IN VIVO AND *IN VITRO* ASPECTS OF IMIDAZOLINE-2 (I_2) SITES WITHIN THE CENTRAL NERVOUS SYSTEM

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Summary

The *in vivo* and *in vitro* characteristics of the I_2 binding site were probed using the technique of drug discrimination and receptor autoradiography.
Data presented in this thesis indicates the I_2 ligand 2-BFI generates a cue in drug discrimination. Further studies indicated agmatine, a proposed endogenous imidazoline ligand, and a number of imidazoline and imidazole analogues of 2-BFI substitute significantly for 2-BFI. In addition to specific I_2 ligands the administration of NRI's (noradrenaline reuptake inhibitors), the sympathomimetic d-amphetamine, the α_1-adrenoceptor agonist methoxamine, but not the β_1 agonist dobutamine or the β_2 agonist salbutamol, gave rise to significant levels of substitution for the 2-BFI cue. The administration of the α_1-adrenoceptor antagonist WB4101, prior to 2-BFI itself significantly reduced levels of 2-BFI appropriate responding. Administration of the reversible MAO-A inhibitors moclobemide and Ro41-1049, but not the reversible MAO-B inhibitors lazabemide and Ro16-6491, gave rise to potent dose dependent levels of substitution for the 2-BFI cue. Further studies indicated the administration of a number of β-carbolines and the structurally related indole alkaloid ibogaine also gave rise to dose dependent significant levels of substitution. Due to the relationship of indole alkaloids to serotonin the 5-HT releaser fenfluramine and a number of SSRI's (selective serotonin reuptake inhibitor) were also administered and these compounds gave rise to significant partial (20-80% responses to the 2-BFI lever) levels of substitution.
The autoradiographical studies reported here indicate [^{3}H]2-BFI labels I_2 sites within the rat arcuate nucleus, area postrema, pineal gland, interpeduncular nucleus and subfornical organ. Subsequent experiments confirmed that the drug discrimination dosing schedule significantly increases levels of [^{3}H]2-BFI I_2 binding within two of these nuclei. However, levels of [^{3}H]2-BFI specific binding were significantly reduced within four of these nuclei after chronic treatment with the irreversible MAO inhibitors deprenyl and tranylcypromine but not pargyline, which only reduced levels significantly in two. Further autoradiographical studies indicated that the distribution of [^{3}H]2-BFI within the C57/B mouse compares favourably to that within the rat. Comparison of these levels of binding to those from transgenic mice who over-express MAO-B indicates two possibly distinct populations of [^{3}H]2-BFI I_2 sites exist in mouse brain.
The data presented here indicates the 2-BFI cue is associated with the selective activation of α_1-adrenoceptors and possibly 5-HT receptors. 2-BFI trained rats recognise reversible MAO-A but not MAO-B inhibitors. However, data within this thesis indicates the autoradiographical distribution of I_2 sites bears a closer resemblance to that of MAO-B not MAO-A and further studies using transgenic mice that over-express MAO-B suggests a non-MAO-B I_2 site exists in mouse brain.

Keywords. imidazoline, MAO, β-carbolines, ibogaine, agmatine, noradrenaline, 5-hydroxytryptamine, drug discrimination, autoradiography.

To Alistair and Jean

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5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HT	5-hydroxytryptamine or serotonin
ARR	Average reinforcement rate
B_{max}	Binding maximum
Ca^{++}	Calcium
cAMP	Cyclic adenosine monophosphate
cCDS	Classic clonidine-displacing-substance
cDNA	Cloned deoxyribonucleic acid
cGMP	Cyclic guanine monophosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
D	Drug
DA	Dopamine
DAO	Diamine oxidase
DD	Drug discrimination
DMT	N,N-dimethyltryptamine
DOM	(-)-2.5-dimethoxy-4-methyl-amphetamine
DOPAC	3, 4-dihydroxyphenylacetic acid
FR	Fixed ratio
GAD	Generalised anxiety disorder
GFAP	Glial fibrillary acidic protein
GLP	Glucagon like peptide
HPLC	High pressure liquid chromatography
HVA	Homovanillic acid
i.c.v.	Intracerebroventricular
i.p.	Intraperitonial
i.v.	Intravenous
IC_{50}	Concentration of ligand that inhibits 50% of radio ligand binding
iCDS	Immunoreactive clonidine-displacing-substance
IP	Inositol phosphate
IRAS-1	Imidazoline Receptor Antisera-Selected cDNA-1
K_D	Radioligand affinity
kDa	KiloDaltons

