiments (Biegon *et al* 1983) or quantitative autoradiography (Cyr *et al* 1998). These latter two studies used [^{3}H]spiroperidol and [^{3}H]ketanserin respectively. The increase observed in 5-HT_{2A} receptor binding sites in response to oestrogen is not strain specific and has been reported in Wistar (Sumner and Fink 1995), Sprague-Dawley and Fischer (Cyr *et al* 1998) strains. In the present study oestrogen treatment produced increases in specific binding site density in the cingulate and frontal cortex, which agrees with previous data (Sumner and Fink 1995). However, no increases were observed in the piriform cortex, nucleus accumbens and olfactory tubercle in this study as had been previously reported (Sumner and Fink 1995). This lack of effect may be attributed to two differences in methodology between the present and earlier study. Firstly, in the earlier study non-specific binding was determined using ketanserin whereas in the present study RP 62203 was used. This may have affected non-specific binding values since ketanserin is known to bind to several receptor sites including 5-$HT_{1B/D}$ receptors (Zgombick *et al* 1995) and two distinct sites in the striatum (Roth *et al* 1987). Secondly, in the present study animals were given a second injection the day after ovariectomy in order to administer progesterone or oil. Stress from handling

has been reported to elevate 5-HT levels within the MnR (Adell *et al* 1997). Adell *et al* (1997) also showed that a single subcutaneous injection of saline can lead to elevated 5-HT levels in the MnR and DR. If 5-HT levels were also increased in the terminal regions this may have affected receptor numbers since it is known that 5-HT_{2A} receptors are rapidly internalised after agonist activation (Berry *et al* 1996). The time between injection and sacrifice may not have allowed full recovery of 5-HT_{2A} receptors in some regions and hence could explain the difference in results.

Using [^{3}H]MDL 100,907 to detect 5-HT_{2A} receptors did not produce any significant changes in binding site density in any area examined with any treatment group. The reason why MDL 100,907 did not show the same increase in 5-HT_{2A} receptor binding with oestrogen treatment in the cingulate and frontal cortex is unclear. The lack of increase in cortical binding site density with [^{3}H]MDL 100,907 is unlikely to be attributable to the tissue since the sections used for MDL 100,907 and ketanserin binding were adjacent sections from the same rats. However, the sections which were processed for ketanserin binding were fixed in 1% paraformaldehyde prior to incubation whereas the sections used for MDL 100,907 binding were not. The prior fixation of sections incubated with [^{3}H]MDL 100,907 was investigated in this chapter and was shown to produce no significant change in binding site density. It should also be noted that although sections from each steroid treatment were processed in parallel, binding experiments using the two ligands were not run simultaneously. This may have affected the incubation conditions but is unlikely to be responsible for the differences in the 5-HT_{2A} binding site density of the oestrogen treated group between the two ligands.

Another possible explanation for the differences observed between ketanserin and MDL 100,907 in binding site density is the reported existence of subtypes of the 5-HT_{2A} receptor (Wright *et al* 1990). The existence of different isoforms or conformations of the 5-HT_{2A} receptor is possible, since both of these have been reported to occur in the closely related 5-HT_{2C} receptor (Burns *et al* 1997 and Berg *et al* 1998 respectively). However, reports which state that MDL 100,907 binds to a single site (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997) make these possible explanations unlikely although the autoradiography study was carried out on tissue fixed with 4% paraformaldehyde (Lopez-Gimenez, personal communication) and this could have affected the binding properties of the receptor.

The results presented here indicate that acute progesterone alone has no effect on 5-HT_{2A} binding site density as detected using [^{3}H]ketanserin. The results also suggest that when progesterone is given in combination with acute oestrogen it attenuates the increase in 5-HT_{2A} receptor binding sites observed with [^{3}H]ketanserin in the cingulate and frontal cortex seen with oestrogen. In chronically treated rats progesterone by itself has been reported to produce a slight increase in 5-HT_2 binding sites (Biegon *et al* 1983). This difference in the effect of progesterone is likely to result from the duration of steroid treatment since an increase in binding site density was only observed in chronically treated rats. In both acute (this study) and chronic (Biegon *et al* 1983) steroid replacement studies in rats the addition of progesterone to the oestrogen treatment attenuated the increase in receptor binding observed in the cortex with oestrogen alone. A possible explanation for the attenuation of the effect of oestrogen by the addition of progesterone is the anti-oestrogenic effect of progesterone itself (reviewed in Graham and Clarke 1997). An example of this action of progesterone is the antagonism of increases in both ER and PR levels induced by oestrogen when both steroids are administered together (Hsueh *et al* 1975, Savouret *et al* 1991).

3.4.3. The interaction between oestrogen and progesterone.

The competing effects of oestrogen and progesterone observed here using [^{3}H]ketanserin is not an exclusive phenomenon of serotonergic neurotransmission. In the VMN and PAG oestrogen produces a decrease in [^{3}H]muscimol binding at the $GABA_A$ receptor whereas progesterone alone or in combination with oestrogen returned binding to control levels (Schumacher *et al* 1989). In the VMN oestrogen has been shown to increase the levels of glutamate and GABA; however, co-administration with progesterone produced a rapid decline in the levels of these two transmitters (Luine *et al* 1997). Precisely how this steroidal modulation of central neurotransmission is mediated is unknown. The timescale used in the present study would allow both genomic and non-genomic mechanisms to occur. In the dorsal raphe short term oestrogen treatment (2 days) has been shown to increase progesterone receptor immunoreactivity but decrease the immunoreactivity for oestrogen receptors (Alves *et al* 1998). Acute oestrogen treatment on the same timescale as used in the present study has been shown to increase 5-HT_{2A} receptor mRNA in the dorsal raphe (Sumner and Fink 1993). However, the study did not find any changes in 5-HT_{2A} receptor mRNA in the terminal regions with the exception of the medial septum and DBB where decreases in silver grain density were observed or

in the MPOA where decreases in the percentage of labelled cells were observed. Since 5-HT_{2A} receptors in the cortex are postsynaptic (Marek and Aghajanian 1994 and 1996) the changes in mRNA expression in the dorsal raphe will only have an indirect effect on 5-HT_{2A} binding site density in these terminal regions, as a consequence of a possible change in raphe firing. Other possible mechanisms for the action of oestrogen and progesterone in modulating 5-HT_{2A} receptors are by regulating phosphorylation of the receptor or by regulating its internalisation or externalisation and therefore its actual surface levels.

The observation that progesterone attenuates the effect of oestrogen both in respect of 5-HT_{2A} receptors and $GABA_A$ receptors (Schumacher *et al* 1989) may have physiological significance. For example, several reports have suggested that progesterone has a negative effect on mood. Administration of progesterone postnatally to women has been shown to increase the risk of developing postpartum depression (Lawrie *et al* 1998). Other studies have examined the effects of oestrogen alone or in conjunction with progesterone and have shown that the addition of progesterone produced more negative moods (Sherwin 1991, Klaiber *et al* 1996). The fact that this study suggests 5-HT_{2A} receptor binding density is modulated by progesterone may help to explain these observations.

In summary, MDL 100,907 and ketanserin produce similar, but not identical, binding patterns throughout the brain. In several cortical regions MDL 100,907 produced significantly higher binding site density values but in the dorsal raphe produced significantly lower values. Oestrogen was shown to produce an increase in binding site density in the cingulate and frontal cortex only when [^{3}H]ketanserin was used. In this situation progesterone returned 5-HT_{2A} binding site density to control levels suggesting progesterone may play an inhibitory role *in vivo*. No significant differences were found in binding site density in any region when MDL 100,907 was used as a ligand.

Figure 3.1:

Flowchart illustrating the methodology used in the [^{3}H]MDL 100,907 development experiments. The protocol sequence runs vertically with subsequent steps in the method being indicated by solid arrows. Steps in the methodology where experimental conditions were altered are shown on the right hand side and are indicated using open arrows.

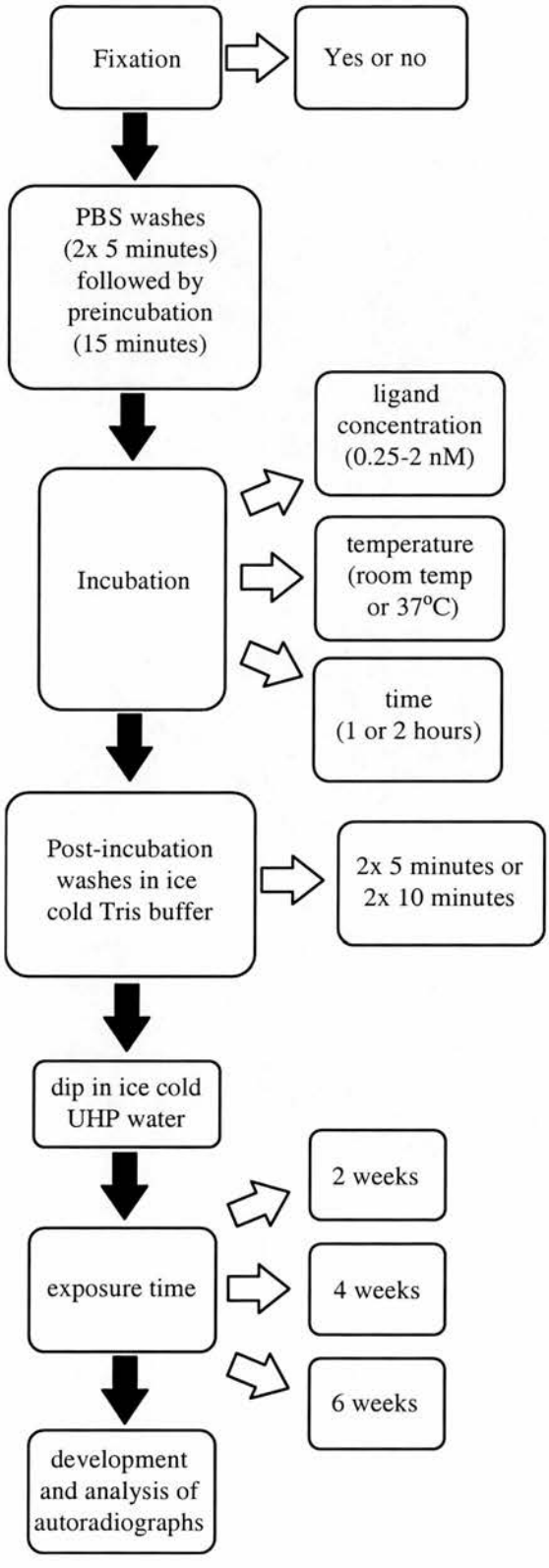

Fixation
Yes or no
PBS washes (2x 5 minutes) followed by preincubation (15 minutes)
Incubation
ligand concentration (0.25-2 nM)
temperature (room temp or 37°C)
time (1 or 2 hours)
Post-incubation washes in ice cold Tris buffer
2x 5 minutes or 2x 10 minutes
dip in ice cold UHP water
exposure time
2 weeks
4 weeks
6 weeks
development and analysis of autoradiographs

Table 3.1:

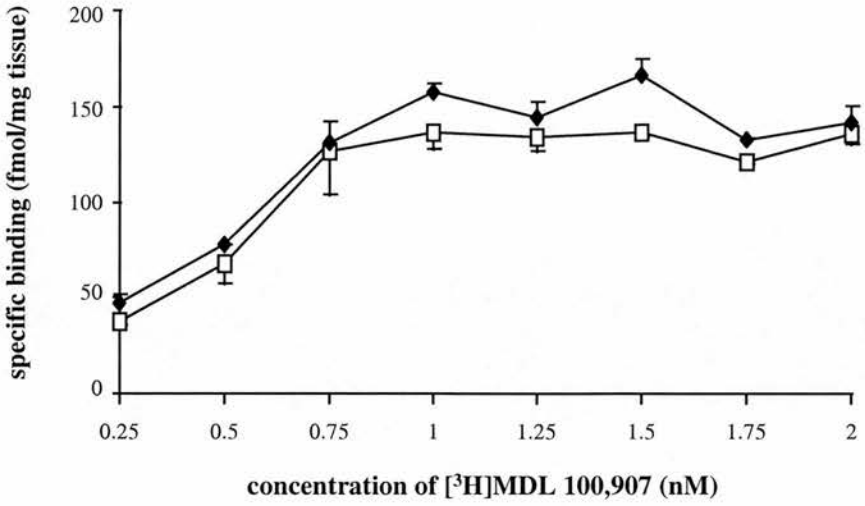
Table showing the four experiments in the [^{3}H]MDL 100,907 methodology development. The table illustrates the set of conditions used in each experiment. In steps where conditions were varied equal numbers of sections were processed in each group. As in figure 3.1 the protocol sequence runs vertically.

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Fixation	no	yes/no	no	no
PBS washes (2x 5 minutes)	yes	yes	yes	yes
Pre-incubation (15 minutes)	yes	yes	yes	yes
Incubation - ligand concentration	0.25-2 nM	0.75 nM	0.75 nM	0.75 nM
- time	1 hour	1 hour	1 or 2 hours	1 hour
- temperature	37°C	37°C	room temp or 37°C	37°C
Post-incubation washes	2x 5 minutes	2x 5 minutes	2x 5 minutes	2x 5 or 10 minutes
dip in ice cold UHP water	yes	yes	yes	yes
exposure time	2, 4 or 6 weeks	4 weeks	4 weeks	N/A

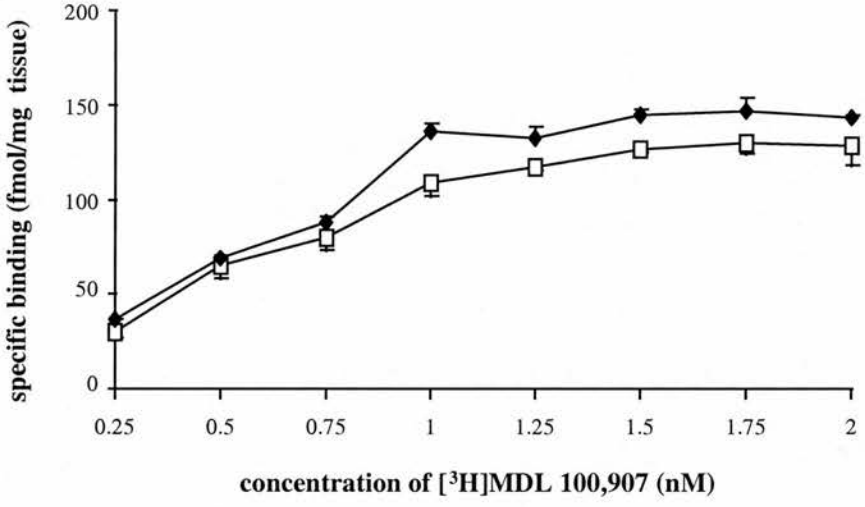
Figure 3.2:

Specific binding of [^{3}H]MDL 100,907 in areas of cortex expressed in fmol/mg tissue after an exposure time of 2 weeks (a), 4 weeks (b) and 6 weeks (c). Binding to the frontal cortex is shown by solid diamonds (◆) and binding in the cingulate cortex is shown by open squares (□). Data are shown as mean ± s.e.m. (n=2).

a)


specific binding (fmol/mg tissue)
200
150
100
50
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
concentration of [3H]MDL 100,907 (nM)

b)


specific binding (fmol/mg tissue)
200
150
100
50
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
concentration of [3H]MDL 100,907 (nM)

c)

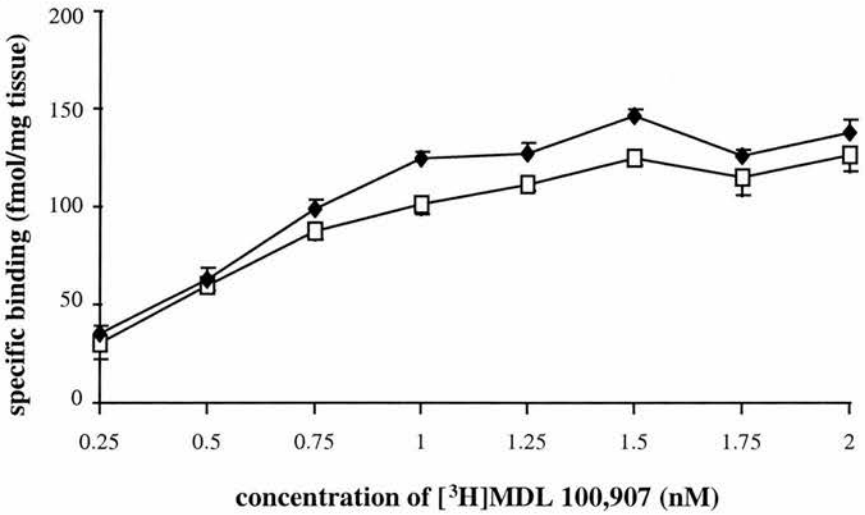
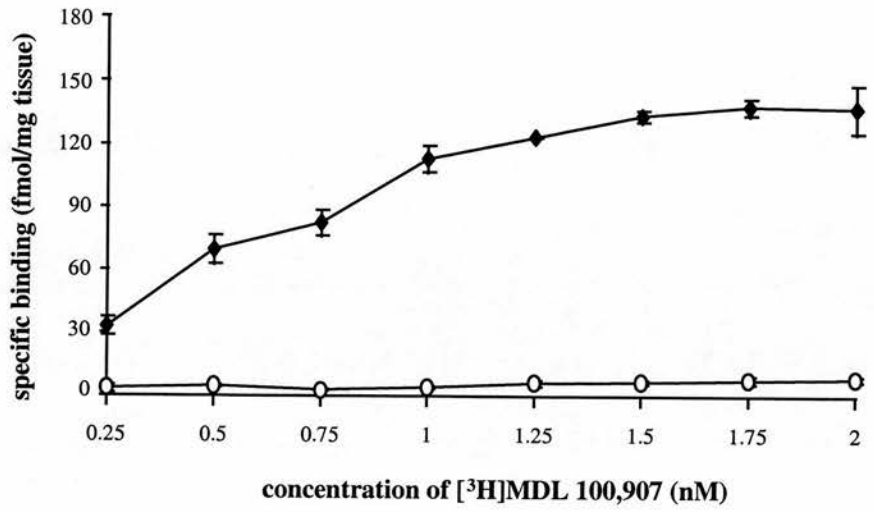

specific binding (fmol/mg tissue)
200
150
100
50
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
concentration of [3H]MDL 100,907 (nM)

Figure 3.3:

Binding of [^{3}H]MDL 100,907 in cingulate cortex (a) and frontal cortex (b) expressed in fmol/mg tissue after an exposure time of four weeks. Total binding is represented by solid diamonds (◆) and non-specific binding is represented by open circles (O). Data are shown as mean ± s.e.m. (n=2).

a)


specific binding (fmol/mg tissue)
180
150
120
90
60
30
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
concentration of [3H]MDL 100,907 (nM)

b)

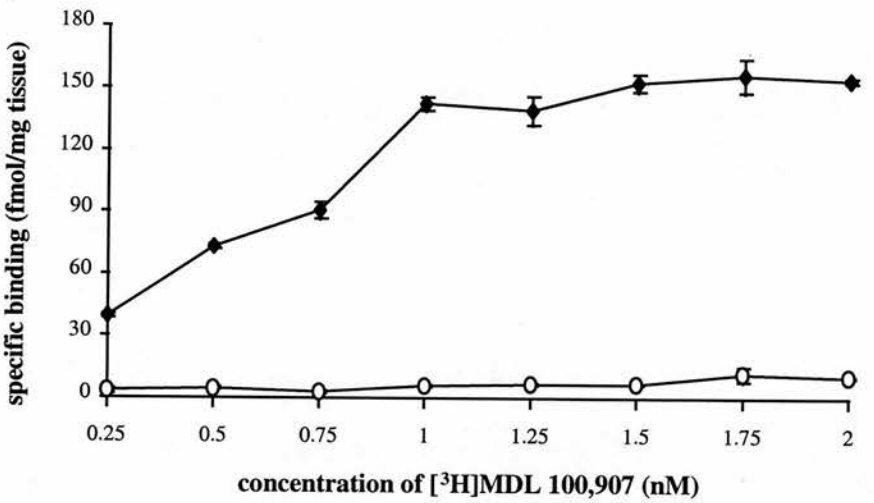

specific binding (fmol/mg tissue)
180
150
120
90
60
30
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
concentration of [3H]MDL 100,907 (nM)

Figure 3.4:

Development of [^{3}H]MDL 100,907 methodology experiments - the effect of fixation, incubation time/temperature and post-incubation wash times.

Figure (a): the effect of fixation prior to incubation on the specific binding of [^{3}H]MDL 100,907 to cingulate and frontal cortex. Unfixed tissue is represented by the open columns and fixed tissue by the solid black columns. Data expressed as mean ± s.e.m. (n=2).

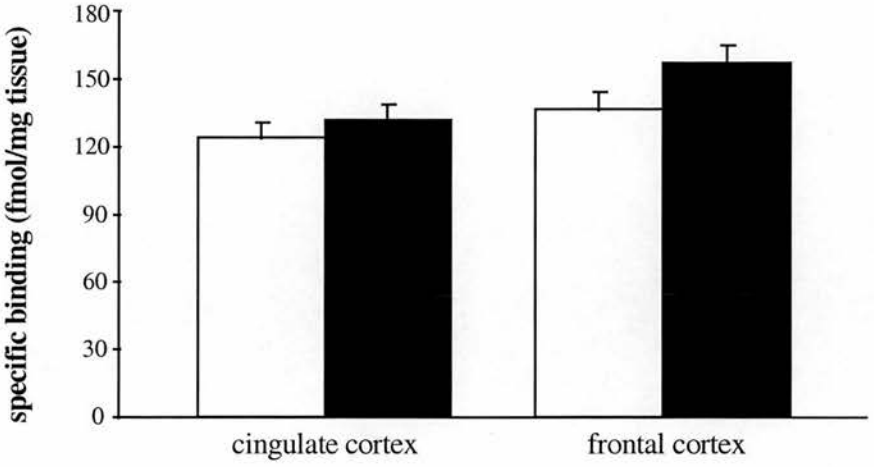
Figure (b): the effect of incubation time and temperature on the specific binding of [^{3}H]MDL 100,907 in frontal and cingulate cortex expressed in fmol/mg tissue after an exposure time of four weeks. Data expressed as mean ± s.e.m. (n=2).