K_I	Affinity of ligand for radiolabelled binding site as described by the Cheng-Prusoff equation (1973)
LSD	Lysergic acid diethylamide
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MCID	Microcomputer imaging device
NA	Noradrenaline
NRI	Noradrenaline reuptake inhibitors
NSB	Non-specific binding
PAC	Para-aminoclonidine
PC-12	Pheochromocytoma cells
RPM	Responses per minute.
S	Saline
s.c.	Subcutaneously
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSNRI	Selective serotonin and Noradrenaline re-uptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
T	Test
VLM	Ventrolateral medulla

Chapter 1

General Introduction

In 1976 Karppanen and colleagues published research indicating that the effects of the centrally acting antihypertensive drug clonidine were blocked by the imidazole histamine-2 (H_2) receptor antagonist metiamide. Previous to this it was believed that the action of clonidine could be attributed to its affinity for central α-adrenoceptors (Haeusler, 1973). Over a decade later Bousquet (1984) and colleagues, after comparing the effects of α_2-adrenoceptor agonists that contained an imidazoline moiety, to non-imidazoline α_2-adrenoceptor agonists, proposed that the centrally mediated hypotensive actions of clonidine could be attributed to the drug's imidazoline moiety rather than its affinity for α_2-adrenoceptors (Bousquet et al, 1984). Further studies using [^{3}H]p-aminoclonidine ([^{3}H]PAC), the tritiated analogue of clonidine, indicated that noradrenaline and other phenylethylamines only displaced 70% of [^{3}H]PAC labelled sites (Ernsberger et al, 1987: see figure 1.1).

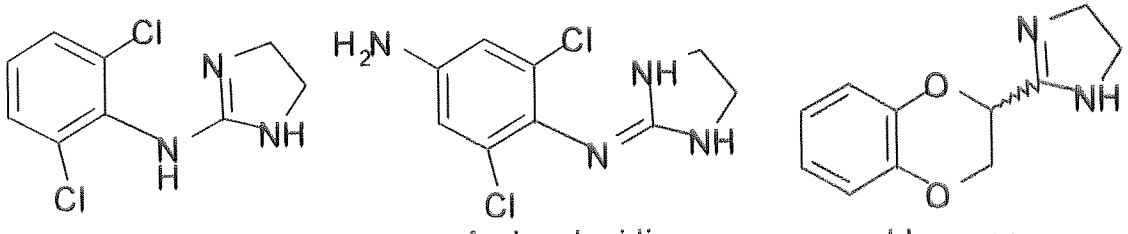


Figure 1.1). *Chemical structures of clonidine, p-aminoclonidine and idazoxan*

In the same year receptor autoradiographical studies indicated the imidazoline α_2-adrenoceptor antagonist [^{3}H]idazoxan, bound to sites other than those labelled by the non-imidazoline α_2-adrenoceptor antagonist [^{3}H]rauwolscine (Boyajian et al, 1987). As a consequence of these findings the first International Symposium on Imidazoline preferring receptors tentatively designated those sites that preferentially bound [^{3}H]PAC (in the presence of 10μM noradrenaline to saturate α-adrenoceptors) as Imidazoline-1 (I_1) sites and those labelled by [^{3}H]idazoxan, under similar conditions, as Imidazoline-2 (I_2) sites (Michel & Ernsberger, 1992).

1.1). Binding sites

1.1.1). Imidazoline-1 (I_1) Binding Sites

Imidazoline-1 (I_1) binding sites are primarily defined by their affinity for radioligands, [^{3}H]PAC or [^{3}H]-clonidine, and associated drug displacement profiles. Both [^{3}H]PAC and [^{3}H]-clonidine, in the presence of saturating concentrations of noradrenaline, show a high affinity for I_1 sites (K_D = 0.6 - 38 nM and 4 - 15 nM respectively; Ernsberger et al, 1994; Ernsberger et al, 1987). Binding (K_i) is inhibited at low concentrations in the rat brain by, clonidine (0.99 nM), p-aminoclonidine (0.93nM), cimetidine (100nm), but higher concentrations are needed for idazoxan (186nM), naphazoline (203nM), noradrenaline (120,000nM) and guanabenz (1,000,000nM) (Ernsberger et al, 1990). Thus I_1 binding sites are classically characterised through their high affinity for clonidine and moderate affinity for idazoxan. Recently, these sites have been further classified in to I_{1A} and I_{1B} based on affinities for cimetidine (Hamilton, 1993 & 1995). Radiochemical ligand binding studies have further suggested that I_1 binding sites may be separated into distinct high and low affinity sites (Ernsberger et al 1994). Ernsberger et al (1994) suggest the low affinity site may represent an uncoupled I_1-receptor. However, analysis of potential secondary messengers has indicated that I_1 sites do not modify levels of cyclic AMP (cAMP or cyclic adenosine monophosphate) or inositol phosphates, but are associated with a delayed and relatively prolonged release of calcium (Ca^{++};

Regunathan et al, 1991a). However, an unconventional cellular signalling pathway has been proposed in choline induced hydrolysis, which gives rise to diaglyceride and arachidonic acid (Ernsberger, 1998 & 1999). As mentioned above a conventional intracellular signalling pathway has not been found, hence the above hypothesis is based on the accumulation of the reaction products, i.e, diaglycerides and phosphocholine, of an unidentified phospholipase C mediated pathway (Ernsberger, 1999).

Two molecular binding proteins, with an apparent molecular weight of 43 and 45 kDa have been identified within humans and rats (Greney et al, 1994; Dontenwill et al, 1999; Escriba et al, 1995). Antibodies raised against radiolabelled imidazoline binding proteins have been used to generate a partial cDNA clone (cloned deoxyribonucleic acid; Ivanov et al, 1998a). The resultant cDNA sequence has been used to generate a full-length gene sequence named IRAS-1 (Imidazoline Receptor Antisera-Selected cDNA-1). IRAS-1 expresses a 167 kDa protein which contains an estimated 5 transmembrane regions. It has been proposed that the 43 and 45 kDa are two fragments from this 167 kDa protein (Piletz et al, 1999).

The clonidine preferring I_1 site has been localised in rats, cows and humans (Kamisaki et al, 1990; Ernsberger et al, 1990; De Vos et al, 1994). Within the central nervous system (CNS) large numbers of I_1 sites have been radio-labelled within the rostral ventrolateral medulla (Ernsberger et al, 1990), dorsomedial medulla and frontal cerebral cortex (B_{max} 49-90 fmol/mg respectively; Ernsberger et al, 1987), olfactory bulb, posterior cortex, striatum, hippocampus, midbrain, hypothalamus, cerebellum, pons and medulla (B_{max} 3.5-27.1 fmol/mg), but not on astrocytes - an area on which α_2-adrenoceptors are expressed (B_{max} 18 fmol/mg; Kamisaki et al, 1990).

One of the most notable effects of drugs that bind to I_1 sites is their ability to reduce blood pressure. The hypotensive effect of clonidine, rilmendine and moxonidine has been attributed to their ability to inhibit the sympathoexcitatory reticulospinal neurons of the ventrolateral medulla (VLM; Bousquet et al, 1984) through choline induced hydrolysis (Ernsberger, 1998 & 1999, Van Zwieten & Peters, 1999). This has the net effect of reducing sympathetic output to the periphery, which results in a drop in blood

pressure. Further evidence suggests that drugs that act at I_1 sites reduce peripheral plasma renin activity, thereby further reducing blood volume and consequently pressure via the angiotensin II pathway (Schafer et al, 1995). Non significant increases in platelet I_1 and α_2 sites has been reported in unipolar depressives but not generalised anxiety disorder (GAD) sufferers. Resultant treatment of these individuals with desipramine, a drug which has little affinity for I_1 and α_2-adrenoceptors (I_1 K_i = 18,000nM, α_2-adrenoceptor K_i = 2600nM), down regulates both I_1 and α_2-adrenoceptors (Piletz et al, 1996). It is likely that the down regulation α_2-adrenoceptors is through increased levels of NA within the synaptic cleft and thus reduction in α_2-adrenoceptor sensitivity and density (Model and Omann, 1998). However, this does not account for the reduction in I_1 sites.