Key for figure b:

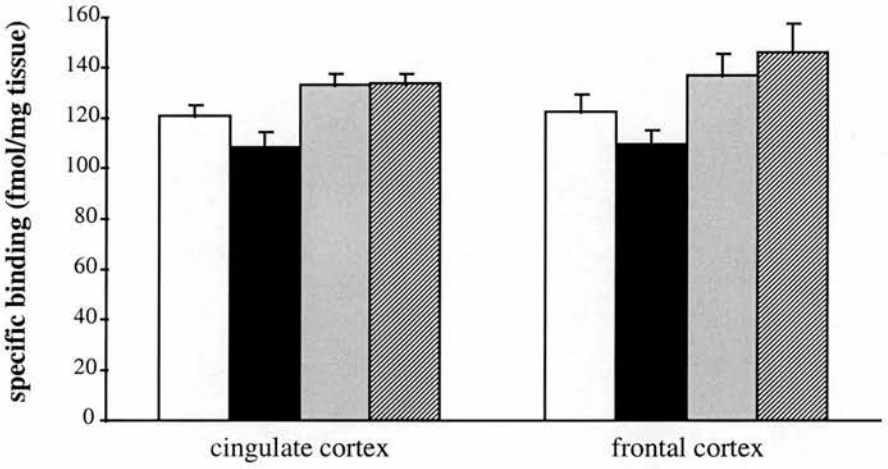
- 1 hour at 37°C
- 2 hours at 37°C
- 1 hour at room temperature
- 2 hours at room temperature

Figure (c): total and non-specific binding levels resulting from different post-incubation wash times after incubation of sections with 0.75 nM [^{3}H]MDL 100,907 (± 1 μM RP 62203). Each slide contained three sections. Scintillation counts are expressed as mean dpm per slide ± s.e.m. (n=4). The open columns represent 2x 5 minute post-incubation washes while the solid black columns represent 2x 10 minute washes.

a)


specific binding (fmol/mg tissue)
180
150
120
90
60
30
0
cingulate cortex
frontal cortex

b)


specific binding (fmol/mg tissue)
160
140
120
100
80
60
40
20
0
cingulate cortex
frontal cortex

c)

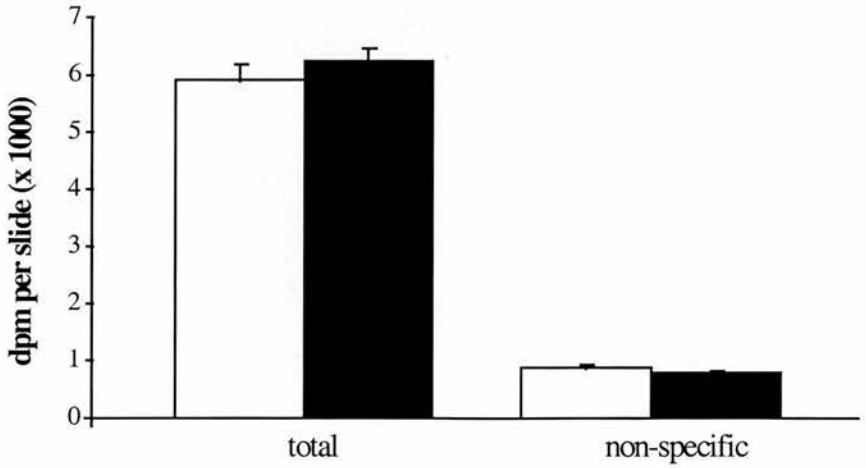

dpm per slide (x 1000)
7
6
5
4
3
2
1
0
total
non-specific

Figure 3.5:

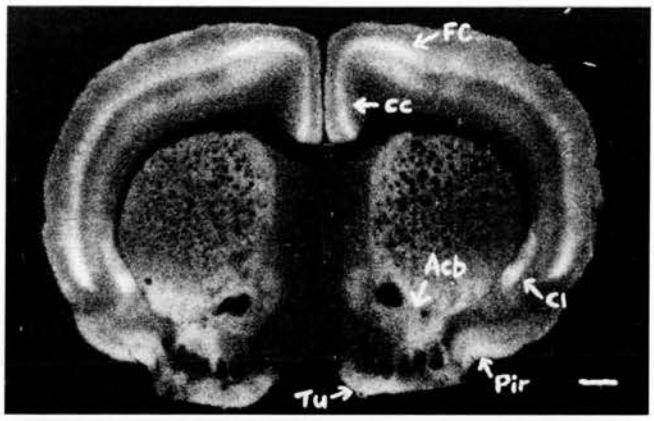
Direct prints from film autoradiographs showing coronal sections of female rat brain containing frontal and cingulate cortex. These sections are from control animals and have been assayed for 5-HT_{2A} receptors using [^{3}H]ketanserin in the presence of prazosin (a and b) and [^{3}H]MDL 100,907 (c and d). Sections (a) and (c) are of total binding while sections (b) and (d) illustrate non-specific binding in the presence of RP 62203.

The region illustrated is +0.70 mm from bregma (Paxinos and Watson 1986). Scale bar represents 1 mm.

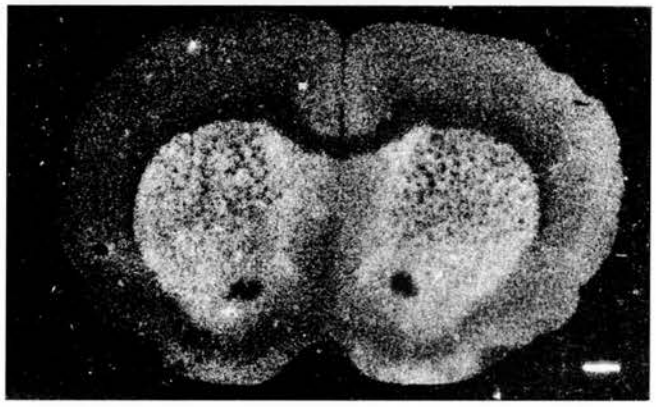
Abbreviations:

Acb	nucleus accumbens
CC	cingulate cortex
Cl	claustrum
FC	frontal cortex
Pir	piriform cortex
Tu	olfactory tubercle

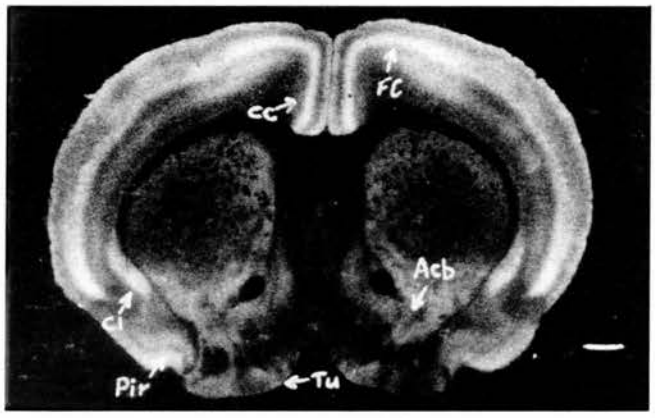
a)



b)



c)



d)

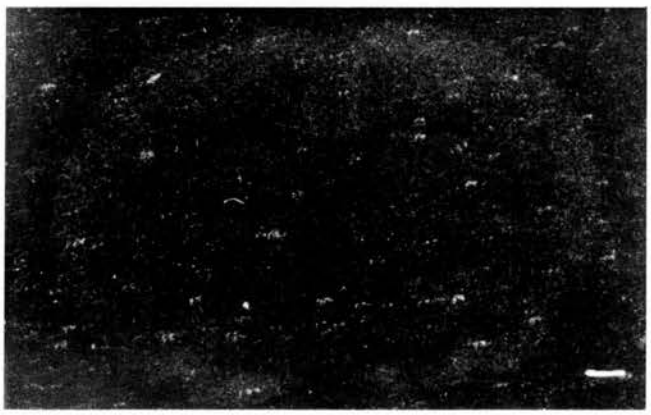


Figure 3.6:

Direct prints from film autoradiographs showing coronal sections of female rat brain containing basolateral amygdala and the VMN. Both sections are from control animals and illustrate total binding. The upper section is a photograph from a section incubated with [^{3}H]ketanserin in the presence of prazosin and the lower is a photograph from a section incubated with [^{3}H]MDL 100,907.

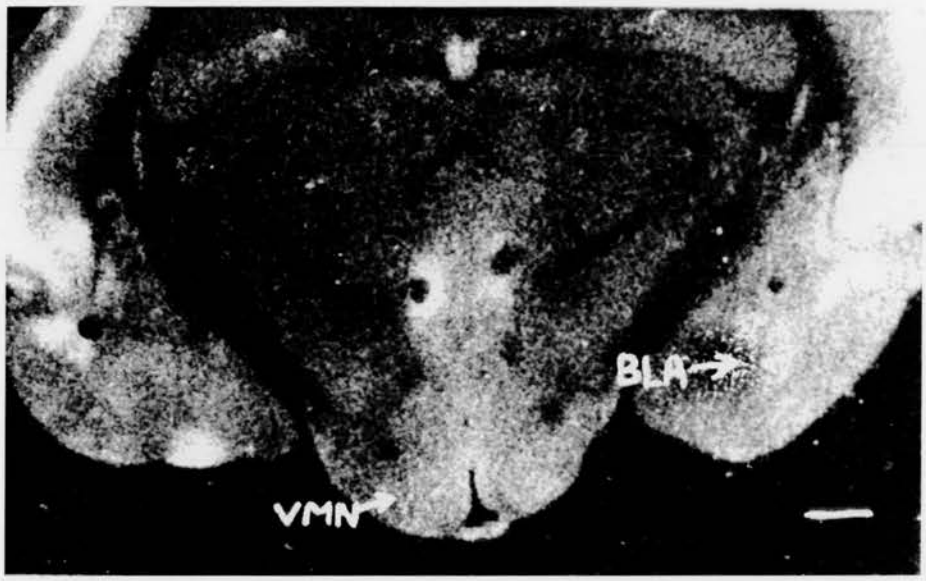
The region illustrated is -2.56 mm from bregma (Paxinos and Watson 1986). Scale bar represents 1 mm.

Abbreviations:

BLA basolateral amygdala

VMN ventromedial nucleus of the hypothalamus

a)



b)

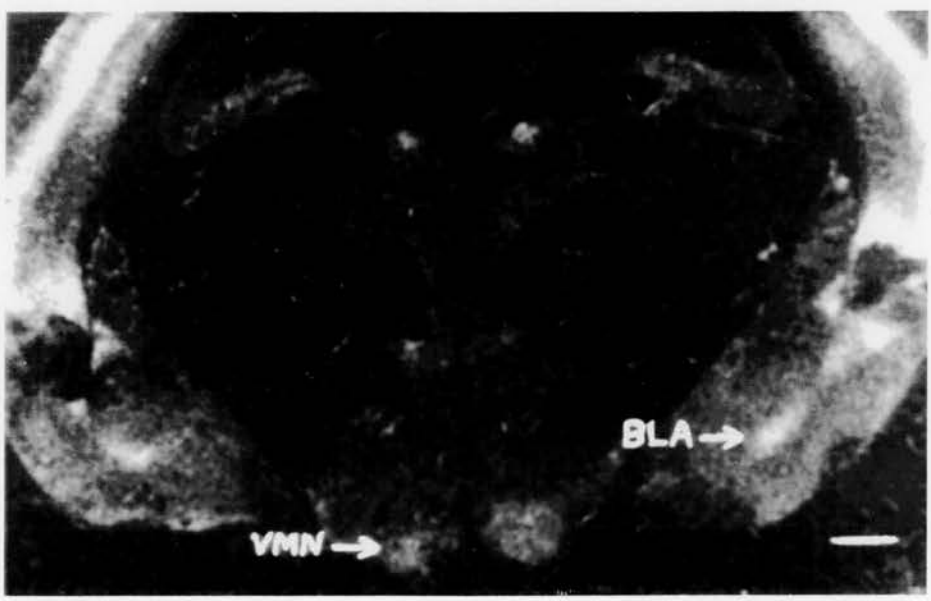


Figure 3.7:

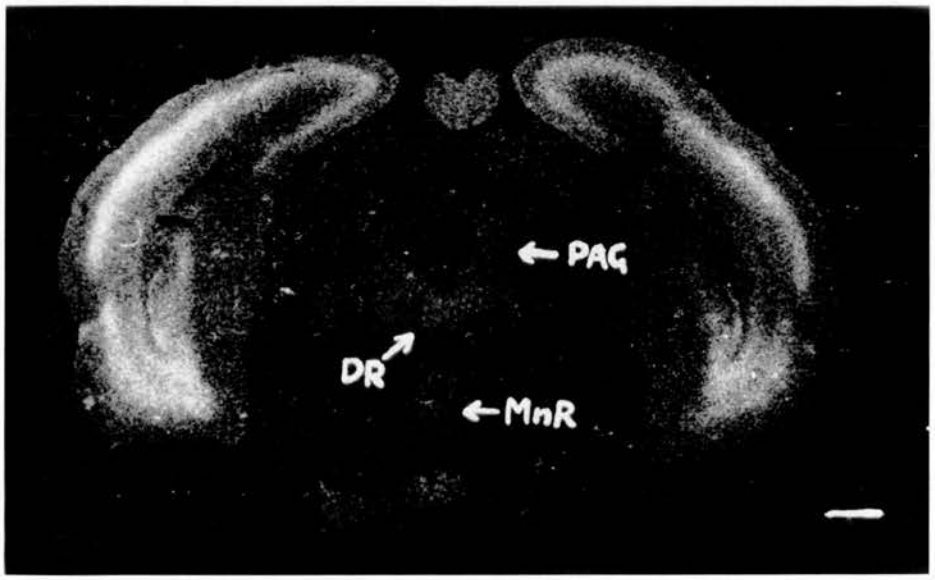
Direct prints from film autoradiographs showing coronal sections of female rat brain containing dorsal raphe and PAG. Both sections are from control animals and illustrate total binding. The upper section is a photograph from a section incubated with [^{3}H]ketanserin in the presence of prazosin and the lower is a photograph from a section incubated with [^{3}H]MDL 100,907.

The region illustrated is -7.64 mm from bregma (Paxinos and Watson 1986). Scale bar represents 1 mm.

Abbreviations:

DR	dorsal raphe
MnR	median raphe
PAG	periaqueductal gray

a)



b)

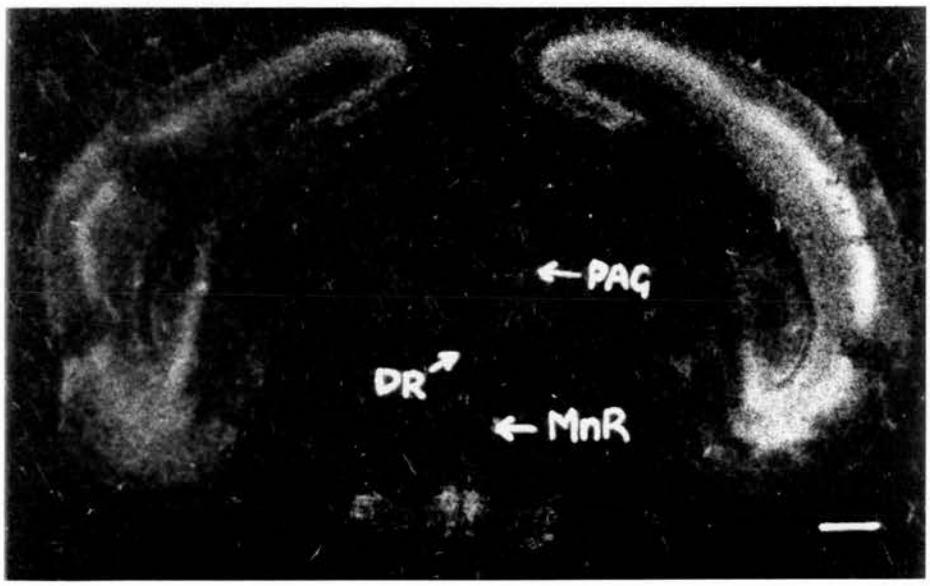
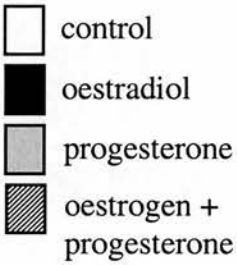


Figure 3.8:

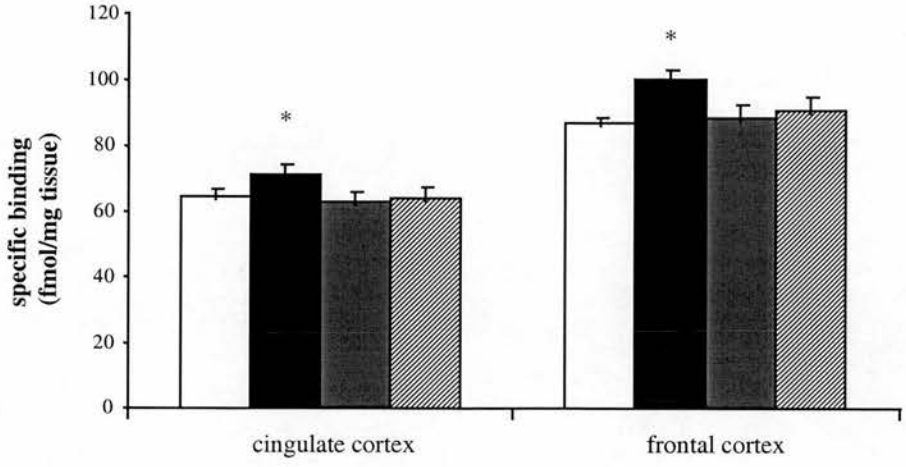
Specific binding to 5-HT_{2A} receptors in coronal sections containing cingulate and frontal cortex. Graph (a) shows specific binding detected by [^{3}H]ketanserin in the presence of prazosin and graph (b) illustrates specific binding detected by [^{3}H]MDL 100,907. Binding site density is shown as fmol/mg tissue ± s.e.m. (n=6).

* $p<0.05$ compared to control; Wilcoxon Rank Sum.

Key:



a)


120
100
80
60
40
20
0
specific binding
(fmol/mg tissue)
*
*
cingulate cortex
frontal cortex

b)

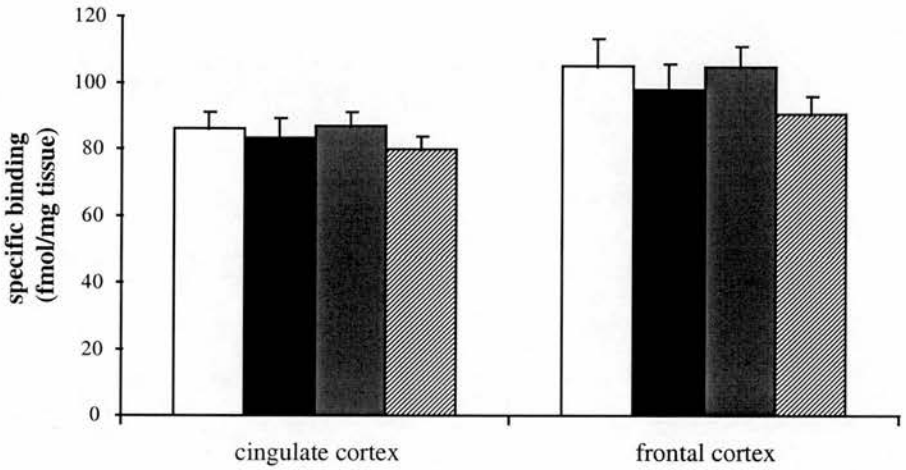
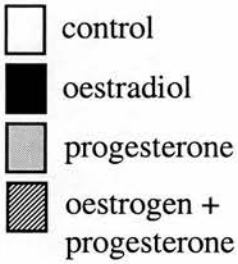

120
100
80
60
40
20
0
specific binding
(fmol/mg tissue)
cingulate cortex
frontal cortex

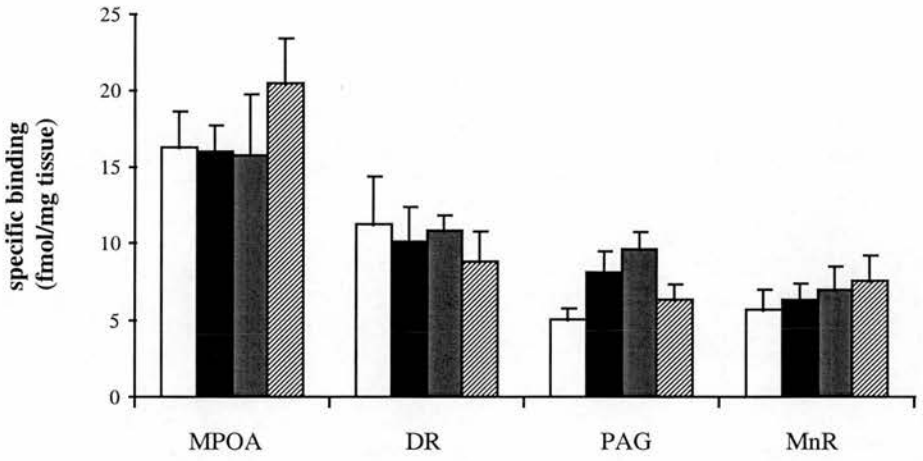
Figure 3.9:

Specific binding to 5-HT_{2A} receptors in coronal sections containing medial preoptic area (MPOA), dorsal raphe (DR), periaqueductal central gray (PAG) and median raphe (MnR). Graph (a) shows specific binding detected by [^{3}H]ketanserin in the presence of prazosin and graph (b) illustrates specific binding detected by [^{3}H]MDL 100,907. Binding site density is shown as fmol/mg tissue ± s.e.m. (n=6).

Key:



a)


specific binding
(fmol/mg tissue)
25
20
15
10
5
0
MPOA
DR
PAG
MnR

b)

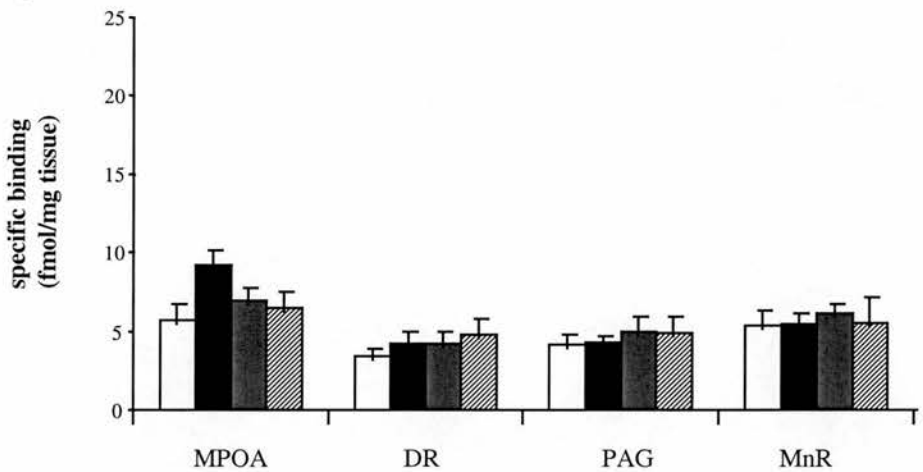

specific binding
(fmol/mg tissue)
25
20
15
10
5
0
MPOA
DR
PAG
MnR

Table 3.2:

Comparison of specific binding site density (expressed as fmol/mg tissue) in several brain regions as determined using either [^{3}H]ketanserin or [^{3}H]MDL 100,907. Data are from control animals and are expressed as mean ± s.e.m. (n=6). Statistical analysis was carried out by Wilcoxon Rank Sum.

* $p<0.05$ between [^{3}H]MDL 100,907 and [^{3}H]ketanserin.