1.1.2). Imidazoline-3 (I_3) Binding Sites

A number of studies have suggested drugs that contain an imidazoline moiety, i.e., efaroxan, antazoline, and phentolamine interact with K^+_{ATP} channels present on β pancreatic cells located within the islets of Langerhans. It has been proposed that these ligands decrease the probability of an open channel conformation in plasma membrane K^+_{ATP} channels (Dunne, 1991; Chan et al, 1994; Chan et al 1995; Morgan et al 1999). This subsequently leads to membrane depolarisation, calcium influx and a rise in insulin secretion (Rustenbeck et al, 1999). However, it is unclear if these actions are a result of the direct action of imidazoline ligands on the potassium channel or these effects are mediated by some remote site that is linked to the channel (Morgan et al, 1999). Furthermore imidazoline ligands have a reported ability to inhibit glucagon secretion from pancreatic α cells (Mourtada et al, 1997). This effect is thought to be independent of K^+_{ATP} channels as α cells do not express the core ion channel forming subunit (Morgan et al, 1999).

The insulin associated site at which imidazoline drugs have been reported to work exhibits a pharmacological profile distinct to that of the classic I_1 and I_2 site. The I_3 site is radiolabelled by [^{3}H]-methoxy-idazoxan under saturating

concentrations of noradrenaline. Drugs that are efficient insulin secretagogues, i.e., efaroxan, displace [^{3}H]-methoxy-idazoxan binding, but those that are not, i.e., idazoxan and UK 14,304 do not. (Chan et al, 1995). Based on this evidence it has been proposed that the pancreatic imidazoline site represents a third site distinct from the I_1 and I_2 sites (Chan et al, 1995; Dunne et al, 1995; Morgan et al, 1999; Zaitesev et al, 1999).

However, a further pharmacologically distinct site has been identified in the rat lung. [^{3}H]atipamezole binding was inhibited by only high concentrations of I_1 specific drugs, e.g. clonidine (IC_{50} = 560,000 nM), para-aminoclonidine (IC_{50} = >1,000,000 nM), and I_2 specific drugs, e.g. idazoxan (IC_{50} = 524,000 nM), cirazoline (IC_{50} = 80,000 nM). Data also indicated that these were not adrenoceptors as binding was insensitive to noradrenaline (IC_{50} = >100,000 nM). However, a number of proposed α_2-adrenoceptor antagonists, that contained an imidazole, rather than imidazoline, moiety showed a high affinity for [^{3}H]atipamezole labelled sites. Notably, detomidine (IC_{50} = 7.3 nM) and dexmedetomide (IC_{50} = 5.3 nM) (Sjöholm et al, 1995).

1.1.3). Imidazoline-2 (I_2) Binding Sites

1.1.3.1). I_2 Ligand Binding Studies

The imidazoline-2 (I_2) site has been classically characterised through its preference for idazoxan over clonidine, in the presence of a 10μM mask of rauwolscine, using [^{3}H]idazoxan as the tritiated ligand. In rat brain the [^{3}H]idazoxan I_2 site has a K_D of 1.75nM and shows the following drug affinity profile, i.e., cirazoline < 2-BFI < idazoxan < clonidine (Boyajian et al, 1987).

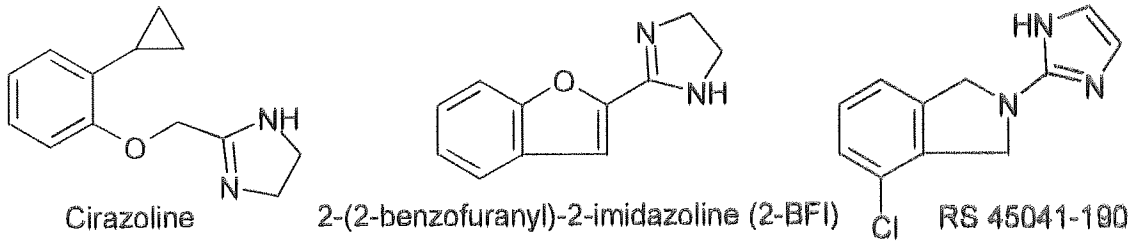


Figure 1.2). *Chemical structures of cirazoline, 2-BFI, RS-45041-190*

Since the inception of the I_2 site and because of [^{3}H]idazoxan's affinity for α_2-adrenoceptors and 5-HT_{1A} receptors more specific radioligands have been sought (Lachaud-Pettiti et al, 1991; Nutt et al, 1995; Llado et al 1996). This has led to the recent synthesis of an irreversible ligand and the tritiated compounds; [^{3}H]-cirazoline, [^{3}H]-RS-45041-190 ([^{3}H]-4-chlo-2-(imidazolin-2-yl) isoindoline) and [^{3}H]-2-BFI ([^{3}H]-2-(-2-benzofuranyl)-2-imidazoline: Coates et al, 2000; Angel et al, 1994; MacKinnon et al, 1995; Lione et al, 1994: see Figure 1.2).