** $p<0.01$ between [^{3}H]MDL 100,907 and [^{3}H]ketanserin.

*** $p<0.005$ between [^{3}H]MDL 100,907 and [^{3}H]ketanserin.

Abbreviations:

MPOA - medial preoptic area

	[^{3}H] ketanserin	[^{3}H]MDL 100,907
cingulate cortex	64.54 ± 2.13	86.09 ± 4.87 ***
frontal cortex	86.76 ± 1.60	105.01 ± 8.18 (p=0.08)
piriform cortex	56.65 ± 5.63	67.94 ± 4.93 (p=0.08)
nucleus accumbens	41.05 ± 6.58	59.12 ± 5.32 *
olfactory tubercle	48.93 ± 3.32	70.11 ± 5.64 **
MPOA	16.28 ± 2.39	5.74 ± 1.02 ***
basolateral amygdala	18.02 ± 1.05	33.28 ± 1.87 **
dorsal raphe	11.25 ± 3.13	3.38 ± 0.53 **

Table 3.3:

Values of specific binding to 5-HT_{2A} receptors in discrete regions of female rat brain after steroid treatment analysed using [^{3}H]ketanserin in the presence of prazosin. Binding site density is shown as fmol/mg tissue ± s.e.m. (n=6). Significance between the multiple treatment groups was assessed using one-way analysis of variance followed by a *post-hoc* Tukey test. Statistical analyses between two treatment groups were carried out using Wilcoxon Rank Sum test with a Bonferonni correction for multiple comparisons with the same mean. For two comparisons the new level of significance becomes $p < 0.025$.

* $p<0.01$ between treated group and control (Wilcoxon Rank Sum).

Abbreviations:

DBB	diagonal band of Broca
MPOA	medial preoptic area
VMN	ventromedial nucleus of the hypothalamus
DR	dorsal raphe
PAG	periaqueductal central gray
MnR	median raphe

	control	oestradiol benzoate (30 μg)	progesterone (2.5 mg)	oestradiol benzoate + progesterone
cingulate cortex	64.54 ± 2.13	71.18 ± 2.87 *	62.85 ± 2.93	63.90 ± 3.45
frontal cortex	86.76 ± 1.60	100.16 ± 2.80 *	88.47 ± 4.11	90.68 ± 4.16
piriform cortex	56.65 ± 5.63	59.86 ± 7.76	64.63 ± 11.34	59.22 ± 8.97
nucleus accumbens	41.05 ± 6.58	50.18 ± 11.08	45.85 ± 10.30	42.86 ± 8.42
claustrum	56.65 ± 6.98	58.60 ± 10.35	62.13 ± 7.60	55.94 ± 7.89
olfactory tubercle	48.93 ± 3.32	53.43 ± 10.66	56.78 ± 13.48	51.78 ± 9.54
DBB	5.05 ± 1.31	6.57 ± 2.87	6.92 ± 5.55	7.89 ± 8.41
medial septum	4.38 ± 3.12	6.39 ± 2.68	8.13 ± 4.42	5.63 ± 3.70
MPOA	16.28 ± 2.39	16.05 ± 1.69	15.75 ± 4.02	20.48 ± 2.98
VMN	10.01 ± 2.01	13.51 ± 2.17	11.63 ± 2.07	12.80 ± 2.09
amygdala	18.02 ± 1.05	19.13 ± 2.54	15.78 ± 3.53	15.93 ± 1.87
DR	11.25 ± 3.13	10.15 ± 2.21	10.87 ± 0.95	8.84 ± 1.94
PAG	5.05 ± 0.71	8.12 ± 1.35	9.59 ± 1.17 *	6.36 ± 1.00
MnR	5.72 ± 1.31	6.36 ± 1.01	7.03 ± 1.45	7.60 ± 1.64

Table 3.4:

Values of specific binding to 5-HT_{2A} receptors in discrete regions of female rat brain after steroid treatment analysed using [^{3}H]MDL 100,907. Binding site density is shown as fmol/mg tissue ± s.e.m. (n=6). Significance between the multiple treatment groups was assessed using one-way analysis of variance followed by a *post-hoc* Tukey test. Statistical analysis between two treatment groups was carried out using Wilcoxon Rank Sum.

Abbreviations:

DBB	diagonal band of Broca
MPOA	medial preoptic area
VMN	ventromedial nucleus of the hypothalamus
DR	dorsal raphe
PAG	periaqueductal central gray
MnR	median raphe

	control	oestradiol benzoate (30 μg)	progesterone (2.5 mg)	oestradiol benzoate + progesterone
cingulate cortex	86.09 ± 4.87	83.38 ± 5.67	86.93 ± 3.97	79.77 ± 3.78
frontal cortex	105.01 ± 8.18	97.76 ± 7.71	104.6 ± 6.15	90.37 ± 5.47
piriform cortex	67.94 ± 4.93	68.63 ± 4.27	71.65 ± 3.92	65.13 ± 1.96
nucleus accumbens	59.12 ± 5.32	60.74 ± 2.70	58.83 ± 3.38	58.19 ± 2.17
claustrum	66.97 ± 6.23	62.55 ± 2.51	73.33 ± 4.02	61.98 ± 2.70
olfactory tubercle	70.11 ± 5.64	63.66 ± 3.37	73.89 ± 4.85	63.83 ± 3.93
DBB	10.37 ± 1.82	10.44 ± 1.52	10.37 ± 1.25	13.26 ± 0.82
medial septum	2.41 ± 0.70	2.07 ± 0.11	2.16 ± 0.54	1.01 ± 0.19
MPOA	5.74 ± 1.02	9.23 ± 0.95 (p=0.08)	6.94 ± 0.86	6.48 ± 1.01
VMN	20.35 ± 1.33	21.54 ± 1.11	22.29 ± 2.02	22.68 ± 1.65
amygdala	33.28 ± 1.87	32.31 ± 0.29	33.53 ± 2.00	33.62 ± 3.33
DR	3.38 ± 0.53	4.26 ± 0.73	4.24 ± 0.74	4.75 ± 1.01
PAG	4.16 ± 0.63	4.29 ± 0.38	5.00 ± 0.95	4.93 ± 1.03
MnR	5.40 ± 0.96	5.44 ± 0.68	6.11 ± 0.62	5.54 ± 1.63

Chapter 4

The effect of acute steroid treatment on 5-HT transporter binding site density in discrete regions of female rat brain as determined by quantitative autoradiography using [^{3}H]paroxetine.

4.1 INTRODUCTION

In the CNS the 5-HT transporter (SERT) is the major site of removal of 5-HT from the synaptic cleft after release (Amara *et al* 1993). Within the CNS the transporter is found presynaptically on neurones and also on glial cells (Bel *et al* 1997). Peripherally the transporter has been detected on platelets and in the adrenal medulla (Schroeter *et al* 1997) where it has been shown to have a similar binding profile to SERT in the CNS (Wren *et al* 1997). Dysfunction of the serotonergic system has been associated with the aetiology of depression (Maes and Meltzer 1995). In relation to depression and SERT it has been reported that susceptibility to depression is linked to the presence of different alleles of a variable number tandem repeat (VNTR) sequence in intron 2 of the SERT gene (Ogilvie *et al* 1996). Many antidepressant drugs target serotonergic neurotransmission by preventing the uptake of 5-HT, including tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRI's) e.g. paroxetine. Depression is not the only CNS disorder which has been linked to changes in the properties of the 5-HT transporter. Decreases in the density of SERT binding sites have been found in the DR of Alzheimer's patients (Tejani-Butt *et al* 1995), in the putamen in Parkinson's disease (Raisman *et al* 1986) and the prefrontal and temporal cortex of suicide victims (Mann *et al* 1996).

Previous studies have examined the effects of female sex steroids on 5-HT synthesis and SERT. It has been shown that mRNA expression of the rate limiting enzyme in 5-HT synthesis, tryptophan hydroxylase, is regulated by ovarian steroids (Pecins-Thompson *et al* 1996). This study in macaques showed that chronic treatment with oestrogen (28 days) in the presence or absence of progesterone (last 14 days) produced an increase in tryptophan hydroxylase mRNA expression in the DR. Another study in macaques examined SERT mRNA expression in the DR after chronic oestrogen treatment in the presence or absence of progesterone using the same timings of the previous study (Pecins-Thompson *et al* 1998). The results of this study demonstrated that oestrogen, with or without progesterone, decreased the levels of SERT mRNA expression, the opposite effect to that seen on tryptophan hydroxylase mRNA. Studies in rats have examined the effect of oestrogen on paroxetine binding (Mendelson *et al* 1993, McQueen *et al* 1997 and 1998) and on SERT mRNA expression (McQueen *et al* 1997 and 1998). Chronic treatment with oestrogen (7 days) in male and female rats produced decreases in SERT binding sites in discrete regions of the hippocampus (CA1-CA4) and no change in binding site density in the cortex (Mendelson *et al* 1993). In some hippocampal regions, namely

CA2, CA4 and dentate gyrus, the density of SERT binding sites was reduced in females compared to males (data pooled from control and oestrogen treated animals). Changes in paroxetine binding and SERT mRNA expression have been studied in female rats treated acutely with oestrogen (30 hours; McQueen *et al* 1997 and 1998). Acute oestrogen was found to produce an increase in the number of cells expressing SERT mRNA in the dorsal raphe (McQueen *et al* 1997) and oestrogen also produced increases in SERT binding site density in discrete brain regions including the lateral septum, BLA and a decrease in binding sites in the PAG. In males acute oestrogen produced increases in SERT binding site density in the arcuate nucleus, BLA, VMN, and a decrease in the PAG relative to vehicle controls (McQueen *et al* 1998). The results in males were comparable to those observed in females.

Taken together, these data suggest that both SERT binding site density and mRNA levels are altered by both acute and chronic administration of female sex steroids. However, these studies have not examined the effect of progesterone alone on the 5-HT transporter nor have they examined whether progesterone affects the response to oestrogen when both steroids are given acutely. Hence the aim of the experiments detailed in this chapter was to examine the effects of acute oestrogen and progesterone on SERT binding site density in discrete regions of female rat brain. This was investigated in acutely ovariectomised rats which were treated with steroids in order to imitate the day of pro-oestrus in intact rats. SERT binding site density was determined using quantitative autoradiography with [^{3}H]paroxetine.

4.2 METHODS

The methodology for paroxetine binding studies are described in chapter 2 sections 2.3.4 and 2.3.5. The protocol to determine plasma LH and prolactin levels are described in 2.3.6 and 2.3.7 respectively.

4.3 RESULTS

4.3.1. Acute treatment with oestrogen and progesterone.

The pattern of distribution of SERT binding sites observed in this study was similar to that previously described in both male (Battaglia *et al* 1991, De Souza and Kuyatt 1987) and in female (McQueen *et al* 1997 and 1998) rat brains. Examples of the binding pattern are shown in figures 4.1 and 4.2. High densities of [^{3}H]paroxetine binding were evident in several structures throughout the brain, in particular in the BLA (figure 4.1b) and the raphe complex (figure 4.2a). Non-specific binding levels varied between 70% of total binding in the cortex to 30% of total binding in the DR. Values of non-specific binding levels are given in table 4.2 and examples of non-specific binding levels are shown in figures 4.1c and 4.2b. The pattern of binding observed was also consistent with the organisation of serotonergic terminals and cell bodies (Steinbusch and Nieuwenhuys 1983).

Results for [^{3}H]paroxetine binding for all four treatment groups are shown in table 4.1 and are illustrated in figure 4.3. Analysis of these results showed oestrogen treatment produced no significant changes in binding site density in any of the regions examined. Progesterone alone or in combination with oestrogen also produced no significant changes in [^{3}H]paroxetine binding site density. Statistical differences were determined between the four treatment groups using one-way analysis of variance followed by a *post-hoc* Tukey test.

In summary, this study showed that acute treatment with oestrogen, progesterone or oestrogen + progesterone produced no significant changes in SERT binding site density as determined by [^{3}H]paroxetine binding. Although there was a tendency towards an increase after oestrogen treatment in the dorsal and median raphe compared to control, the difference was not significant ($p=0.1$, Wilcoxon Rank Sum).

4.3.2 Effect of sex steroids: plasma LH and prolactin levels and *post-mortem* uterine weights.

The plasma LH levels were sampled at the time of the LH surge (see methods 2.3.1) and show that rats treated with oestradiol or oestradiol + progesterone had significantly higher plasma LH levels compared to oil treated controls ($p<0.05$; one way ANOVA with a *post-hoc* Tukey test). These data also show that plasma LH

levels in the oestradiol + progesterone group are similar to those from the pro-oestrous rats (figure 4.5a). The uterine weights of the pro-oestrous rats (figure 4.5c) are also similar to those of the rats in the oestrogen + progesterone group confirming that (1) the pro-oestrous rats had been exposed to both steroids and (2) the doses of the two steroids given to ovariectomised (OVX) rats produced increases in LH levels and uterine weights which mimicked physiological conditions (figure 4.5a and c). No changes were observed in the prolactin levels between the pro-oestrous rats and any of the steroid treatment groups (figure 4.5b).

4.4 DISCUSSION

4.4.1 The effect of oestrogen and progesterone on SERT binding site density.

These experiments show that in the 13 regions examined the distribution of [^{3}H]paroxetine binding sites was similar to that observed in both male (Battaglia *et al* 1991, De Souza and Kuyatt 1987) and female (McQueen *et al* 1997 and 1998) rats with the highest binding being observed in the dorsal raphe. No changes were found in [^{3}H]paroxetine binding in the cingulate or frontal cortex after oestrogen treatment and this finding agrees with previous findings in both male and female rats (Mendelson *et al* 1993, McQueen *et al* 1997 and 1998). In the dorsal and median raphe increases in binding were seen in 5 out of 6 animals after oestrogen treatment compared to controls in this study; however, the increase was not significant overall. It should also be noted that there was a high degree of variability in the results presented here. This variability could have masked a small difference between the treatment groups.

The results described here differ from those reported by McQueen *et al* (1997 and 1998) where acute oestrogen produced increases in [^{3}H]paroxetine binding in the BLA and VMN with a decrease being observed in the PAG. These changes were found in both studies by McQueen *et al* although different doses of oestradiol-17β were administered in each study, these being 10 μg and 30 μg respectively. The present study also used 30 μg of oestradiol-17β however no changes were observed in the density of SERT binding sites in either the BLA, VMN or PAG. A possible explanation for these differences may be due to a slight difference in methodology. In both studies the oestradiol was administered immediately after ovariectomy, however in the present study a second injection was given to all rats the following

day in order to administer progesterone or oil. Stress from handling has been reported to elevate 5-HT levels within the MnR and in the amygdala (Adell *et al* 1997). This study also showed that a single subcutaneous injection of saline can lead to elevated 5-HT levels in the MnR and DR and increased levels of 5-HT in the prefrontal cortex. Since the injection of oil or progesterone occurred approximately 5 hours prior to the animals being sacrificed the increases in 5-HT levels may have affected the 5-HT transporter and may account for the differences observed in binding site density results between this study and that of McQueen *et al* (1998). The firing of DR neurones in ovariectomised rats after acute oestrogen is discussed in chapter 5. The lack of effect of progesterone alone may reflect the need for pre-treatment with oestrogen in order to stimulate the production of progesterone receptors (Rainbow *et al* 1982). However, the increase in progesterone receptor mRNA produced by oestrogen has been reported to be transient (Shughrue *et al* 1997b) and this may explain the lack of effect seen in the oestrogen + progesterone group.

In relation to the treatment of depression it is interesting to note that acute administration of SSRI's produce a local increase in 5-HT levels in the DR whilst chronic administration of the same compounds results in no change in 5-HT levels (Bel and Artigas 1992 and 1993). Acute and chronic oestrogen treatment has been shown to produce a similar response with acute administration producing increased 5-HT levels in the DR and chronic administration resulting in no effect (Di Paolo 1983). These corresponding observations suggest that SSRI's and oestrogen might produce their antidepressant effect, at last in part, through a similar mechanism. It is unlikely, however; the mechanism being identical since oestrogen is used to successfully treat depression which is resistant to conventional drug treatments (Klaiber *et al* 1979).

In conclusion, no significant changes in SERT binding site density were detected between any of the four treatment groups in any of the regions examined.

4.4.2. Plasma hormone levels and *post-mortem* uterine weights.

The plasma LH levels in oestrogen, oestrogen + progesterone and pro-oestrous rats were all elevated above control levels suggesting that these animals had experienced a LH surge. The plasma prolactin levels however did not change between any of the treatment groups and would appear to be a poor indication of the steroid status of the

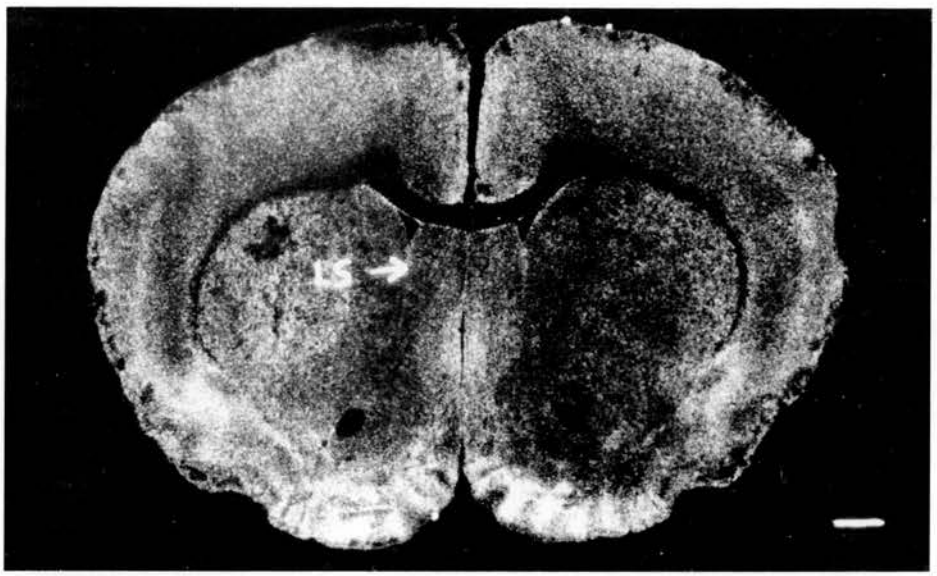
animal. Examination of uterine weights again showed increases in the oestrogen treated, oestrogen + progesterone treated and pro-oestrous rats compared to controls. It has been previously reported that oestrogen increases both plasma LH levels and uterine weights (Aiyer and Fink 1974) and this was confirmed in the present study. It should be noted that the LH and uterine weight values for the pro-oestrous rats were most similar to those in the oestrogen + progesterone group suggesting that (a) the pro-oestrous rats had been exposed to both hormones and (b) that the doses of oestrogen and progesterone given to the OVX rats were such that they produced a response similar to normally cycling rats. These results would suggest that the doses of hormones given to the steroid treated rats were such that they reliably mimicked the conditions found in the pro-oestrous rats.

Figure 4.1:

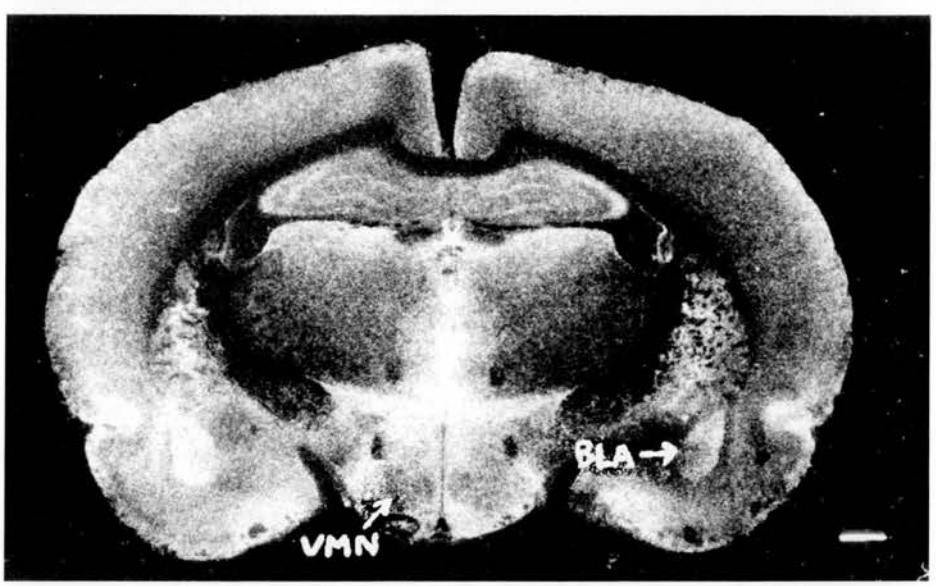
Direct prints from film autoradiographs showing coronal sections of female rat brain illustrating [^{3}H]paroxetine binding in forebrain regions. All three sections are from control animals with sections (a) and (b) illustrating total binding and section (c) illustrating non-specific binding in the presence of citalopram. Section (a) illustrates binding in the cingulate cortex, frontal cortex and lateral septum (LS). Sections (b) and (c) are adjacent sections and illustrate binding in the basolateral amygdala (BLA) and ventromedial nucleus of the hypothalamus (VMN).

The region illustrated in (a) is +0.48 mm from bregma and in (b) and (c) is -2.56 mm from bregma (Paxinos and Watson 1986). Scale bar represents 1 mm.

a)



b)



c)

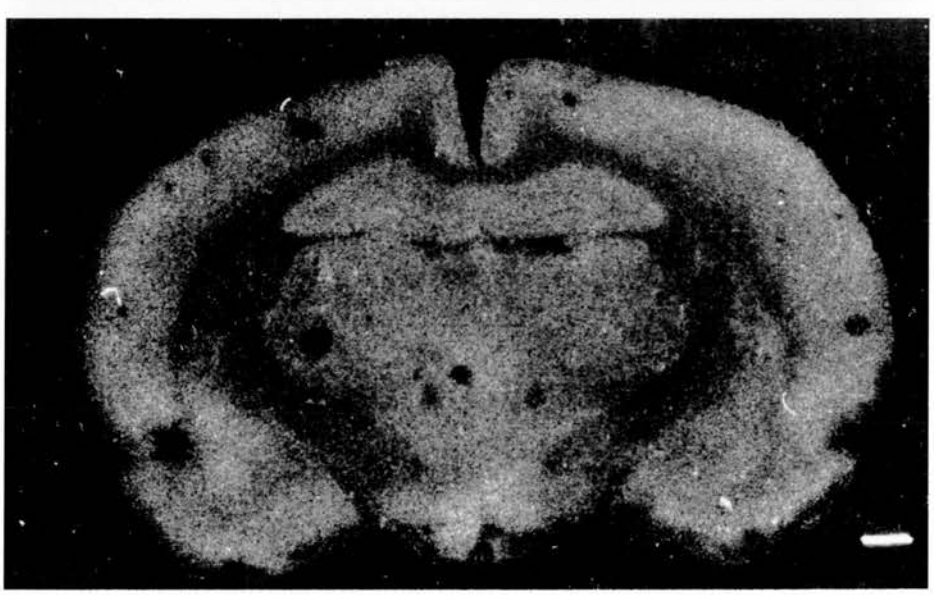


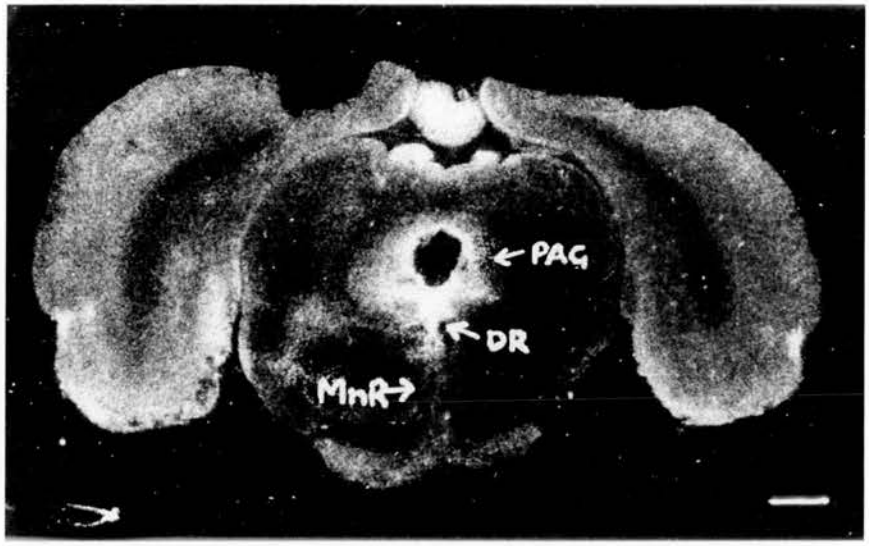
Figure 4.2:

Direct prints from film autoradiographs showing coronal sections of female rat brain illustrating [^{3}H]paroxetine binding in midbrain regions. All three sections are from control animals with sections (a) and (c) illustrating total binding and section (b) illustrating non-specific binding in the presence of citalopram. Sections (a) and (b) are adjacent sections and illustrate binding in the dorsal and median raphe. Section (c) illustrates binding in the locus coeruleus and the caudal region of the dorsal raphe. The region illustrated in (a) and (b) is -7.64 mm from bregma and in (c) is -9.30 mm from bregma (Paxinos and Watson 1986). Scale bar represents 1 mm.

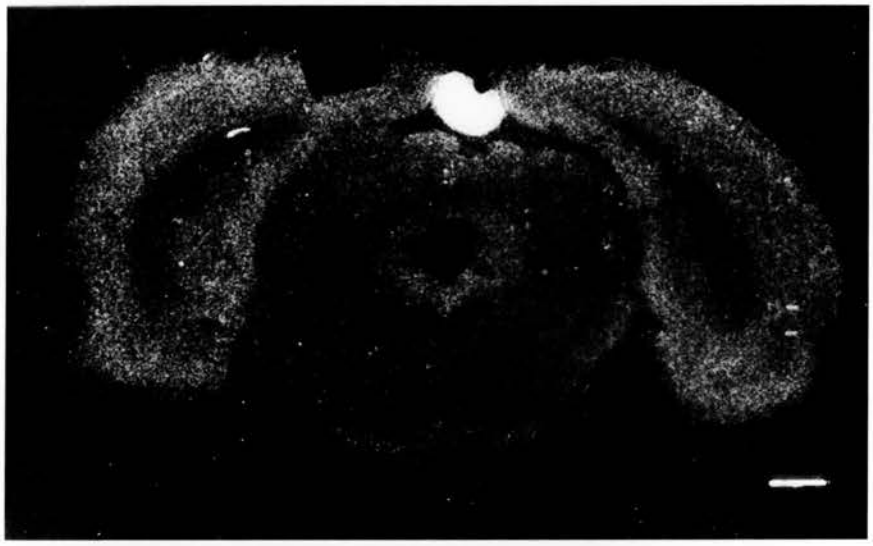
Abbreviations:

DR	dorsal raphe
DRY	The DRY refers to the dorsal raphe at the level of the locus coeruleus where it is Y-shaped in appearance.
LC	locus coeruleus
MnR	median raphe
PAG	periaqueductal central gray

a)



b)



c)

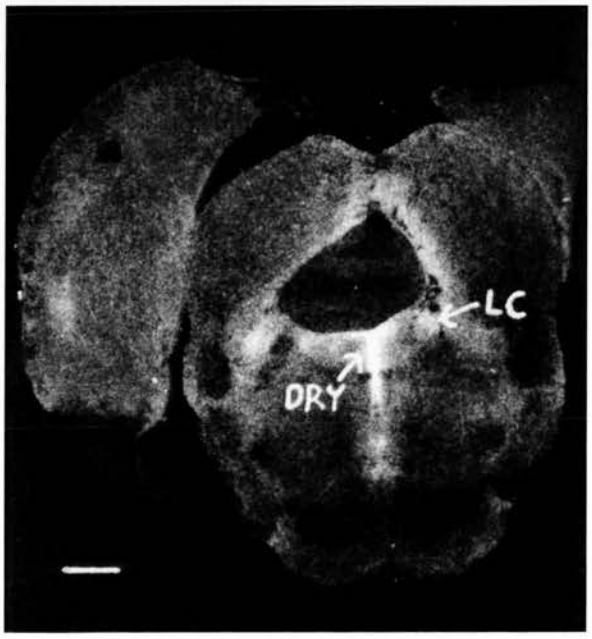


Figure 4.3:

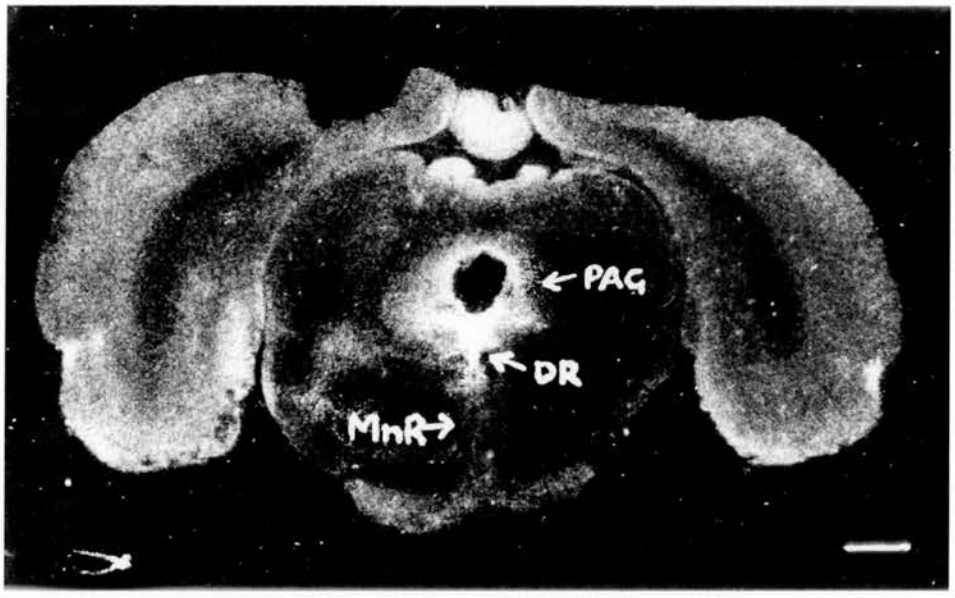
Direct prints from film autoradiographs showing coronal sections of female rat brain illustrating [^{3}H]paroxetine binding in the dorsal and median raphe nuclei. Section (a) is from a control animal and section (b) is from an oestrogen treated animal. Both sections illustrate total binding The region illustrated in (a) and (b) is -7.64 mm from bregma (Paxinos and Watson 1986).

Scale bar represents 1 mm.

Abbreviations:

DR	dorsal raphe
MnR	median raphe
PAG	periaqueductal central gray

a)



b)

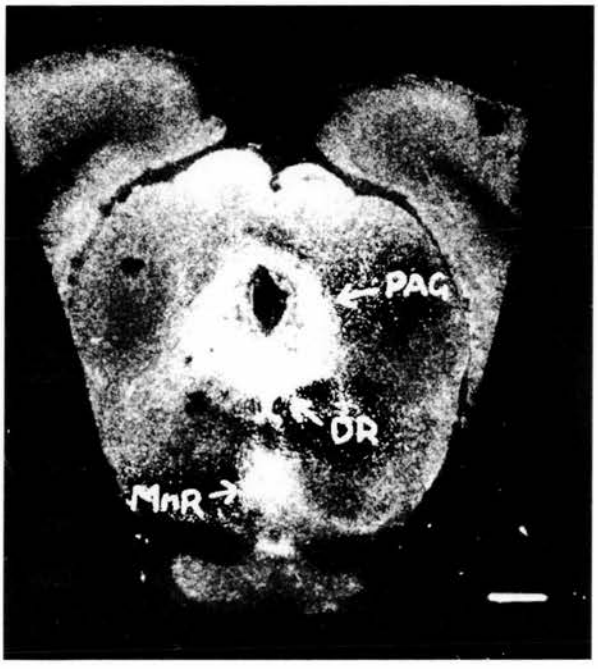


Figure 4.4:

Specific binding to the 5-HT transporter receptors in discrete nuclei in female rat brain. The graph illustrates the specific binding detected in these regions by [^{3}H]paroxetine.

Binding site densities are illustrated as mean ± s.e.m. (n=6) and are expressed in fmol/mg tissue.

Abbreviations:

DR	dorsal raphe
DRY	The DRY refers to the dorsal raphe at the level of the locus coeruleus where it is Y-shaped in appearance.
MnR	median raphe
PAG	periaqueductal central gray
VMN	ventromedial nucleus of the hypothalamus

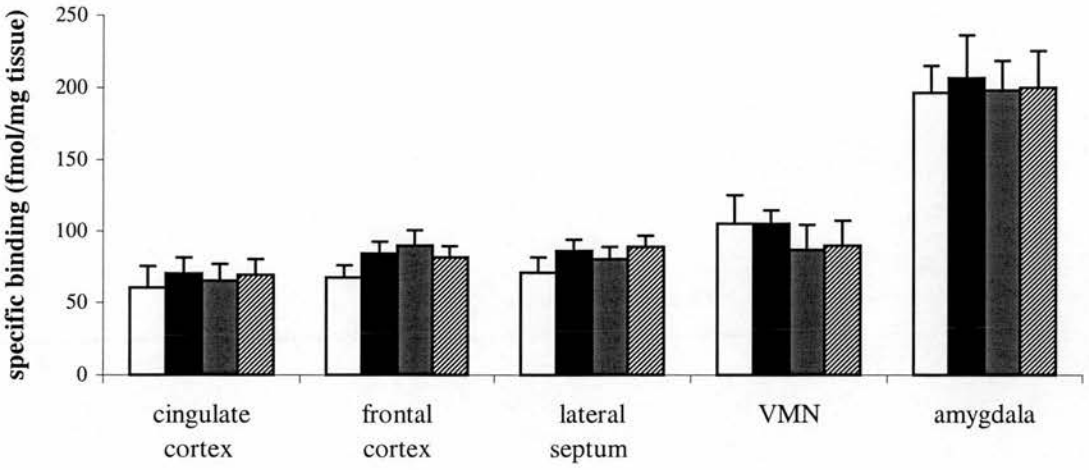
Key:

- control (OVX + oil)
- oestradiol (30 μg)
- progesterone (2.5 mg)
- oestradiol (30 μg) + progesterone (2.5 mg)

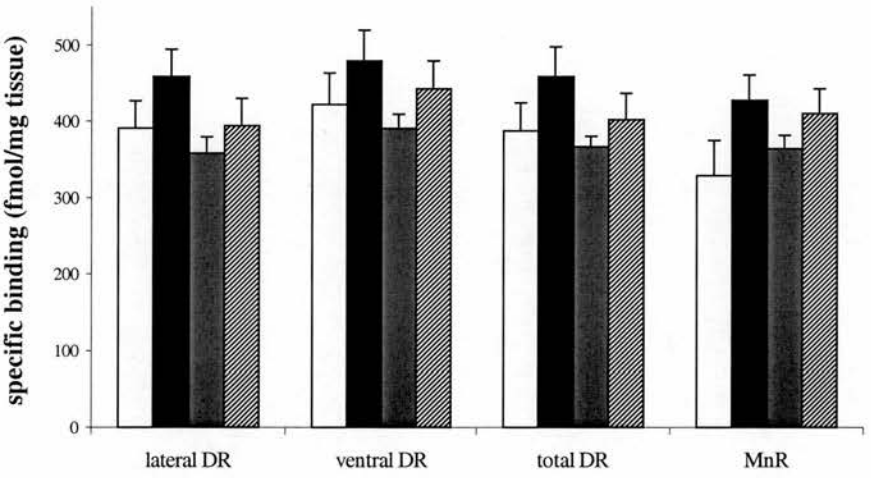
Details of steroid treatments are shown below. Rats were sacrificed at the time of the LH surge (see methods 2.3.1).

Treatment	**after OVX**	**noon next day**
control	oil	oil
oestradiol	oestradiol benzoate (30 μg)	oil
progesterone	oil	progesterone (2.5 mg)
oest + prog	oestradiol benzoate (30 μg)	progesterone (2.5 mg)

a)


specific binding (fmol/mg tissue)
250
200
150
100
50
0
cingulate cortex
frontal cortex
lateral septum
VMN
amygdala

b)


specific binding (fmol/mg tissue)
500
400
300
200
100
0
lateral DR
ventral DR
total DR
MnR

c)

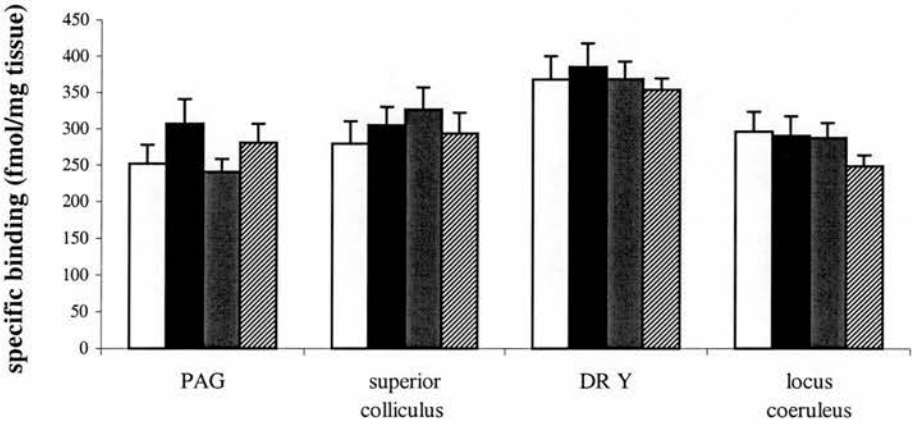

specific binding (fmol/mg tissue)
450
400
350
300
250
200
150
100
50
0
PAG
superior colliculus
DR Y
locus coeruleus

Table 4.1:

Table showing SERT binding site density in discrete regions of OVX female rat brain after treatment with oestrogen, progesterone and oestrogen + progesterone. The experiments were carried out on coronal sections containing the various regions.
The data show mean specific binding ± s.e.m. (n=6) and are expressed in fmol/mg tissue. Specific binding was determined by subtracting non-specific binding (evaluated by [^{3}H]paroxetine in the presence of citalopram) from total binding (evaluated by [^{3}H]paroxetine).

Abbreviations:

VMN	ventromedial nucleus of the hypothalamus
DR	dorsal raphe
lateral DR	lateral wings of the dorsal raphe nucleus
ventral DR	ventral region of the dorsal raphe nucleus
DR total	whole dorsal raphe including the lateral and ventral regions
DRY	The DRY refers to the dorsal raphe at the level of the locus coeruleus where it is Y-shaped in appearance.
PAG	periaqueductal central gray
MnR	median raphe

	control	oestradiol benzoate (30 μg)	progesterone (2.5 mg)	oestradiol benzoate + progesterone
cingulate cortex	60.56 ± 14.66	70.08 ± 11.18	65.38 ± 11.56	69.55 ± 10.70
frontal cortex	67.37 ± 8.67	84.28 ± 8.39	90.00 ± 10.90	81.82 ± 7.73
lateral septum	71.03 ± 10.68	86.38 ± 7.65	80.30 ± 8.39	88.93 ± 7.55
VMN	105.21 ± 19.74	105.08 ± 9.34	86.81 ± 17.60	90.01 ± 17.41
amygdala	196.10 ± 18.87	206.20 ± 29.66	197.67 ± 20.73	199.60 ± 25.48
lateral DR	376.62 ± 32.82	458.55 ± 34.76	358.45 ± 18.05	394.67 ± 35.15
ventral DR	422.24 ± 40.60	478.50 ± 40.06	391.02 ± 13.61	442.65 ± 36.09
DR total	386.79 ± 37.43	458.35 ± 38.68	366.27 ± 16.60	401.65 ± 35.38
MnR	328.80 ± 45.87	427.27 ± 33.55	364.57 ± 25.74	410.24 ± 32.41
PAG	251.77 ± 26.25	306.85 ± 33.64	240.60 ± 18.26	281.00 ± 25.62
superior colliculus	279.73 ± 30.83	305.21 ± 24.80	326.66 ± 30.08	293.80 ± 28.18
DR Y	367.64 ± 32.37	385.36 ± 32.50	368.52 ± 23.80	353.84 ± 15.16
locus coeruleus	296.27 ± 27.42	290.48 ± 26.84	287.93 ± 20.24	248.92 ± 14.37

Table 4.2:

Table showing the proportion of non-specific binding as a percentage of total binding in discrete regions of female rat brain. Values were calculated from data from control rats (treated with vehicle) and are expressed as mean ± s.e.m. (n=6 except superior colliculus where n=4).

Abbreviations:

VMN	ventromedial nucleus of the hypothalamus
DR	dorsal raphe
DRY	The DRY refers to the dorsal raphe at the level of the locus coeruleus where it is Y-shaped in appearance.
PAG	periaqueductal central gray
MnR	median raphe

	non-specific/ total binding (%)
cingulate cortex	77.2 ± 4.6
frontal cortex	73.6 ± 2.5
lateral septum	70.1 ± 3.4
VMN	63.2 ± 3.9
amygdala	46.7 ± 2.0
lateral DR	31.4 ± 1.2
ventral DR	29.2 ± 1.5
DR total	30.8 ± 1.3
MnR	33.5 ± 3.4
PAG	39.3 ± 1.4
superior colliculus	37.1 ± 1.0
DR Y	32.8 ± 0.7
locus coeruleus	36.7 ± 1.3

Figure 4.5:

Plasma luteinising hormone (a), prolactin levels (b) and uterine weights (c) for the rats used in the binding experiments. Data shown as mean ± s.e.m. where n=6 for all groups except uterine weights for pro-oestrus rats where n=4.

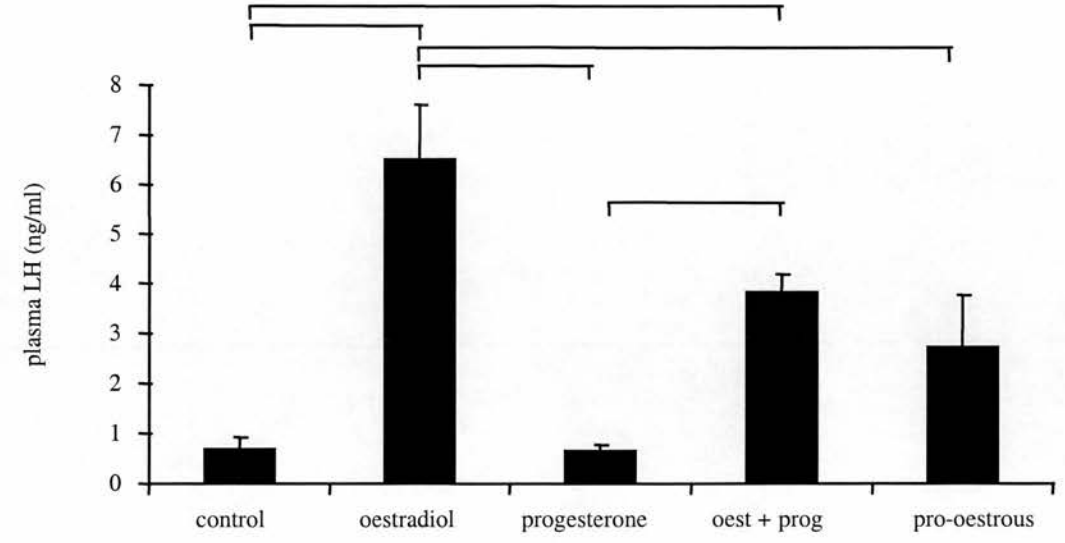
Treatment groups were as follows:

Treatment	**after OVX**	**noon next day**
control	oil	oil
oestradiol	oestradiol benzoate (30 μg)	oil
progesterone	oil	progesterone (2.5 mg)
oest + prog	oestradiol benzoate (30 μg)	progesterone (2.5 mg)
pro-oestrous	pro-oestrous rat with no steroid treatment	

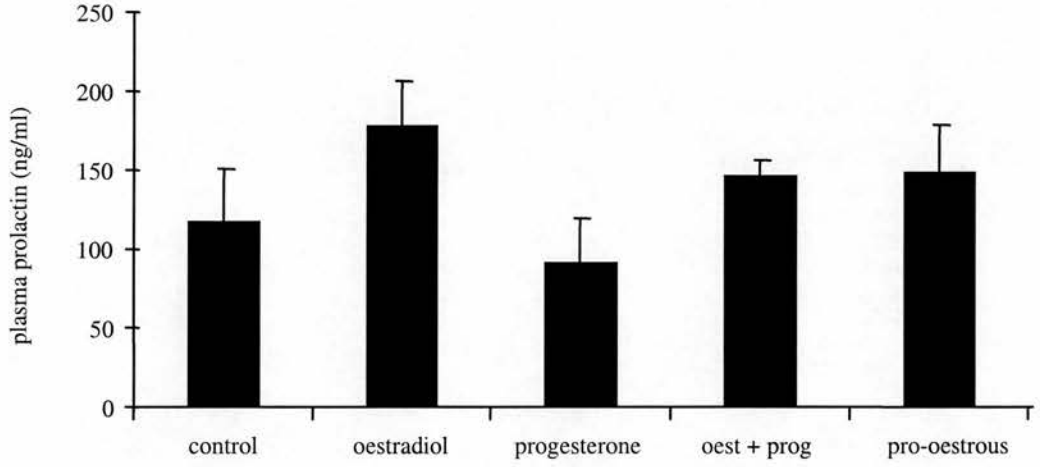
Significant differences in LH levels between groups are indicated by lines ($P<0.05$; one-way analysis of variance followed a by *post-hoc* Tukey test).

There were no significant differences in prolactin levels between any of the groups.