[^{3}H]-RS-45041-190 binding within the rat brain is of high affinity, reversible and saturable. The rat [^{3}H]-RS-45041-190 I_2 site has a K_d of 2.71nM and drug displacement profile of, RS-45041-190 < cirazoline < idazoxan < guanabenz < amiloride << clonidine. In the rabbit and rat [^{3}H]-2-BFI shows similar pharmacological characteristics i.e., it binds reversibly and with high affinity. Similarly to [^{3}H]-RS-45041-190 [^{3}H]2-BFI has a nM affinity (K_D = 1.71) for the I_2 site and shows a similar drug displacement profile, i.e., (K_i < 10 nM), RS-45041-190 < naphazoline < 2-BFI < BU224 < BU239 < cirazoline < idazoxan << (K_i < 100 nM), clorgyline < guanabenz < amiloride << clonidine << (K_i < 100,000nM) efaroxan (Lione et al, 1996; Alemany et al, 1997; Lione et al, 1998). 2-BFI also has a similar high affinity for I_2 sites in species other than the rabbit and rat, i.e., the mouse, dog, rabbit, calf and human (Vauquelin et al, 1999). Both [^{3}H]-2-BFI and [^{3}H]-RS-45041-190 have a high affinity (<10nM) for I_2 sites, with little affinity for α_2-adrenoceptors (>1.5 μM; Brown et al, 1995; Nutt et al, 1995; Alemany et al, 1997).

One of the most notable features of radioligand binding assays at I_2 sites is that some drugs exhibit biphasic displacement curves, i.e., the competing ligand is displacing the radioligand from two differing sites or one site that displays two differing affinity states (Boyajian et al, 1987; Wikberg et al 1992; Wikberg, 1995). At the rat CNS [^{3}H]2-BFI and [^{3}H]idazoxan I_2 site, 2-BFI, BU224, BU239, cirazoline, LSL 60101, LSL 60101, RS-45041-190, clonidine, bromoxidine, idazoxan, naphazoline, guanabenz, UK-14,304, amiloride, medetomidine and the MAO inhibitors, clorgyline, deprenyl, pargyline, tranylcypromine and phenelzine exhibit biphasic displacement

curves (see table 1.2), whilst efaroxan, rauwolscine, moxonidine, agmatine, desipramine, and the reversible monoamine oxidase inhibitors Ro 41-1049, moclobemide and Ro 16-6491 have monophasic curves (see table 1.1). A similar pharmacological profile is also obtained within rabbit brain, i.e, BU224, clonidine, idazoxan, naphazoline, clorgyline, deprenyl, pargyline exhibit biphasic displacement curves (see table 1.3). Moxonidine, rauwolscine agmatine, guanabenz and the reversible monoamine oxidase inhibitors R0 41-1049 have monophasic curves, see table 1.1. Correspondingly, saturation studies (K_D) have indicated tritiated ligands bind to two differing affinity components. Tritiated cirazoline labels the high affinity site with a K_D of 0.39nM and the low affinity site a K_D of 5.61nM (Angel et al, 1994); [^{3}H]-2-BFI, high affinity site K_D = 0.29 nM and the low affinity site K_D = 8.97 nM (Lione et al, 1996) and [^{3}H]idazoxan itself labelling a high affinity site with a K_D of 0.22nM and a low affinity site with a K_D of 0.84nM (Boyajian et al, 1987). It has been suggested that [^{3}H]-RS-45041-190 also binds to two affinity components - a high affinity site (K_D = 0.57nM) and the low affinity site (K_D = 0.89nM; MacKinnon et al, 1995). However, these affinity values do not look significantly diffferent from each other.

1.1.3.2). Signal Transduction

Ernsberger et al, (1994) has suggested that the differing affinity components could be accounted for by a coupled or uncoupled G protein. However, evidence has indicated that intracellular signalling molecules, cAMP (cyclic adenosine monophosphate), IP (inositol phosphate), cGMP (cyclic guanine monophosphate) and Ca^{++} (calcium) are not regulated by ligands that bind at I_2 sites (Regunathan et al, 1991a; Reis et al, 1992).