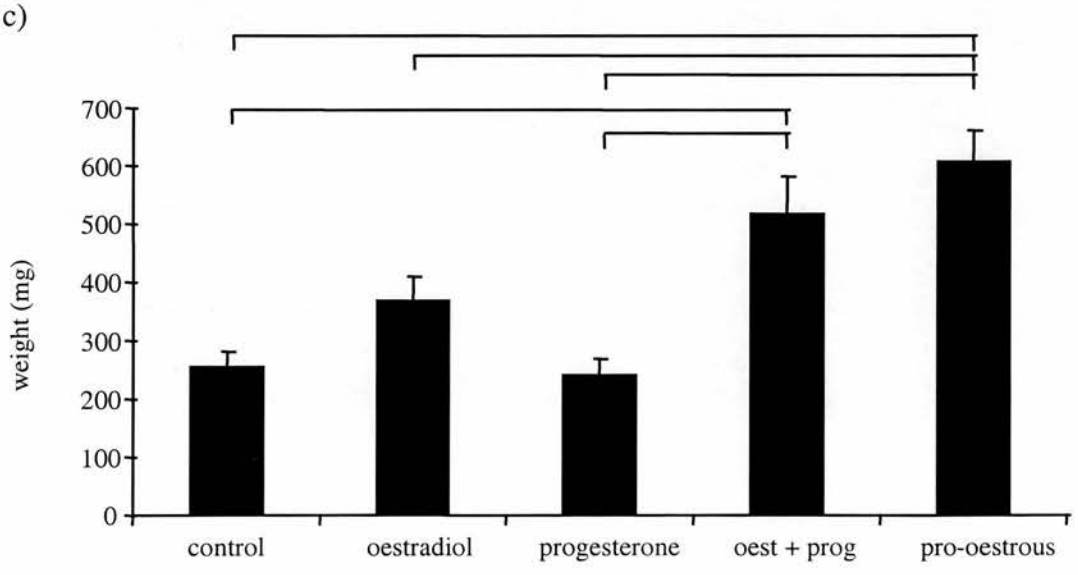
a)


plasma LH (ng/ml)
0
1
2
3
4
5
6
7
8
control
oestradiol
progesterone
oest + prog
pro-oestrous

b)


plasma prolactin (ng/ml)
0
50
100
150
200
250
control
oestradiol
progesterone
oest + prog
pro-oestrous

c)


weight (mg)
0
100
200
300
400
500
600
700
control
oestradiol
progesterone
oest + prog
pro-oestrous

Chapter 5

The effect of acute oestrogen treatment on the firing characteristics of putative 5-HT neurones in the rat dorsal raphe.

5.1 INTRODUCTION

The dorsal raphe nucleus is one of the main nuclei containing serotonergic cell bodies in the brain (Dahlstrom and Fuxe 1965, Steinbusch and Nieuwenhuys 1983) and gives rise to an extensive innervation of the forebrain including the amygdala, cortex, hippocampus, hypothalamus and thalamus (Azmitia and Segal 1978). In some regions innervated by the DR serotonergic neurotransmission has been shown to be altered during the oestrous cycle. In female rats, there is a decrease in 5-HT release in the hypothalamus during oestrus, a time when oestrogen levels are high (Gundlah *et al* 1998). Changes in serotonergic neurotransmission have also been observed after oestrogen treatment. For example, acute oestrogen treatment produces an increase in 5-HT_{2A} receptor binding sites in the terminal regions of DR projections including the frontal and cingulate cortex, olfactory tubercle and nucleus accumbens (Sumner and Fink 1995, Fink and Sumner 1996). Studies have also shown increased 5-HT transporter (SERT) binding site density in the BLA and the VMN after oestrogen treatment (McQueen *et al* 1997 and 1998). In the dorsal raphe itself, oestrogen has been shown to increase 5-HT levels (Di Paolo *et al* 1983), increase 5-HT_{2A} receptor binding site density (Sumner and Fink 1995) and increase the number of cells expressing SERT mRNA (McQueen *et al* 1997). These studies illustrate that oestrogen produces effects both at the level of the cell body and terminals and that oestrogen affects several aspects of serotonergic neurotransmission (i.e. release, reuptake and receptors).

Serotonergic neurones fire in a characteristic manner which was first described by Wang and Aghajanian (1977). These neurones fire spontaneously with a slow, regular discharge rate of 0.2-3 spikes s^{-1}. More recently, a second firing pattern has been reported for 5-HT neurones. This second type of 5-HT neurones, termed burst-firing neurones, were described as firing with the same action potential pattern as classical 5-HT neurones but occasionally a doublet or triplet was observed where only a single spike was expected (Hajos *et al* 1995b). It is believed these burst-firing cells are serotonergic since their firing is inhibited by paroxetine and they respond to denervation of the DR in a manner similar to that of traditional 5-HT neurones (Hajos *et al* 1995b, Hajos and Sharp 1996a). Burst-firing cells have been recorded throughout both the DR and the median raphe (MnR) and are reported to account for approximately 30% of 5-HT neurones in the DR (Hajos *et al* 1995b and 1996). The evidence so far suggests burst-firing neurones are a discrete subpopulation of 5-HT

neurones since cells do not alternate between being regularly firing and "burst-firing" (Hajos *et al* 1996).

The firing of 5-HT neurones is under autoregulatory control such that activation of somatodendritic 5-HT_{1A} receptors decrease the firing of the neurone (Sprouse and Aghajanian 1987). The presence of these autoreceptors could be due to the existence of 5-HT collaterals within the raphe nuclei. Studies have reported the existence of collaterals and suggested their involvement in a recurrent inhibitory pathway (Wang and Aghajanian 1977 and 1978). Stimulation of efferent 5-HT axonal pathways has been shown to produce inhibition of firing of 5-HT neurones in the DR (Wang and Aghajanian 1977). This inhibition was abolished after the destruction of 5-HT neurones using 5,7-dihydroxytryptamine (Wang and Aghajanian 1977) and was eliminated or reduced after a reduction in 5-HT levels using either parachlorophenylalanine (tryptophan hydroxylase inhibitor) or reserpine (depletes vesicles of transmitter) (Wang and Aghajanian 1978).

Previous studies have examined the effects of oestrogen on neuronal firing. However, these studies have centred around regions of the brain known to contain classical oestrogen receptors (i.e. ERα). Oestrogen has been shown to increase the spontaneous activity of striato-nigral neurones (Tansey *et al* 1983) and neurones in the medial nucleus of the amygdala (Schiess *et al* 1988). However, no change in firing rate was observed for neurones in various hypothalamic regions (including the MPOA, VMN and arcuate nucleus) after chronic oestrogen treatment (Bueno and Pfaff 1976). *In vitro* oestrogen has been shown to rapidly reduce calcium currents in neostriatal cells with the reduction observed being greater in cells from female rats than from male rats (Mermelstein *et al* 1996). Other studies have also shown rapid effects of oestrogen on electrical currents. In the CA1 region, oestrogen has been shown to produce a rapid (onset <1 minute) and reversible depolarisation in pyramidal neurones (Wong and Moss 1991) and rapidly potentiate kainate induced currents in these neurones (Gu and Moss 1996). This potentiation of kainate induced currents is reported to be mediated via G-protein coupled receptors (Gu and Moss 1998) and is independent of activation of oestrogen receptors ERα or ERβ (Gu *et al* 1999). Although oestrogen has been shown to affect kainate currents in CA1 neurones, neither acute (4/5 days) or chronic (2/3 weeks) treatment with oestrogen produced any change in 5-HT_{1A} mediated outward currents in these neurones (Dijcks *et al* 1994). This study also reported no change in several cell properties such as resting membrane potential and membrane capacity after acute or chronic oestrogen

treatment (although acute oestrogen did produce an increase in the size of the afterhyperpolarisation).

The aim of the experiments detailed in this chapter was to examine the effects of acute oestrogen on the firing characteristics of 5-HT neurones in the DR *in vivo*. This study examined two different aspects of serotonergic neurotransmission. The first part of the study investigated whether the firing patterns and *in vivo* discharge rates of DR neurones were similar in females to those previously reported in male rats (Hajos *et al* 1995b) and whether these characteristics were altered by oestrogen. The second part examined the activation of the recurrent inhibitory pathway (Wang and Aghajanian 1978) by sub-threshold stimulation of the MFB and determined whether the response of this pathway was altered by oestrogen treatment.

5.2 METHOD

The methodology used for extracellular recording of putative 5-HT neurones in the dorsal raphe is described in chapter 2 section 2.4. Plasma hormone levels were determined as detailed in chapter 2 section 2.4.5 for oestradiol and 2.5 for LH.

Pontamine sky blue (PSB) dye (2%) was included in the recording electrode in order to leave a dye mark showing the position of the recording electrode. A previous report has shown that the presence of PSB (2.5%) in the recording electrode has no affect on firing of 5-HT or noradrenaline neurones (Boakes *et al* 1974). At the end of the experiment, the position of both the stimulating and recording electrodes were verified histologically *post-mortem*. Only those neurones confirmed to be within the DR were included in this study. Cells in 2/74 (2.7%) animals were excluded on this basis. A schematic diagram of an electrode track is shown in figure 5.1 and illustrations of sections containing recording electrode marks are shown in figure 5.2.

5.3 RESULTS

5.3.1 Verification of steroid treatment

These experiments and subsequent analysis were carried out blind. In order to verify the treatment of the animal, whether oil or oestradiol, uterine weights and plasma oestradiol levels were examined. Oestrogen treatment is known to increase uterine weights (Aiyer and Fink 1974) and this was confirmed as animals in the OB-treated group had significantly higher uterine weights than oil controls (444.6 ± 34.3 mg vs. 270.8 ± 16.4 mg; mean ± s.e.m. n=13 in both groups; $P<0.005$ Wilcoxon Rank Sum). Animals in the OB-treated group also had significantly higher plasma oestradiol levels than oil controls (178.5 ± 33.8 pg ml^{-1} vs. 62.3 ± 28.9 pg ml^{-1}, n=13 in each group; $P<0.001$ Wilcoxon Rank Sum).

5.3.2 Cell classification

The cells examined in this study were identified as putatively serotonergic on the basis of firing patterns described by Wang and Aghajanian (1977) (i.e. they fire spontaneously with a slow, regular discharge rate of 0.2-3 spikes s^{-1}). Examples of a recording of the firing rate from a typical 5-HT neurone (approximately 1.5 Hz) and a non-serotonergic neurone (approximately 20 Hz) are shown in figure 5.3. Cells which were defined as non-serotonergic constituted 4.8% of all cells recorded. Any putative serotonergic cells found in the DR were characterised by their firing pattern and by stimulation of the medial forebrain bundle (MFB) to establish that the neurones projected to the forebrain. Only antidromically-activated cells (85.2% of putative serotonergic cells) were included in this study. An illustration of an antidromically-activated cell is shown in figure 5.4.

5.3.3 Resting Firing characteristics

Cells in the OB-treated group had a longer antidromic spike latency in response to MFB stimulation (10.4 ± 0.4 ms, n=100) than cells in the oil control group (9.5 ± 0.4 ms, n=153; $P<0.05$ Wilcoxon Rank Sum) (table 5.1). No differences were observed between cells in the oil control group and those in the OB-treated group with reference to the threshold voltage required to initiate an antidromic spike. The absolute values for threshold voltages were 31.1 ± 1.7 V (n=153) for oil controls and 29.9 ± 2.1 V (n=100) in the OB-treated group (table 5.1).

Of the antidromically-activated cells, 98% (150/153) of oil controls and 92% (92/100) of cells in the OB-treated group were found to be spontaneously active. The resting firing rate of stable spontaneously active cells was not significantly different between cells in the oil control (1.4 ± 0.1 spikes s^{-1}, n=145) and OB-treated groups (1.3 ± 0.1 spikes s^{-1}, n=92) (table 5.1). Five cells in the oil control group were lost too early in the recording period for firing rates to be estimated.

As described by Hajos *et al* (1995b), the spontaneously active cells in both groups could be separated into two subtypes on the basis of their action potential firing pattern. These two cell types were regularly firing cells, which fired single action potentials at regular time intervals, and doublet-firing cells which also fired regularly but occasionally fired a doublet or triplet where a single action potential was expected (figure 5.5). In both groups, approximately two thirds of cells were regularly firing (69% of oil controls and 67% of cells in the OB-treated group), agreeing with previous data from male rats (Hajos *et al* 1995b, Hajos and Sharp 1996a). Although the proportion of doublet-firing cells was similar in the two groups, the behaviour of these cells differed. In the OB-treated group, the doublet-firing cells fired fewer doublets per minute (3.7 ± 0.5 doublets min^{-1}, n=30) than cells in the oil control group (9.3 ± 1.7 doublets min^{-1}, n=47; $P<0.05$, Wilcoxon Rank Sum) (Table 5.1).

There was no difference in antidromic spike latency between regularly firing cells and doublet-firing cells within the same treatment group (table 5.1). Similarly, no differences were found between the threshold voltages of regularly firing cells and doublet-firing cells in either treatment group (table 5.1). Also, no obvious pattern was observed in either the time from which doublet-firing cells were recorded or their depth and position within the DR, suggesting these cells are spread throughout this nucleus.

5.3.4 Effect of sub-threshold stimulation

The effect of sub-threshold stimulation of the MFB was tested in 86 cells from the oil control group and 56 cells in the OB-treated group. A schematic representation of the experimental set-up is shown in figure 5.6. The purpose of sub-threshold stimulation of the MFB was to activate the recurrent inhibitory pathway in the DR (Wang and Aghajanian 1978). In the majority of cells tested, 47 (55%) of oil

controls and 37 (66%) of OB-treated cells, inhibition was observed. Most of the remaining cells showed no effect (37 oil controls, 18 OB-treated), with 2 cells in the oil control group and one cell in the OB-treated group showing excitation (table 5.2). Examples of post-stimulus time histograms illustrating no effect (a) and inhibition (b) in response to sub-threshold stimulation of the MFB are shown in figure 5.7.

In the cells which showed inhibition, there was no difference in the latency of effect between the two groups (26.2 ± 4.1 ms in oil controls, n=47 and 23.4 ± 3.3 ms in the OB-treated group, n=37) (table 5.2). The mean duration of inhibition was not significantly different between the oil controls and the OB-treated group, 104.6 ± 12.6 ms, n=47 and 121.0 ± 11.7 ms, n=37 respectively. However 17/47 (36%) of oil control cells showed a duration of inhibition <50 ms whereas only 3/37 (8%) of cells in the OB-treated group did and this difference in distribution (figure 5.8) was found to be significant ($P<0.005$, Fisher's Exact Test). In the oil control group both regularly and doublet-firing cells displayed a duration of inhibition <50 ms, while only regularly firing cells in the OB-treated group did so (figure 5.8).

In order to examine the effect of drugs on the duration of inhibition, the cell would need to be stimulated for a second time. Initially, a second stimulation was given to ensure this process alone did not affect the duration of inhibition. These experiments showed that repeated sub-threshold stimulation produced no significant changes in either latency or duration of inhibition in either treatment group (figure 5.9a). Initial experiments examined the effect of the 5-HT_{1A} partial agonist NAN-190 (Hodgkiss *et al* 1992) (5 μg kg^{-1} i.v. in saline) on both the latency and duration of inhibition. Administration of NAN-190 one hundred seconds prior to the repeated stimulation produced no change in either the latency or duration of the inhibition (figure 5.9b). Subsequent experiments examined the effect of the 5-HT_{1A} antagonist WAY 100,635 (Fletcher *et al* 1996) (1 mg kg^{-1} i.v. in saline) on sub-threshold stimulation. Administration of WAY 100,635 one hundred seconds prior to the repeated stimulation produced an abolition of inhibition in 2 oil control cells and one cell in the OB-treated group, a reduction in the duration of inhibition in 3 cells in both treatment groups, and had no effect on one cell in the oil control group (figure 5.10). Administration of saline (vehicle) one hundred seconds prior to repeated sub-threshold stimulation had no effect on either the latency or duration of the inhibition.

5.4 DISCUSSION

Firstly, the results from the present study of ovariectomised female rats show that oestrogen treatment significantly increased the mean antidromic latency in response to MFB stimulation. Secondly, the study showed that doublet-firing cells occur in female rats in similar proportions to their occurrence in male rats (Hajos *et al* 1995b) and that the frequency of doublet-firing was reduced by oestrogen treatment. Thirdly, sub-threshold stimulation of the MFB produced inhibition of firing in the majority of cells tested with fewer cells displaying short duration of inhibition to sub-threshold stimulation after oestradiol treatment.

5.4.1 Firing Characteristics

In these experiments, oestrogen treatment produced an increase in the mean antidromic latency to MFB stimulation which suggests a reduction in the axonal conduction velocity in oestrogen OB-treated animals. A possible mechanism for this decrease in conduction velocity is modulation of potassium channels. At $GABA_B$ and μ-opioid receptors, oestrogen has been shown to decrease the potency of agonists resulting in the attenuation of coupling of these receptors to potassium channels (Lagrange *et al* 1994, Lagrange *et al* 1996). However, oestradiol has also been reported to increase potassium conductance in CA1 neurones (Nabekura *et al* 1986) and in T84 (human colonic epithelial) cells (Condliffe *et al* 1998). However, an alteration of potassium currents would be expected to affect the resting firing rate, but this was not observed in the OB-treated group in this study. Similarly, oestrogen has previously been found to have no effect on the firing rate of cells in the medial preoptic area and medial anterior hypothalamus (Bueno and Pfaff 1976). A second possible mechanism for this decrease in conduction velocity is a steroidal interaction with the axon membrane since a previous study has shown progesterone increases orthodromic latency in medial preoptic neurones (Watanabe 1992).

The ratios of doublet-firing cells to regularly firing cells found in both the oil control and OB-treated groups were similar to those previously described in male rats (Hajos *et al* 1995b, Hajos and Sharp 1996a). It has been previously reported (Hajos and Sharp 1996b) that doublet-firing cells have a shorter antidromic latency compared to regularly firing cells. This difference was not observed in our study and may reflect a sex difference. At present, there is no evidence to suggest regularly and doublet-firing cells innervate different regions of the brain (Hajos and Sharp 1996b). Despite

this, due to the short interspike interval between doublets, doublet-firing cells may have a role in transmitter release. Evidence supporting this idea comes from the hippocampus where two pulses which occur within a short time interval (8 ms) can produce reliable transmitter release at a synapse (Stevens and Wang 1995). Also, a recent study examining 5-HT release in the DR has shown that two stimuli given within a short time interval (10 ms apart) released twice as much transmitter as a single pulse (Bunin and Wightman 1998). The decrease in the frequency of doublet-firing produced by oestradiol (34% of control values) may cause a decrease in 5-HT release at the terminal regions of DR neurones. A decrease in 5-HT release has been reported in the hypothalamus of female rats during oestrus when oestradiol levels are high (Gundlah *et al* 1998). However, other studies examining 5-HT in the forebrain have found no change in the 5-HT content (Di Paolo *et al* 1983) or a change in the extracellular 5-HT concentration (Farmer *et al* 1996).

The mechanism underlying the production of doublets in 5-HT neurones in the DR is unknown. However, doublet-firing in other neurones has been linked to low threshold calcium potentials and currents (Eustache and Gueritaud 1995, Chen *et al* 1996 respectively). Oestradiol has previously been shown to inhibit a high voltage-activated calcium current in rat neostriatal neurones (Mermelstein *et al* 1996). Hence, a reduction in calcium currents could be one possible explanation for the decrease in frequency of doublet-firing observed in 5-HT neurones in this study. However, observations in the dentate gyrus and olfactory bulb suggest that burst firing is regulated by cholinergic neurones. In these regions it has been reported that centrally acting muscarinic antagonists blocked burst firing (Heale and Vanderwolf 1994). This raises the possibility that burst firing is linked to afferent inputs to the burst firing cells. This hypothesis would help to explain why doublet-firing 5-HT neurones are not observed in dorsal raphe slices *in vitro* (Hajos personal communication).

5.4.2 Sub-threshold stimulation of the MFB

The overall effect in both the oil control and OB-treated groups to sub-threshold stimulation of the MFB was similar, with the majority of cells in each treatment group showing inhibition. However, there are fewer cells displaying a short duration of inhibition (<50 ms) in the OB-treated group. Oestradiol may be affecting doublet-firing cells preferentially since a short duration of inhibition was displayed by both cell types in the oil control group but only displayed by regularly firing cells in the

OB-treated group. However, this observation may be a result of the small number of cells with a short duration of inhibition in the OB-treated group (n=3).

The firing of DR neurones is regulated by the action of 5-HT on 5-HT_{1A} autoreceptors. Activation of the 5-HT_{1A} somatodendritic autoreceptor results in an inhibition of cell firing (Sprouse and Aghajanian 1987). A decrease in the potency of the 5-HT_{1A} agonist 8-OH DPAT at somatodendritic 5-HT_{1A} autoreceptors has been reported in female rats treated with oestrogen for 48 hours (Lakoski 1988) and in rats during pro-oestrus and oestrus (Uphouse *et al* 1991). This reduced effectiveness of 8-OH DPAT may be due to receptor desensitisation, as acute oestrogen treatment (12 and 24 hours) increases 5-HT levels in the DR (Di Paolo *et al* 1983), or a change in the proportion of active receptors, as 8-OH DPAT preferentially binds to receptors which are linked to G-proteins (Hall *et al* 1985). The decreased potency of agonists at the 5-HT_{1A} receptor suggests the firing rate of DR neurones should have increased after oestrogen treatment, but this was not observed in this study.

Stimulation of the MFB has been suggested to cause the local release of 5-HT from recurrent collaterals within the DR. Evidence to support this comes from the observation that stimulating the 5-HT axonal pathway in this region produces inhibition of dorsal raphe cell firing (Wang and Aghajanian 1997) and that blocking 5-HT synthesis or storage results in a reduction or abolition of this inhibition (Wang and Aghajanian 1978). It should be noted that not all cells in this study showed inhibition in response to sub-threshold stimulation. The lack of effect in some cells may be due to the sub-threshold stimulus not being strong enough to elicit a response. It may also be the result of the cell which was activated not having synaptic contact with the cell under study. The fact that inhibition was reproducible, as shown by repeated sub-threshold stimulation, suggests the mediators of the inhibition are rapidly removed from their site of action. The lack of effect of NAN-190 on the latency or the duration of inhibition may result from the drug displaying different properties at postsynaptic and somatodendritic 5-HT_{1A} receptors. At the postsynaptic 5-HT_{1A} receptor, NAN-190 behaves as an antagonist and has been shown to antagonise the inhibition of phosphoinositide turnover produced by 8-OH DPAT in hippocampal slices (Claustre *et al* 1991). Similarly, *in vivo* NAN-190 has been shown to antagonise 8-OH DPAT induced behaviour in reserpinised rats (Hjorth and Sharp 1990). However, in the dorsal raphe NAN-190 has a partial agonist action producing hyperpolarisation of 5-HT neurones when given alone, but reducing the response to application of 5-HT or 8-OH DPAT (Hodgkiss *et al* 1992).

In vivo NAN-190 has been shown to inhibit 5-HT release in the hippocampus although the level of inhibition was 50% of that produced by 8-OH DPAT (Hjorth and Sharp 1990).

The attenuation of the duration of inhibition seen with most cells (9/10) after the administration of WAY 100,635 suggests that the inhibition produced by sub-threshold stimulation is, at least in part, due to the action of 5-HT on 5-HT_{1A} autoreceptors. However, it has been suggested that the inhibition of DR neuronal firing produced by stimulation of the medial prefrontal cortex (mPFC) is not due to the activation of 5-HT autoreceptors, but to the action of glutamate on GABA interneurones (Hajos *et al* 1998). GABA interneurones have been shown to occur within the DR (Gallager 1978, Nanopolous *et al* 1982, Wang *et al* 1992). Activation of these GABA interneurones by recurrent collaterals may contribute to the inhibition observed and hence explain why WAY 100,635 was only partially effective in blocking the inhibition. Previous studies have shown that oestrogen reduces GABAergic inhibitory postsynaptic currents in the hippocampus (Murphy *et al* 1998) and reduces agonist potency at $GABA_B$ receptors in the hypothalamus (Lagrange *et al* 1996). These findings suggest modulation of GABAergic neurotransmission within the DR could also be a factor in the differences in duration of inhibition observed between the two treatment groups in this study.

5.4.3 Mechanism of action of oestrogen

The experiments in this study were conducted 24-32 hours after oestradiol treatment. Therefore, the effects observed could be due to either genomic or non-genomic mechanisms. The genomic actions of oestrogen are mediated by intracellular oestrogen receptors. It is now known that two types of intracellular oestrogen receptor exist, namely the classical oestrogen receptor (ERα) and the recently discovered ERβ (Kuiper *et al* 1996). The DR contains few oestrogen concentrating cells (Pfaff and Keiner 1973). However, it is unclear which of the two oestrogen receptors this nucleus contains. In the DR, mRNA for ERβ but not ERα was detected at low levels by Shughrue *et al* (1997a). However, a second study detected ERα but not ERβ immunoreactivity in this nucleus (Alves *et al* 1998). These findings suggest ERα mRNA may be produced outside the DR, but that the receptor protein is transported to this region. Evidence to support this hypothesis comes from the observation that ERα immunoreactivity was not found on 5-HT neurones and may be located on GABA interneurones (Alves *et al* 1998). If oestrogen is not

having a direct genomic effect on 5-HT neurones within the DR, it may be acting on GABA interneurones located in the DR via either ERα or ERβ. There also remains the possibility that the genomic effects of oestrogen are the result of oestrogen acting outside the DR, on neurones which project to the DR. The PAG contains GABA interneurones which project to the DR and also contains both intracellular oestrogen receptors, ERα and ERβ (Shughrue *et al* 1997a). The locus coeruleus (LC) in rats also contains both ERα and ERβ (Shughrue *et al* 1997a) and noradrenergic neurones from the LC are known to innervate the DR and drive 5-HT firing (Baraban *et al* 1978, Baraban and Aghajanian 1980). The involvement of the LC in the genomic actions of oestrogen could be ascertained by carrying out DR recordings in rats with lesions in the LC itself or the pathway from the LC to the DR.

A second mechanism of action for oestrogen is via non-genomic actions. Membrane binding sites for oestrogen have been reported in crude synaptosomal membrane preparations from the cerebellum, olfactory bulb and hypothalamus of female rat brain (Zheng and Ramirez 1997). The action of oestrogen on membrane sites has been shown to reduce calcium currents in neostriatial neurones probably via G-protein activation (Mermelstein *et al* 1996) and may relate to the observed reduction in doublets fired by the doublet-firing cells in the DR in this study. Oestrogen has also been shown to affect second messenger systems and has been reported to potentiate kainate-induced currents in CA1 neurones via activation of the cAMP cascade (Gu and Moss 1996). The above evidence suggests doublet-firing could be linked to the effect of oestradiol on a membrane site on DR neurones themselves. This theory would require testing using *in vitro* or *in vivo* intracellular recording techniques. However, it should be remembered that the timing protocol used in this study has previously produced genomic changes in the DR with increases in the expression of mRNA for the 5-HT_{2A} receptor (Sumner and Fink 1993) and SERT (McQueen *et al* 1997). The increase in 5-HT_{2A} receptor mRNA expression is accompanied by a corresponding increase in 5-HT_{2A} receptor density in the DR, which suggests the mRNA is translated to functional receptors. Whether this increase is a consequence of, or a factor in, the generation of the observed electrophysiological changes is unknown. Activation of 5-HT_{2A} receptors on 5-HT neurones in the DR causes depolarisation (Davies *et al* 1988), hence activation of these 5-HT_{2A} receptors would increase the likelihood of DR neurones firing. The increase in 5-HT within the DR after oestrogen treatment (Di Paolo *et al* 1983) would be expected to act on 5-HT_{1A} receptors to decrease firing, hence the increase in 5-HT_{2A} receptors may be opposing this effect. This may help to explain why no

change in firing was seen in this study. The increased levels of 5-HT in the DR could also be counteracted by removal by SERT. However, no increase in SERT binding sites is seen in the DR after oestradiol (McQueen *et al* 1997 and 1998). The overall picture of changes in serotonergic neurotransmission in response to oestrogen is complex and some of the effects seen may be a reaction to maintain homeostasis.

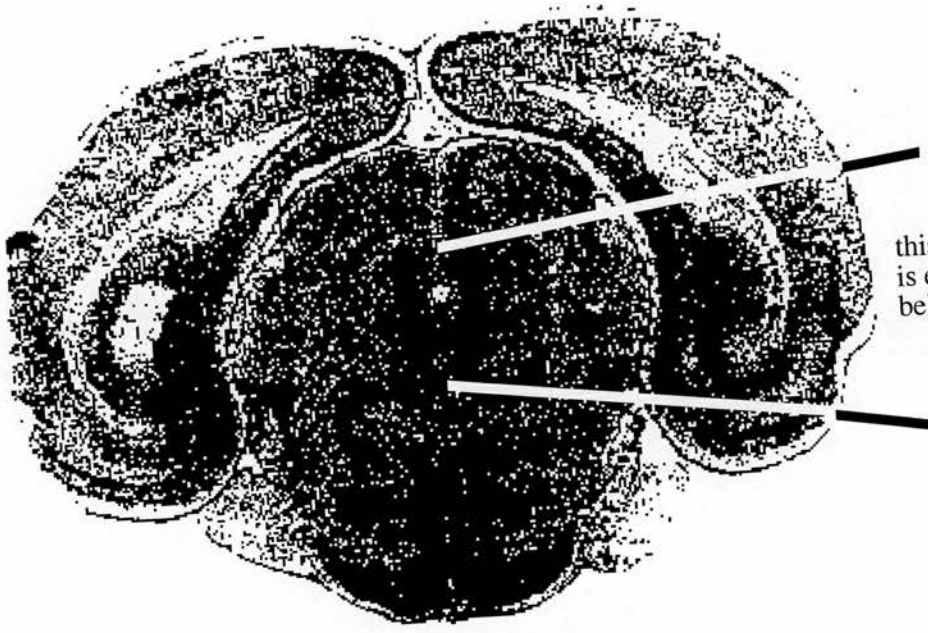
In conclusion, the present study demonstrates that doublet-firing cells are found in the DR in female rats in similar proportions to those reported previously in males and that oestrogen treatment produces a decrease in the number of doublets fired per minute by these cells. It is possible that the decrease in doublets fired reported here may have practical implications for transmitter release in the projection areas of the DR. Sub-threshold stimulation of the MFB produces inhibition of firing in the majority of cells and appears to be, at least in part, the result of 5-HT_{1A} receptor activation. The observation that fewer cells in the OB-treated group show a short duration of inhibition in response to sub-threshold stimulation of the MFB suggests an enhanced recurrent inhibition of DR neuronal firing with oestrogen treatment. The effects of oestrogen treatment on dorsal raphe neurones shown here could result from either non-genomic or genomic actions of oestrogen. These changes in 5-HT neurotransmission could originate within the dorsal raphe itself by the action of oestrogen on GABA interneurones or on the 5-HT neurones themselves. However it is also possible that the effects of oestrogen could originate in regions which are outside the DR but which project to it such as the PAG or the LC.

Figure 5.1:

Diagrams of coronal sections of rat brain which contain the dorsal raphe, adapted from Paxinos and Watson (1986). The top diagram shows a coronal section through rat brain at the level of the dorsal raphe (-7.64 mm from bregma). The lines indicate the area which is expanded in the bottom diagram. The bottom diagram is a diagrammatic representation of a track through the DR in one brain, each cell is represented by a number (1-5) and the position of each cell is marked by a line crossing the electrode track. In this particular track cells 1 and 5 were doublet-firing and cells 2 to 4 were regularly firing cells. An illustration of an actual electrode track is shown in figure 5.2 (b).

Abbreviations:

CLi	caudal linear nucleus of the raphe
DRD	dorsal region of dorsal raphe nucleus
DRV	ventral region of dorsal raphe nucleus
DRVL	ventrolateral region of dorsal raphe nucleus
mlf	medial longitudinal fasciculus
PAG	periaqueductal gray
xscp	decussation of the superior cerebellar peduncle


this region
is expanded
below

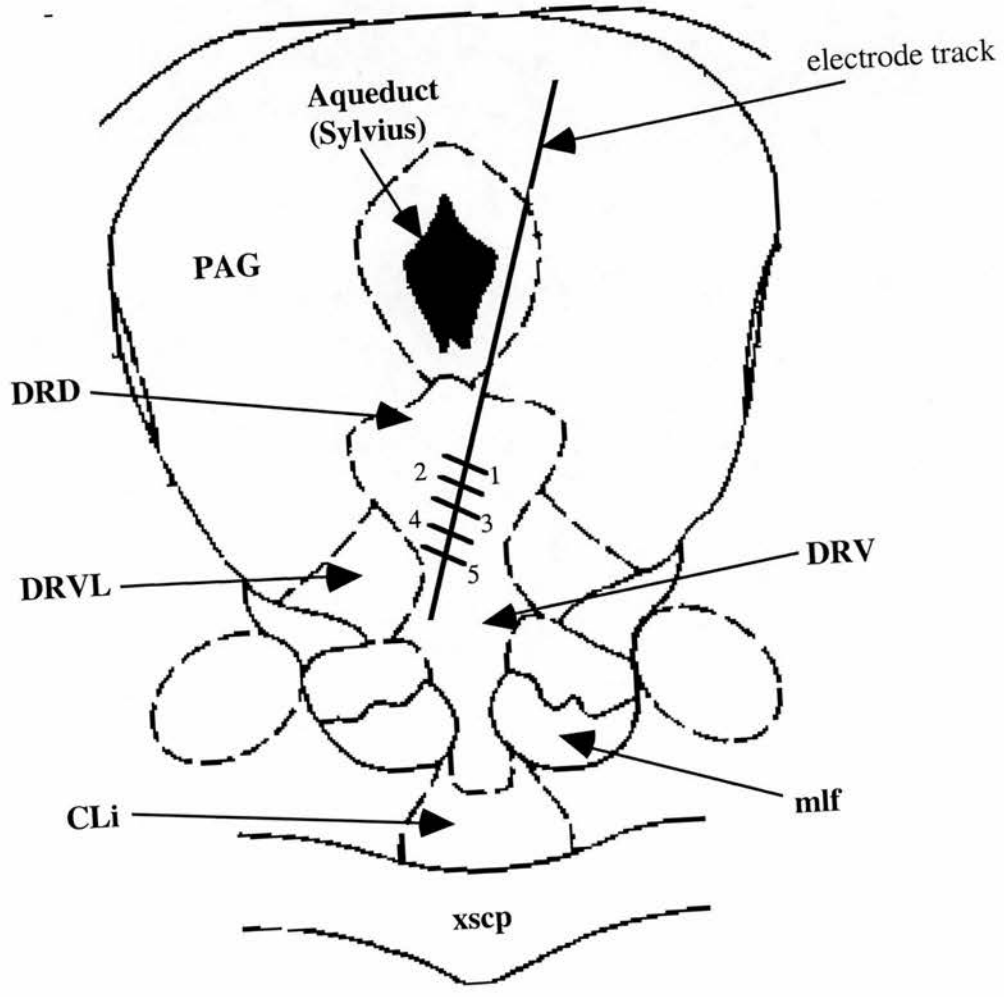
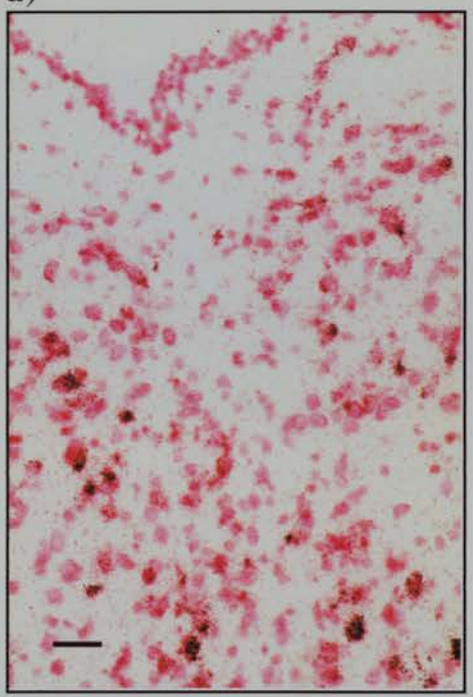

electrode track
Aqueduct
(Sylvius)
PAG
DRD
2
1
4
3
5
DRV
DRVL
mlf
CLi
xscp

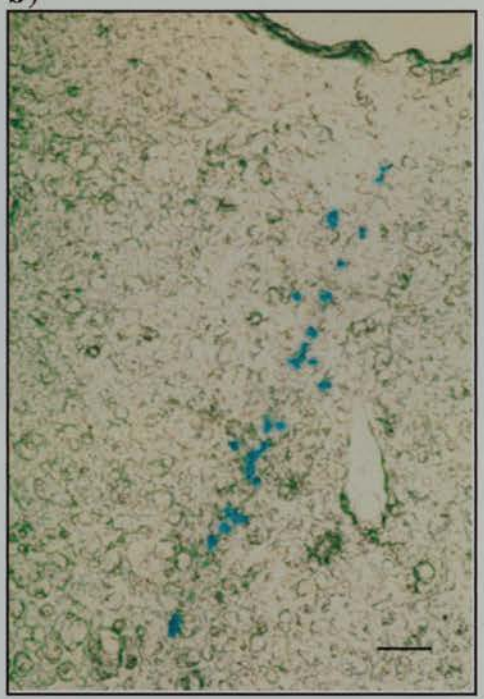
Figure 5.2:

Examples of tissue sections containing the dorsal raphe. These images are taken from slides of coronal sections through rat brain at -7.5 to -7.64 mm from bregma (Paxinos and Watson 1986). Sections are orientated such that the aqueduct (see figure 5.1) is located at the top of each picture. Slide (a) is from a pyronin counterstained tissue section which has undergone *in situ* hybridisation to detect mRNA for the 5-HT transporter and illustrates the presence of serotonergic cell bodies. Slides (b) to (d) show sections taken from the brains of rats from this study. Slide (b) shows a dye track left by pontamine sky blue indicating the electrode passed through the DR. The section in slide (b) has not been counterstained. Slides (c) and (d) show the localisation of blue dye spots, indicated by the arrows, within the DR of neutral red counterstained sections. The blue dye spots indicate the position of tip of the recording electrode. In slides (a) to (c) the scale bar indicates 50 μm and in slide (d) the scale bar indicates 400 μm.

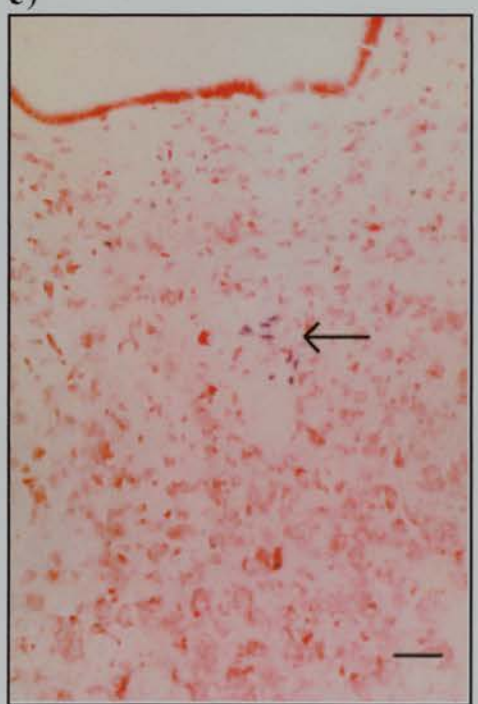
a)



b)



c)



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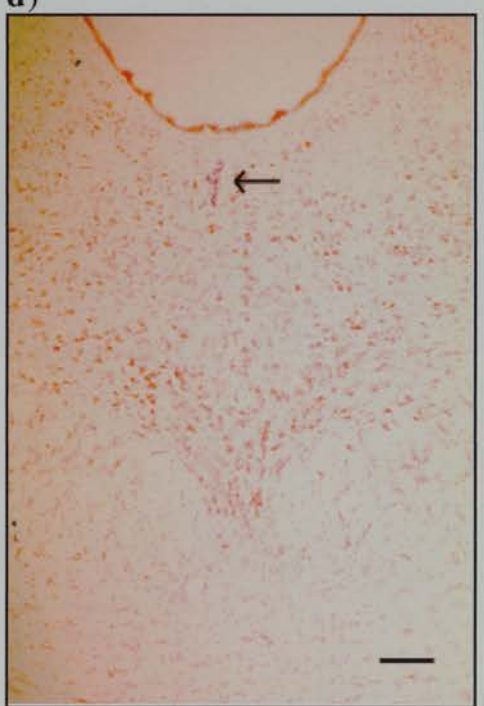
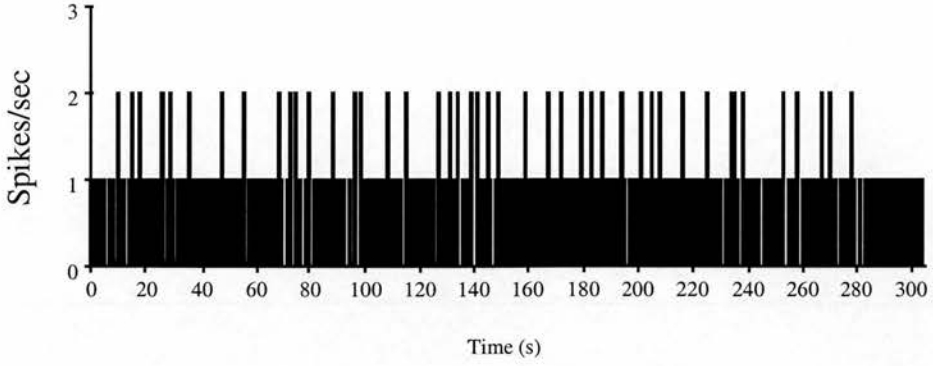


Figure 5.3:

Examples of firing rates of a serotonergic cell and non-serotonergic cell recorded within the dorsal raphe in an ovariectomised female rat treated with oil and anaesthetised with 1.1g kg^{-1} urethane i.p. Diagram (a) shows a serotonergic cell firing at approximately 1.5 Hz. Diagram (b) shows a non-serotonergic cell which had a frequency of approximately 20 Hz.

a)


Spikes/sec
3
2
1
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Time (s)

b)

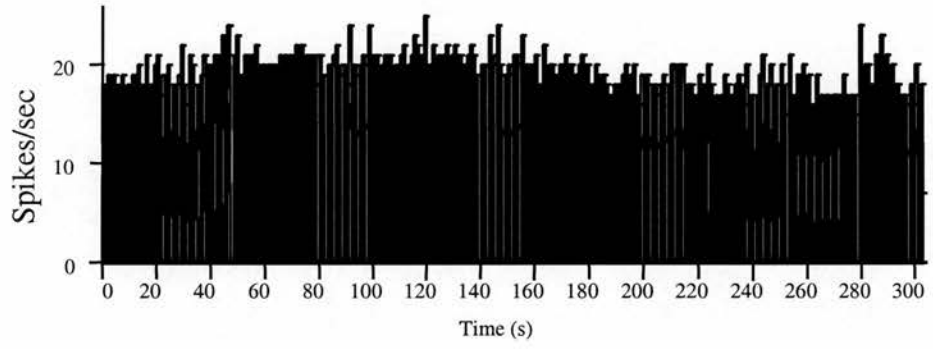

Spikes/sec
20
10
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Time (s)

Figure 5.4:

Diagram illustrating an antidromic spike resulting from stimulation of the MFB in an oil control animal. The black arrow indicates the point at which stimulation was applied. A stimulus artefact was produced and was followed by the antidromic spike, also indicated by an arrow.

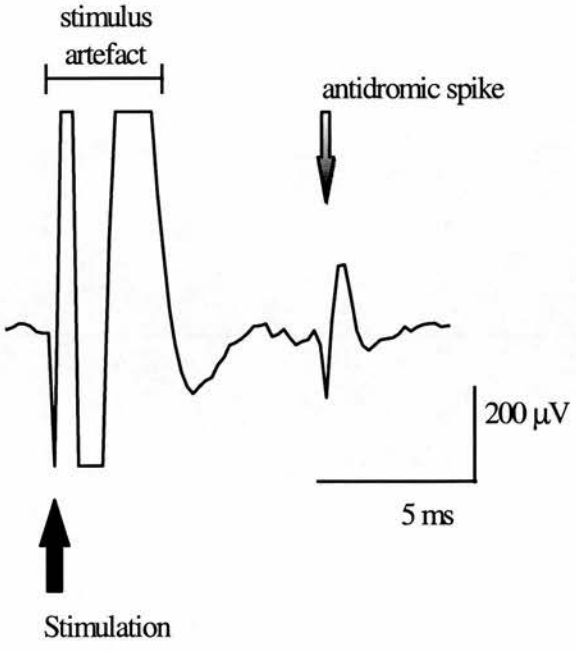

stimulus
artefact
antidromic spike
200 μV
5 ms
Stimulation

Table 5.1:

Table showing resting firing characteristics of antidromically-activated cells in the oil control and OB-treated groups.

Data are shown as mean ± s.e.m.

* indicates $p<0.05$ (Wilcoxon Rank Sum) compared to control

		oil control	OB-treated
Antidromic spike latency		9.5 ± 0.4 (n= 153)	10.4 ± 0.4 (n=100) *
Threshold voltage (V)		31.3 ± 1.7 (n=153)	29.9 ± 2.1 (n=100)
% antidromic cells which were spontaneously active		98 (150/153 cells)	92 (92/100 cells)
resting firing rate (spikes s^{-1})		1.4 ± 0.1	1.3 ± 0.1
doublets/minute fired by doublet-firing cells		9.3 ± 1.7 (n=47)	3.7 ± 0.5 (n=30) *
Antidromic spike latency (ms)	regular	9.5 ± 0.5 (n=103)	10.1 ± 0.5 (n=62)
	doublet	9.6 ± 0.5 (n=47)	10.8 ± 0.7 (n=30)
Threshold voltage (V)	regular	31.5 ± 2.0 (n=103)	31.8 ± 3.1 (n=62)
	doublet	31.2 ± 3.3 (n=47)	27.0 ± 3.1(n=30)

Figure 5.5:

Characterisation of resting firing characteristics in regularly firing and doublet-firing putative 5-HT neurones in the DR. Typical firing patterns of a regularly firing cell (A) and a doublet-firing cell (B) are illustrated. The differences in timescale between trace (A) and (B) reflects a difference in the firing rate of the two neurones. The asterisks on trace B indicate doublets which due to the timescale only appear as single spikes, the doublets are shown in expanded form in (C). (D) and (E) illustrate interval spike histograms for a regularly firing cell (D) and a doublet-firing cell (E) which have similar firing rates. These histograms illustrate the time difference between adjacent action potentials, the arrow in (E) highlights the short interspike interval between doublets.

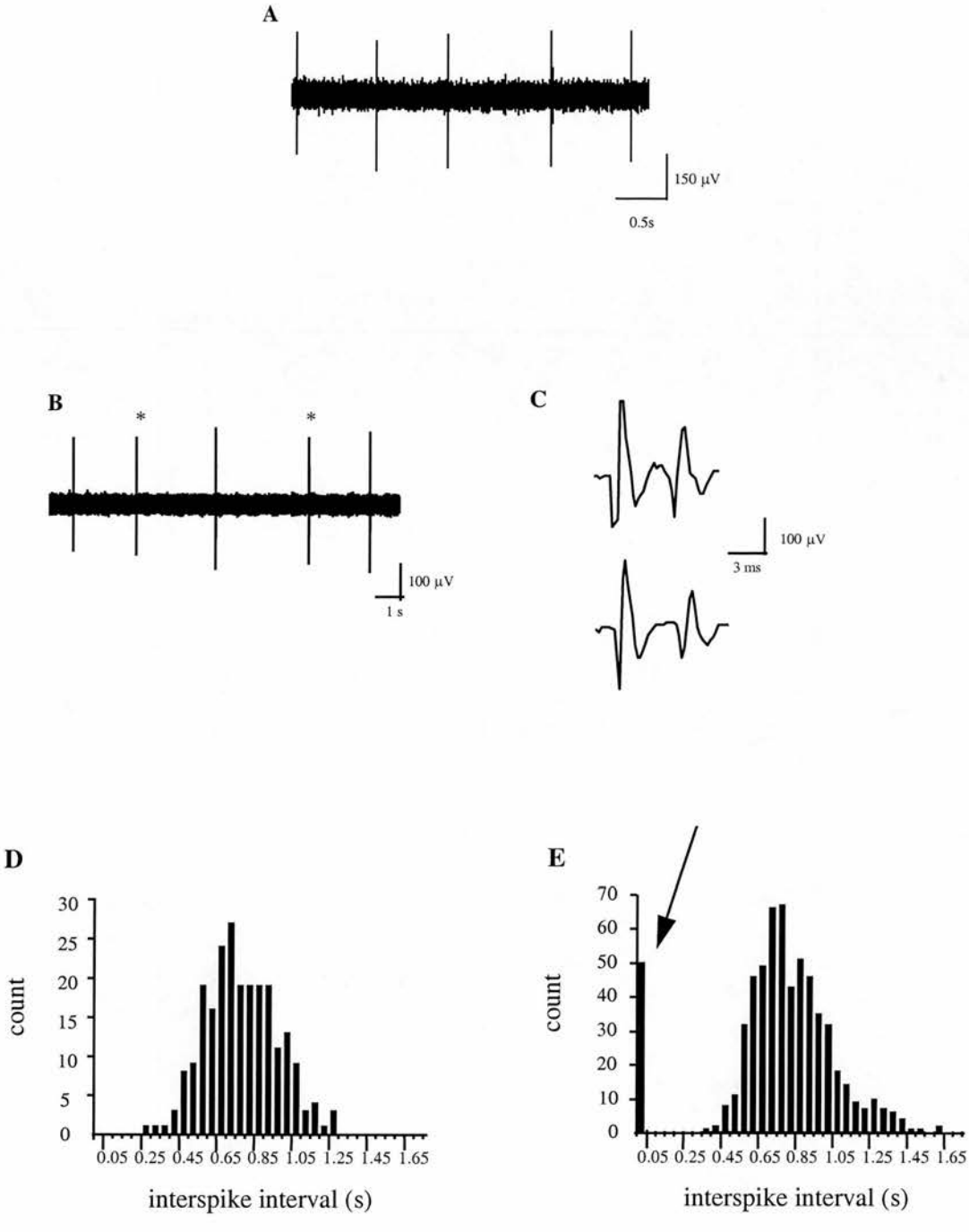

A
150 µV
0.5s
B
*
*
100 µV
1 s
C
100 µV
3 ms
D
count
30
25
20
15
10
5
0
0.05 0.25 0.45 0.65 0.85 1.05 1.25 1.45 1.65
interspike interval (s)
E
count
70
60
50
40
30
20
10
0
0.05 0.25 0.45 0.65 0.85 1.05 1.25 1.45 1.65
interspike interval (s)

Figure 5.6:

Schematic diagram showing the placement of the stimulating electrode in relation to the axons originating from dorsal raphe 5-HT neurones. The recording electrode was placed in the DR and the stimulating electrode was placed in the MFB. The diagram illustrates recurrent collaterals originating from DR neurones which synapse onto the cell bodies of 5-HT neurones within the DR itself.

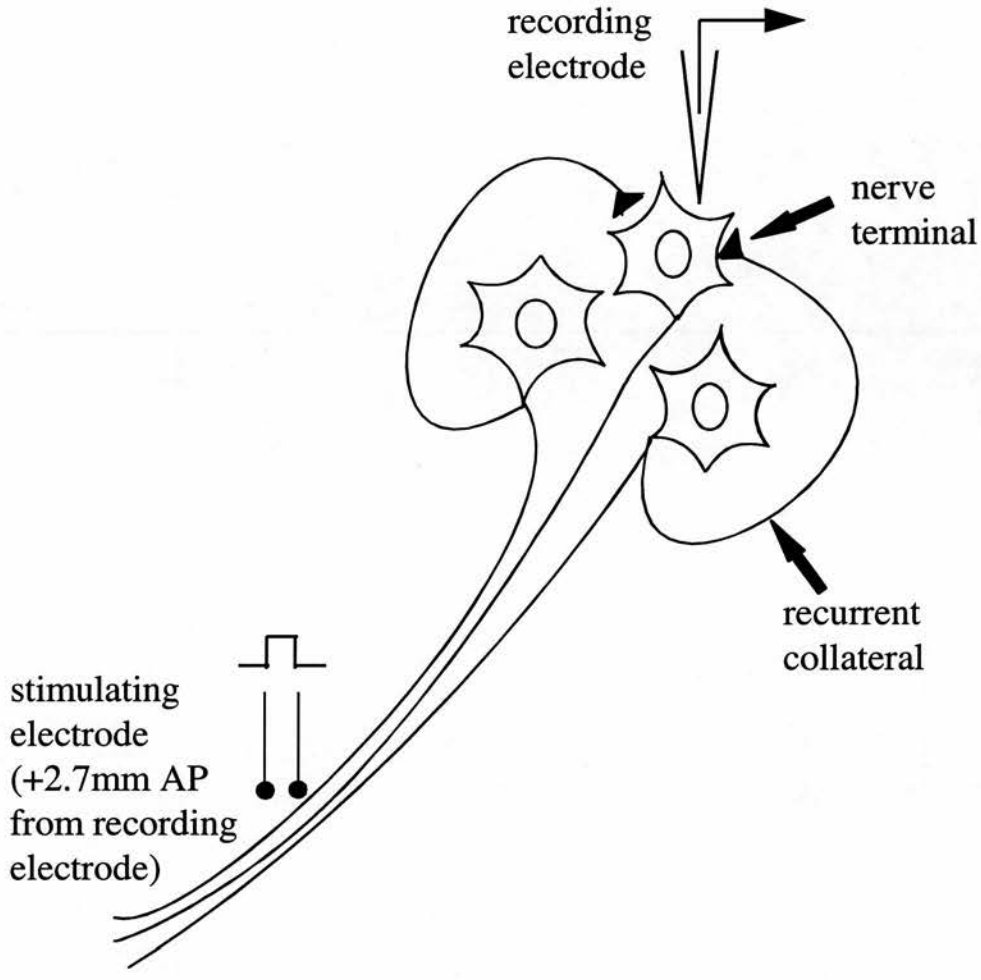

recording
electrode
nerve
terminal
recurrent
collateral
stimulating
electrode
(+2.7mm AP
from recording
electrode)

Table 5.2:

The effect of subthreshold stimulation of the MFB on the firing of cells in the oil control and OB-treated groups.

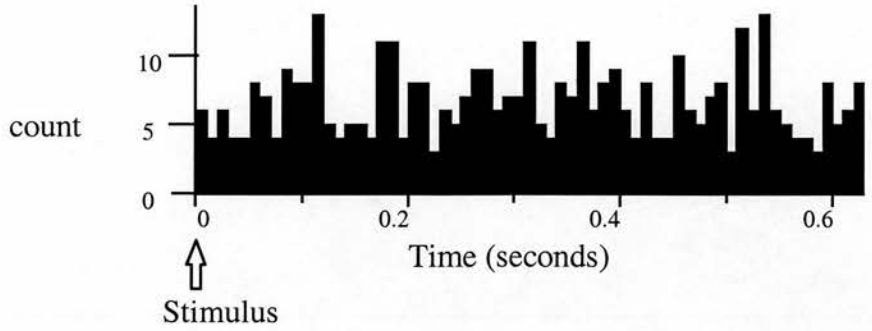
Data shown as mean ± s.e.m.

	oil control	OB-treated
number of cells in which subthreshold stimulation tested in	86	56
no. of cells where inhibition was observed	47 (55%)	37 (66%)
no. of cells where no effect was observed	37 (43%)	18 (32%)
no. of cells where excitation was observed	2 (2%)	1(2%)
latency of effect for inhibition (ms)	26.2 ± 4.1	23.4 ± 3.3
mean duration of inhibition (ms)	104.6 ± 12.6	121.0 ± 11.7

Figure 5.7:

Examples of post-stimulus time histograms (PSTH) from cells in the control group illustrating no effect (a) and inhibition (b) in response to sub-threshold stimulation of the MFB. These histograms show the time between the stimulus and the next action potential fired by the cell. If the stimulus has no effect on cell firing the next action potential after the stimulus will occur at a random point in time hence the bin size in the histogram will be random, as illustrated in (a). In diagram (b) there is a time window where the cell has been inhibited from firing. The inhibition produced does not occur directly after the stimulus but has a lag time known as the latency.

a)


count
10
5
0
0
0.2
0.4
0.6
Time (seconds)
Stimulus

b)

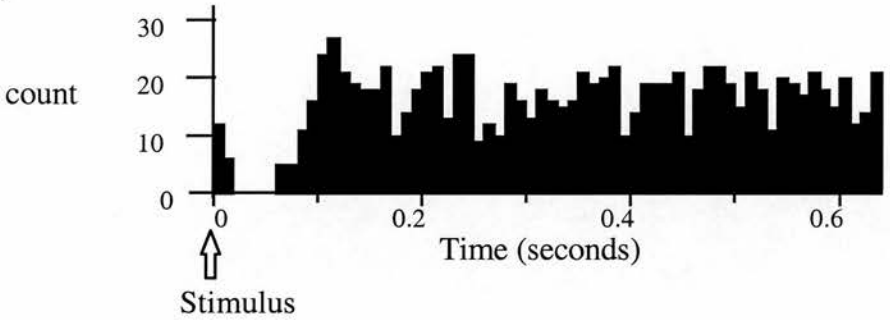

count
30
20
10
0
0
0.2
0.4
0.6
Time (seconds)
Stimulus

Figure 5.8:

Analysis of the effect of sub-threshold stimulation of the MFB on cell firing in the control and OB-treated groups. The histogram illustrates the distribution of the duration of inhibition to sub-threshold stimulation of the MFB. The duration of inhibition is plotted on a log scale, cells in the oil control group are represented by the open bars and cells in the OB-treated group by the solid bars. After OB-treatment there were significantly fewer cells with a short duration of inhibition ($p<0.005$ Fisher's Exact test).

Key:

- oil control - regularly firing
- oil control - doublet-firing
- OB-treated - regularly firing
- OB-treated - doublet-firing

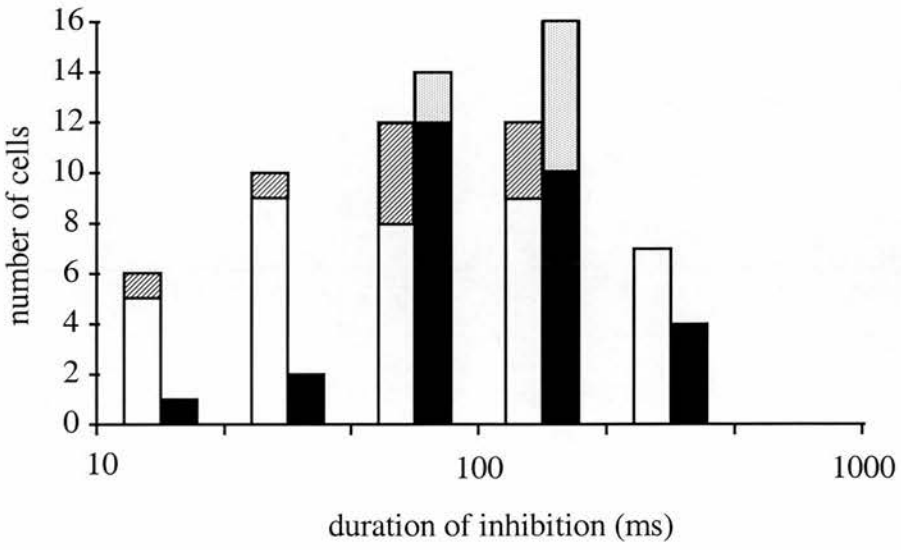

16
14
12
10
8
6
4
2
0
number of cells
10
100
1000
duration of inhibition (ms)

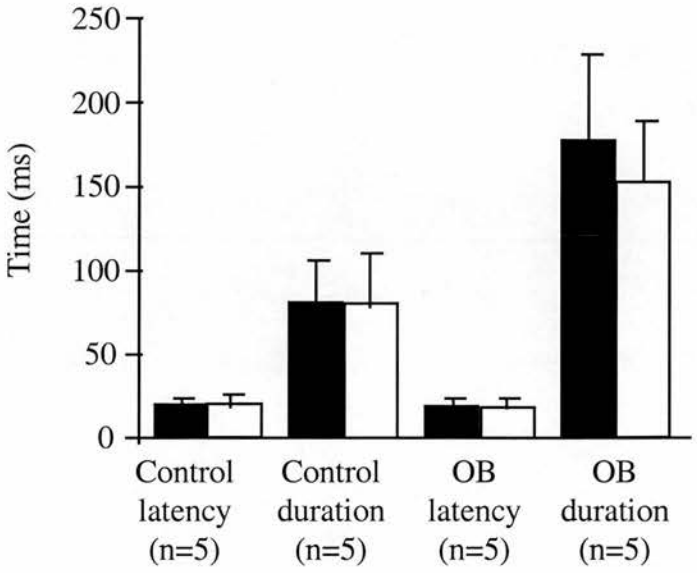
Figure 5.9:

The effect of repeating subthreshold stimulation in the absence or presence of NAN-190 (5 μg kg^{-1}).

(a) shows the summary of results for latency and duration of inhibition seen when sub-threshold stimulation of the MFB was repeated in the absence of NAN-190. The first stimulation is represented by the solid bars and the second stimulation is represented by the open bars. The values for latency and duration are shown as mean ± s.e.m. (n=5). A ten minute interval separated the two stimulations.

(b) shows the effect of repeating sub-threshold stimulation in the presence of NAN-190 (5 μg kg^{-1}, i.v.). The first stimulation is represented by hatched bars and the second stimulation, in the presence of NAN-190, is represented by the solid bars. Ten minute intervals separated the two stimulations and NAN-190 was administered i.v. 100 seconds prior to the second stimulation. Data are shown as mean ± s.e.m., n=3 for oil controls and n=5 for the OB-treated group.

(a)


250
200
150
100
50
0
Time (ms)
Control latency (n=5)
Control duration (n=5)
OB latency (n=5)
OB duration (n=5)

(b)

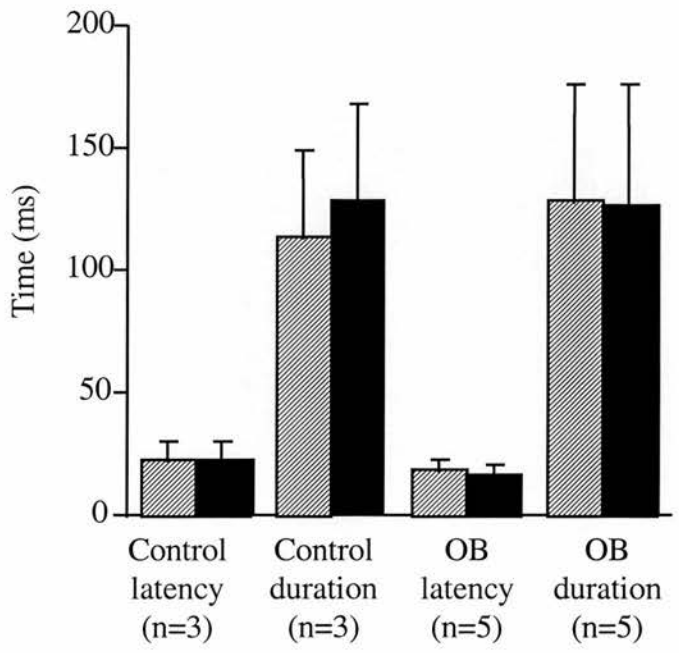
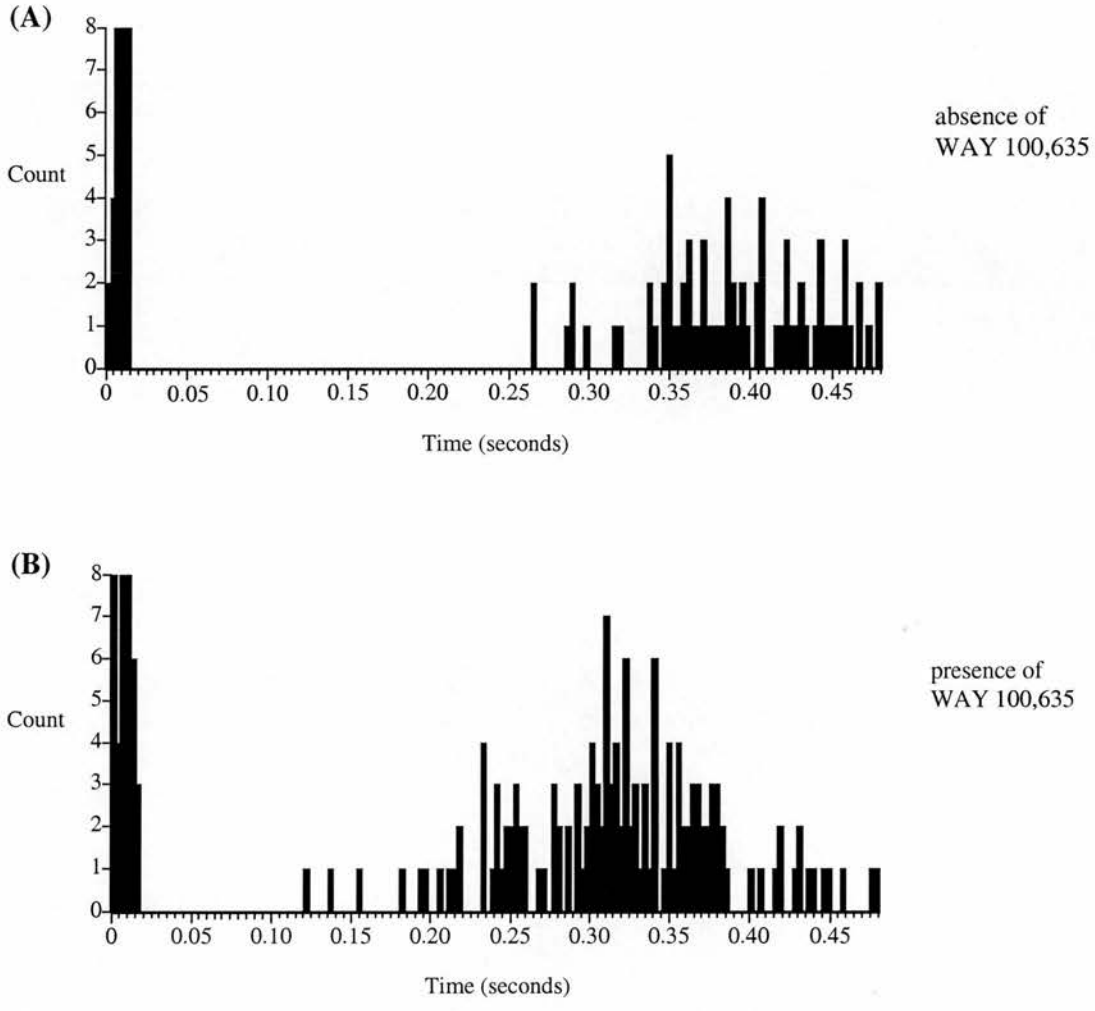

200
150
100
50
0
Time (ms)
Control latency (n=3)
Control duration (n=3)
OB latency (n=5)
OB duration (n=5)

Figure 5.10:

Illustration of inhibition to sub-threshold stimulation in the presence and absence of WAY 100,635. Both diagrams are post-stimulus histograms from an oil treated animal. The two histograms shown were produced from data obtained at different timepoints within the same experiment. Diagram (A) shows the inhibition produced in response to the first sub-threshold stimulation. Diagram (B) shows the result of a second sub-threshold stimulation where WAY 100,635 (1 mg kg^{-1}, i.v.) was administered 100 seconds prior to the second stimulation. The PSTH in (B) shows a reduction in the duration of inhibition in response to the second sub-threshold stimulation in the presence of WAY 100,635. The two stimulations were separated by ten minutes.


(A)
Count
absence of
WAY 100,635
Time (seconds)
(B)
Count
presence of
WAY 100,635
Time (seconds)

Chapter 6

General Discussion

These studies examined the effect of acute treatment with oestrogen and progesterone on several aspects of serotonergic neurotransmission. The effect of oestrogen and progesterone on the binding site density of 5-HT_{2A} receptors and the 5-HT transporter (SERT) was investigated. In order to examine changes in the 5-HT_{2A} receptor an experimental protocol for using [^{3}H]MDL 100,907 as a ligand was first developed. The effect of acute oestrogen treatment on the firing characteristics of presumed 5-HT neurones in the DR nucleus was also examined. Part of this study examined the effect of activation of the recurrent inhibitory pathway within the dorsal raphe (DR) and investigated whether activation of 5-HT_{1A} receptors was involved in the inhibition observed.

6.1 HORMONE TREATMENT.

All animals used in both the binding (5-HT_{2A} receptor and SERT) studies and the electrophysiology experiments, with the exception of the animals used in the experiments to develop a binding assay methodology using [^{3}H]MDL 100,907, had been ovariectomised and treated with steroid(s) or vehicle. Plasma samples were taken from the animals used in all the binding experiments and the level of luteinising hormone (LH) in the plasma was determined. The results of the LH assay indicated that the dose of oestradiol (30 μg) plus progesterone (2.5 mg) given to ovariectomised rats resulted in similar plasma levels of LH to those found in pro-oestrous rats (Chapter 4). In both the binding experiments and in the electrophysiology experiments uterine weights were examined *post-mortem*. As expected animals which had been given oestrogen had higher uterine weights than oil controls (Aiyer and Fink 1974) (Chapters 4 and 5). Ovariectomised animals which had received oestrogen plus progesterone had similar uterine weights to intact pro-oestrous rats. The results from the LH assay and examination of uterine weights suggest that the dose of oestrogen and progesterone used in these experiments produced LH plasma levels similar to those found in pro-oestrous females and that hormone replacement produced similar uterine weight increases to those seen in intact cycling female rats. Hence it would appear that the steroid treatment model used in these experiments is a reliable model of the pro-oestrous rat.

6.2 5-HT_{2A} RECEPTOR AND SERT BINDING STUDIES.

In order to use [^{3}H]MDL 100,907 to detect 5-HT_{2A} receptors, experiments were required to define the optimal experimental conditions for this ligand. The results from these experiments suggested that [^{3}H]MDL 100,907 binds to a single saturable binding site in the cingulate and frontal cortex of female rat brain. These results agree with previous reports which have also suggested [^{3}H]MDL 100,907 binding to a single site (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997). The previous binding studies were carried out using tissue homogenates (Johnson *et al* 1996) and quantitative autoradiography (Lopez-Gimenez *et al* 1997), the latter being carried out on sagittal sections from male rat brain. Low levels of non-specific binding (<5% of total binding) were observed using [^{3}H]MDL 100,907 in these preliminary experiments and this finding agrees with other reports (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997 and 1998, Rioux *et al* 1999). These previous studies used either 10 μM spiperone (Johnson *et al* 1996, Lopez-Gimenez *et al* 1997 and 1998) or 10 μM cianserin (Rioux *et al* 1999) to detect non-specific binding. The present study confirms the finding of low non-specific binding found with [^{3}H]MDL 100,907 and is the first study using RP 62203 for evaluation of non-specific binding.

The results from the study examining the effect of oestrogen and progesterone on 5-HT_{2A} binding site density demonstrate differences between the two ligands used, MDL 100,907 and ketanserin. Using [^{3}H]MDL 100,907 no changes were observed with any treatment group in any of the areas examined. On the other hand, using [^{3}H]ketanserin, increases were observed in binding site density in the cingulate and frontal cortex after oestrogen treatment, although changes were not observed in any of the other regions examined. The addition of progesterone attenuated the increase in cortical binding site density observed using [^{3}H]ketanserin in the oestrogen treated group. Regional differences in specific binding were observed between MDL 100,907 and ketanserin with higher specific binding in the cortex and lower specific binding in the DR with [^{3}H]MDL 100,907. A possible explanation for the increased specific binding in the cortex with [^{3}H]MDL 100,907 is the higher B_{max} previously reported for this ligand (Johnson *et al* 1996). The higher specific binding seen with ketanserin in the DR may result from ketanserin binding to tetrabenzamine sites in this region (Leysen *et al* 1987). In summary, the results from this study indicate that although the two ligands, MDL 100,907 and ketanserin, produce a similar binding pattern throughout the brain they do not bind to identical sites.

Some previous studies have shown differences between the effects of these two ligands (MDL 100,907 and ketanserin); however, the majority of studies show the two compounds produce similar results both *in vitro* and *in vivo*. Studies which have shown differences between the two ligands have been carried out *in vivo* hence the present study is the first to show differences between these two ligands *in vitro*. One *in vivo* study which has shown differences between the two ligands has examined a model of learning. In this study (Meneses *et al* 1997) MDL 100,907 was reported to abolish the cognitive effects of both DOI (5-$HT_{2A/2C}$ agonist) and ketanserin (5-$HT_{2A/2C}$ antagonist). In a second *in vivo* study it was shown that classical 5-HT_{2A} receptor antagonists, e.g. ketanserin, block the release of luteinising hormone-releasing hormone (LHRH), but MDL 100,907 failed to block the release of this hormone (Fink *et al* 1997, Fink *et al* 1999 and personal communication). However, numerous studies *in vitro* and *in vivo* have suggested both compounds producing similar effects. For example, using *in vitro* electrophysiology both MDL 100,907 and ketanserin have been shown to inhibit the release of GABA in the rat frontal cortex (Cozzi and Nichols 1996). Experiments *in vivo* have shown the two 5-HT_{2A} antagonists produce the same effect in behavioural studies examining head twitches (Schreiber *et al* 1995, Willins and Meltzer 1997) and in studies examining hyperlocomotion (Gleason and Shannon 1997).

The lack of effect of oestrogen on 5-HT_{2A} receptor binding sites as detected using [^{3}H]MDL 100,907 does not agree with findings from previous studies. Using [^{3}H]ketanserin increases in binding site density were detected in the frontal, cingulate and piriform cortex, in the nucleus accumbens and in the olfactory tubercle of female (Sumner and Fink 1995, Fink and Sumner 1996) and male (Sumner and Fink 1998) rats treated acutely with oestrogen. In the present study increases in binding site density, detected using [^{3}H]ketanserin, were only observed in the cingulate and frontal cortex after oestrogen treatment. In female rats chronically treated with oestrogen, increases in 5-HT_{2A} receptor binding have been detected in tissue homogenates of cerebral cortex using [^{3}H]spiroperidol (Biegon *et al* 1983) and in the frontal cortex using quantitative autoradiography with [^{3}H]ketanserin (Cyr *et al* 1998). No increase in 5-HT_{2A} receptor binding site density after oestrogen treatment was detected in the DR in this study using either ligand. Previously, an increase was shown using [^{3}H]ketanserin although non-specific binding was detected using unlabelled ketanserin (Sumner and Fink 1995). As mentioned above, ketanserin is known to bind to a tetrabenzamine sites in the DR (Leysen *et al* 1987) and it is possible that the increase observed by Sumner and Fink (1995) is due to an increase

in tetrabenzamine sites not 5-HT_{2A} receptors. It should be noted that this previously reported increase was confined to the lateral DR and that in the present study the low level of binding observed in the DR made the measurement of divisions within the DR impractical hence the DR as a whole was measured.

Acute progesterone treatment alone produced no change in 5-HT_{2A} receptor binding site density with either ligand used in the current study. This is in contrast to chronic progesterone treatment which has been previously shown to produce a slight increase in 5-HT_{2A} receptors in the cerebral cortex (Biegon *et al* 1983). This suggests that the effect of progesterone is time dependent having no effect given acutely (5 hours) but producing an increase in 5-HT_{2A} receptor binding when given chronically for 2 weeks (Biegon *et al* 1983). Co-administration of oestrogen and progesterone in the present study attenuated the increase in binding seen in the cortex using ketanserin in the oestrogen treated group. The attenuation of an oestrogen-induced increase in 5-HT_{2A} receptor binding sites by progesterone has previously been observed in chronically steroid treated rats (Biegon and McEwen 1982, Biegon *et al* 1983). The fact that progesterone attenuates the effects of oestrogen after both acute and chronic administration but only increases 5-HT_{2A} receptor binding after chronic treatment suggests two different mechanisms could be involved.

The effect of sex steroids on SERT binding sites were examined using [^{3}H]paroxetine. The distribution of [^{3}H]paroxetine binding sites seen in this study was similar to that observed in both male (Battaglia *et al* 1991, De Souza and Kuyatt 1987) and female (McQueen *et al* 1997 and 1998) rats with the highest binding being observed in the DR. In the present study no changes were observed in [^{3}H]paroxetine binding in the cortex after oestrogen treatment and this agrees with previous studies in both male and female rats (Mendelson *et al* 1993, McQueen *et al* 1997 and 1998). The results from the present study differ from those reported by McQueen *et al* (1997 and 1998) which showed increases in [^{3}H]paroxetine binding in the BLA and VMN with a decrease being observed in the PAG after acute oestrogen treatment. The differences between the two studies could be explained by a slight difference in methodology. In the present study animals received a second injection and it is known that both handling and subcutaneous injections stress animals resulting in increased 5-HT levels within the raphe nuclei (Adell *et al* 1997). This study also demonstrated increased 5-HT levels in the amygdala as a result of handling and in the medial prefrontal cortex after saline injection. Changes in 5-HT levels in terminal regions 5 hours before the animals were sacrificed could produce

increased internalisation or desensitisation of SERT leading to the lack of apparent effect of oestrogen observed in this study.

In both the 5-HT_{2A} and SERT binding experiments progesterone had little effect alone but appeared to attenuate the effects of oestrogen on 5-HT_{2A} binding in discrete regions. The modulation of oestrogenic effects by administration of progesterone is not a phenomenon confined to the serotonergic system. For example, in the VMN and PAG oestrogen decreased [^{3}H]muscimol binding at the $GABA_A$ receptor whereas progesterone alone or in combination with oestrogen returned binding to control levels (Schumacher *et al* 1989). Part of this effect may be explained by the observation that oestrogen increase the levels of glutamate and GABA in the VMN and that co-administration of progesterone resulted in a rapid decline in the levels of these two transmitters (Luine *et al* 1997). The modulation of the effect of oestrogen by progesterone is also observed in *in vivo* behavioural models. For example in epilepsy oestrogen has been shown to produce a decrease in seizure frequency, progesterone had no effect but a combination of the two steroids resulted in an increase in seizure duration (Hom *et al* 1993).

In conclusion, progesterone produces no significant effect on either 5-HT_{2A} receptor or SERT binding site density when given alone and when given in conjunction with oestrogen it appears to attenuate the increases in 5-HT_{2A} binding site density produced by oestrogen in some brain regions.

6.3 EVALUATION OF THE EFFECT OF OESTROGEN ON THE FIRING OF 5-HT NEURONES IN THE DORSAL RAPHE.

These experiments examined the effect of oestrogen on different firing characteristics of presumed 5-HT neurones in the DR and also investigated the effect of oestrogen on the recurrent inhibitory pathway in the DR, activated by sub-threshold stimulation of the medial forebrain bundle (MFB).

This study showed that oestrogen increased the mean antidromic latency to MFB stimulation but had no effect on resting basal firing rate. The lack of change in firing rate observed here agrees with a previous study (Bueno and Pfaff 1976) which showed that oestrogen had no effect on the firing rate of cells in the medial preoptic area and medial anterior hypothalamus. The mechanism involved in the increase in

latency is unknown but may be the result of a steroidal interaction with the axonal membrane since it has been shown that progesterone increases orthodromic latency of neurones in the medial preoptic area on stimulation of the median eminence-arcuate nucleus (Watanabe 1992). Another possible mechanism is via modulation of potassium channels. Oestrogen has been reported to increase potassium conductance in CA1 neurones (Nabekura *et al* 1986) and in T84 (human colonic epithelial) cells (Condliffe *et al* 1998). However, changing potassium conductances would alter firing rate and this was not observed in this study.

The proportion of doublet-firing cells found in this study was similar in both treatment groups such that approximately one third of the cells were doublet-firing. This proportion is similar to that reported to occur in male rats (Hajos *et al* 1995b, Hajos and Sharp 1996a). Examination of the antidromic latencies of doublet-firing cells did not confirm that doublet-firing cells had a longer antidromic latency as previously reported for these cells in male rats (Hajos and Sharp 1996b). These two findings suggest that although numbers of doublet-firing cells may be the same in both males and females the properties of these cells may differ between the sexes. Doublet-firing cells have been detected in both the dorsal and median raphe nuclei *in vivo* but at present there is no evidence to suggest that doublet-firing cells preferentially innervate particular brain regions (Hajos *et al* 1995b, Hajos and Sharp 1996b). Doublet-firing cells have not been recorded *in vitro* suggesting afferent inputs to the raphe nuclei are involved. Indeed in the dentate gyrus and olfactory bulb it has been shown that burst firing is regulated by cholinergic neurones (Heale and Vanderwolf 1994).

There is increasing evidence that doublet-firing cells have a role to play in transmitter release. It has been shown in the hippocampus that two pulses given within a short time interval (8 ms) can produce reliable transmitter release (Stevens and Wang 1995). Two recent studies have examined the effect of two pulses 10 ms apart on 5-HT release. The first study, which examined slices containing the DR or substantia nigra, showed that two stimuli given 10 ms apart released twice as much transmitter as a single pulse (Bunin and Wightman 1998). A second study examined [^{3}H]5-HT release in various brain regions in response to either single or twin pulses. In slices containing medial prefrontal cortex (mPFC), caudate/putamen or the dorsal raphe nucleus twin pulses 10 ms apart released twice as much transmitter as single pulses (Hajos *et al* 1999). Further experiments were carried out in the mPFC and dorsal raphe which demonstrated that single pulses at 4 Hz and twin pulses at 2 Hz released

the same amount of 5-HT. The second part of this study was conducted *in vivo* and showed that twin stimuli produced longer inhibition of firing of neurones in the mPFC than single pulses and that this effect could be partially blocked by WAY 100,635 suggesting it was mediated by 5-HT (Hajos *et al* 1999, Hajos personal communication). These recent findings would suggest that the decrease in doublet-firing after oestrogen treatment reported here could result in a decrease in 5-HT release. This theory is supported by the finding that there is a decrease in 5-HT levels in the hypothalamus of female rats during oestrus when oestradiol levels are high (Gundlah *et al* 1998). However, no change was observed in the 5-HT concentration in tissue homogenate samples from several forebrain regions in oestrogen treated animals (Di Paolo *et al* 1983) although 5-HT release could still have been reduced. The lack of effect of oestrogen on 5-HT in the study by Di Paolo *et al* (1983) may suggest that oestrogen preferentially affects 5-HT levels in particular brain regions but the difference in findings may also be methodological, since the study by Di Paolo *et al* (1983) examined 5-HT content in tissue homogenate samples compared to 5-HT release by microdialysis (Gundlah *et al* 1998).

Activation of the recurrent inhibitory pathway (Wang and Aghajanian 1978) by sub-threshold stimulation of the MFB produced similar effects in both the control and treated groups. In both groups the majority of cells showed inhibition although fewer cells displayed a short duration of inhibition (< 50 ms) in the treated group. In the control group both regularly firing and doublet-firing cells displayed a short duration of inhibition however in the treated group only regularly firing cells did so. This observation suggests that oestrogen is preferentially affecting doublet-firing cells although this finding may have arisen as a result of the small number of cells with a short duration of inhibition in the treated group (n=3). In order to determine the mediator of the inhibition observed the stimulus was repeated after administration of WAY 100,635 (1 mg kg^{-1} i.v.). The reduction in the duration of inhibition seen with most cells (9/10) after the administration of WAY 100,635 suggests that the inhibition produced by sub-threshold stimulation is, at least in part, due to the action of 5-HT on 5-HT_{1A} autoreceptors. Other studies have examined the receptors involved in afferent and efferent pathways of the DR. Stimulation of the efferent pathway to the mPFC has been shown to produce inhibition of neuronal firing which was, like the present study, partially inhibited by WAY 100,635 (Hajos *et al* 1999, Hajos personal communication). However, stimulation of the afferent pathway from the DR to the mPFC produced a different effect. Although stimulation of the pathway resulted in inhibition of DR cell firing the inhibition was not blocked by

WAY 100,635 and it was suggested that the inhibition observed involved glutamate and GABA (Hajos *et al* 1998). It should be noted that WAY 100,635 only partially blocked the inhibition observed in the present study. It is possible that the remaining inhibition may involve the activation of GABA interneurones which have been shown to occur within the DR (Gallager 1978, Nanopolous *et al* 1982, Wang *et al* 1992).

In conclusion, the present study demonstrates that doublet-firing cells are found in the DR in female rats in similar proportions to those reported previously in males and that oestrogen treatment produces a decrease in the number of doublets fired per minute by these cells. It is possible that the decrease in doublets fired reported here may produce a physiologically relevant reduction in 5-HT release in the projection areas of the DR. Activation of the recurrent inhibitory pathway by sub-threshold stimulation of the MFB produced inhibition of firing in the majority of cells and appears to be, at least in part, the result of 5-HT_{1A} receptor activation. The observation that fewer cells in the treated group show a short duration of inhibition in response to sub-threshold stimulation of the MFB suggests an enhanced recurrent inhibition of DR neuronal firing with oestrogen treatment.

6.4 THE MECHANISM OF ACTION OF AND THE INTERACTIONS BETWEEN OESTROGEN AND PROGESTERONE.

These studies have examined the effects of acute oestrogen and progesterone treatment on serotonergic neurotransmission. The time point examined in these studies, 24-32 hours post-oestrogen and 5 hours post-progesterone, would allow effects to be a result of either genomic (McMahon and Sabban 1992) or non-genomic (Bression *et al* 1986, Tischkau *et al* 1993) mechanisms. Indeed increases in the expression of mRNA for the 5-HT_{2A} receptor (Sumner and Fink 1993) and SERT (McQueen *et al* 1997 and 1998) have been found with oestrogen within this experimental time frame.

The genomic actions of oestrogen and progesterone are mediated through intracellular receptors. In the case of oestrogen two types of intracellular receptor are now known to exist: these are the ERα (classical oestrogen receptor) and ERβ receptors (Kuiper *et al* 1996). These two receptors have a different distribution throughout the brain although their distributions overlap in some areas e.g. in the

preoptic area and locus coeruleus (Shughrue *et al* 1997a). Shughrue *et al* (1997a) reported that ERα was found exclusively in the VMN whereas ERβ was detected in the olfactory bulb, nucleus accumbens and cerebellum. The DR contains very few oestrogen concentrating cells (Pfaff and Keiner 1973) although as yet it is unclear whether these are ERα or ERβ. The uncertainty derives from two reports, the first of which showed the DR contained only ERβ mRNA (Shughrue *et al* 1997a) whereas Alves *et al* (1998) detected only ERα and not ERβ immunoreactivity within the DR.

A second mechanism of action for oestrogen is via non-genomic actions. Membrane binding sites for oestrogen have been reported in crude synaptosomal membrane preparations from the cerebellum, olfactory bulb and hypothalamus of female rat brain (Zheng and Ramirez 1997). The action of oestrogen on membrane sites has been shown to reduce calcium currents in neostriatial neurones probably via G-protein activation (Mermelstein *et al* 1996) and this mechanism may relate to the observed reduction in doublets fired by the doublet-firing cells in the DR in this study. This theory would require testing, preferably *in vitro*, however doublet-firing cells have not been detected in the dorsal or median raphe *in vitro* (Hajos personal communication).

Membrane binding sites have also been detected for progesterone (Tischkau *et al* 1993) and would allow non-genomic effects of progesterone to occur. The mRNA for classical progesterone receptors, which mediate genomic effects, has been detected in the cortex, hippocampus and hypothalamus (Hagihara *et al* 1992). In regions of the hypothalamus classical progesterone receptors are oestrogen-inducible whereas in the cortex they are not (Rainbow *et al* 1982, Parsons *et al* 1982). Progesterone receptor immunoreactivity has been detected in the DR (Alves *et al* 1998) and here too oestrogen has a regional effect on progesterone receptors. In the lateral wings of the DR, progesterone receptors were not oestrogen-inducible, although elsewhere in the DR oestrogen treatment increased progesterone receptor immunoreactivity (Alves *et al* 1998). The interaction between oestrogen and progesterone in the modulation of 5-HT_{2A} and SERT binding sites suggested by the data presented here may reflect interactions between these two steroids. As mentioned above oestrogen is known to induce the synthesis of progesterone receptors in some regions of the brain but not in others. The converse is true for progesterone since it has been shown to antagonise the increases in both oestrogen and progesterone receptor levels induced by oestrogen in oestrogen-primed animals (Hsueh *et al* 1975, Savouret *et al* 1991). In addition progesterone also displays anti-

oestrogenic properties; for example, progesterone inhibits, whereas oestrogen stimulates, progesterone receptor expression through the same promoter region (Savouret *et al* 1991). This may explain the attenuation of the effect of oestrogen observed in the 5-HT_{2A} receptor seen here. Modulation of other transmitter systems by these two steroids has been shown and is discussed in 6.2.

The negative effect of progesterone, observed here, may also be related to observations of steroid replacement therapy in depression. Several reports have suggested that progesterone has a negative effect on mood. Postnatal administration of progesterone has been shown to increase the risk of the women developing postnatal depression (Lawrie *et al* 1998). In other studies which have examined the effects of oestrogen alone or in conjunction with progesterone, the addition of progesterone was shown to produce more negative moods (Sherwin 1991, Klaiber *et al* 1996).

In conclusion, the present study has shown that, in female rat brain, the binding patterns of [^{3}H]ketanserin and [^{3}H]MDL 100,907 are similar but not identical. This finding would suggest further investigation of the differences in binding sites between these two ligands is required. This study has also shown that oestrogen and progesterone have subtle effects on serotonergic neurotransmission. Both the 5-HT_{2A} receptor and SERT binding studies have suggested that progesterone has no effect alone; however, progesterone can attenuate the oestrogen-induced increase in 5-HT_{2A} binding sites in some regions. This finding may help explain why progesterone exacerbates depression. The electrophysiology study shows that the proportion of cells which are doublet-firing is similar to that in males and that oestrogen decreases the frequency of doublet-firing in these cells. In the context of recent reports, the observed decrease in doublet-firing suggests a possible mechanism for the observed decrease in 5-HT levels observed when oestrogen levels are high. This study also suggested that recurrent inhibition of DR neuronal firing is enhanced after oestrogen treatment and that the inhibition is partly due to activation of 5-HT_{1A} receptors.

6.5 SUGGESTIONS FOR FUTURE WORK

1. Further studies to examine the effects of chronic steroid treatment with oestrogen and progesterone on 5-HT_{2A} receptor and 5-HT transporter binding sites in female rats using quantitative autoradiography to examine the interaction between the two steroids.
2. Examine the effect of acute and chronic oestrogen treatment on 5-HT_{1A} receptor binding sites in the dorsal and median raphe nuclei using both [^{3}H]8-OH DPAT and [^{3}H]WAY 100,635 to determine any changes in binding sites and proportion of receptors coupled to G-proteins.
3. A study to examine the effect of acute progesterone on 5-HT neuronal firing and ascertain whether it normalises the oestrogen response when given in combination.
4. To examine the involvement of the noradrenergic input from the locus coeruleus (LC) to the dorsal raphe in mediating the effects of oestrogen on dorsal raphe firing *in vivo* using rats with LC lesions.
5. An investigation to examine the effect of chronic oestrogen treatment on the firing characteristics of 5-HT neurones *in vivo* and to determine whether oestrogen treatment produces similar results to chronic treatment with antidepressant drugs.
6. To examine whether the firing characteristics of DR 5-HT doublet-firing cells are altered in the chlorimipramine-induced model of depression since DR neuronal firing is reportedly decreased in this model (Yavari *et al* 1993).

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