In support of the related high affinity/low affinity hypothesis Wikberg et al (1992 & 1995), has suggest that the biphasic pattern of binding cannot be accounted for by a simple two non-interacting site model. If the binding of I_2 ligands was to individual non-interacting sites then the relative proportion of binding to each site should be the same regardless of the drug. Cirazoline binds to 42% of the available high affinity sites, whereas UK-14,304 shows a

much higher preference i.e., 72% of total binding was for the high affinity site.

Table 1.1). *Monophasic inhibitory concentrations (K_I) for [^{3}H]2-BFI and [^{3}H]idazoxan ([^{3}H]IDZ) in brain labelled I_2 sites*

	Rat [^{3}H]2-BFI (nM)	Rat [^{3}H]IDZ (nM)	Rabbit [^{3}H]2-BFI (nM)	Human [^{3}H]IDZ (nM)
Moxonidine	10,000[2],	5,000[2]	16,614[3]	
(-)-noradrenaline	60,075[8]			
BU 239			1.74[3]	
2-methoxy-idazaxon (RX821002)		45,000[6]		32,000[6]
(-)-adrenaline		106.2[4]		
Efaroxan	>100 μM[8]	58,000[5]		
Rauwolscine	>100 μM[8]		20,170[3]	
Agmatine	>100 μM[8]	>100 μM[2]	>100 μM[3]	
Histamine	>100 μM[8]		23,385[3]	
Desimipramine		6,400[1]		
Moclobemide		>100 μM[7]		
Ro 41-1049		79,000[7]	>100 μM[3]	
Ro 16-6491		1,900[1]		
Guanabenz			27.5[3]	28[6]
Medetomidine				985[6]
Rilmendine				2,100[6]

Sources: [1] Olmos et al (1993), [2] Alemany et al (1997), [3] Lione et al (1996), [4] Mallard et al (1992), [5] Alemany et al (1995a), [6] Miralles et al (1993), [7] Alemany et al (1995b), [8] Lione et al (1998).

Analysis of rat and rabbit CNS I_2 ligand binding, see table 1.2 & 1.3 shows that there is no discernible consistency in the proportion of binding to the high and low site between I_2 specific compounds, i.e., 80 % of BU224 binding is to the high site, whilst only 59% of BU239 is (Lione et al, 1998). The proportion of high affinity binding also shows inconsistency between different research on the same tissue. For idazoxan, in rat brain, Lione et al (1998) report 68 % binding to the high affinity component, whilst Alemany et al (1997) report 83 %. Furthermore, in the same tissue the proportion of high affinity binding is not consistent when different radioligands are used. When the rat I_2 site is identified with [^{3}H]2-BFI, 39% of LSL 60101 binding is to the high affinity site, whilst when [^{3}H]idazoxan is used 79% is evident (Alemany et al, 1997). Such data has led Wikberg to propose that the I_2 receptor has

multiple binding sites, which exhibit cooperativity, i.e. as one I_2 ligand binds this reduces the affinity of another I_2 ligand to another binding site on the same protein (Wikberg, 1992).

Table 1.2). *Inhibitory concentrations (K_i) for [^{3}H]2-BFI and [^{3}H]idazoxan labelled sites in rat brain for I_2 ligands that exhibit biphasic displacement curves*

	Rat^H [^{3}H] 2-BFI (nM)	Rat^L [^{3}H] 2-BFI (nM)	Rat $^{\%H}$ [^{3}H]2-BFI	Rat^H [^{3}H] IDX (nM)	Rat^L [^{3}H] IDX (nM)	Rat $^{\%H}$ [^{3}H]IDX
RS-45041-190	0.0068[6]	2.58[6]	27[6]			
*Medetomidine	50[2]	37,000[2]	72[2]	45[2]	56,000[2]	76[2]
D-medetomidine	66[6]	11,436[6]	46[6]			
L-medetomidine	56.4[6]	>100 μM[3]	44[6]			
Naphazoline	1.68[6]	849[6]	55[6]			
2-BFI	1.71[6]	242[6]	82[6]	7.5[2]	80,000[2]	73[2]
BU 224	2.08[6]	561[6]	80[6]			
BU239	4.30[6]	789[6]	59[6]			
Cirazoline	5.3[2]	4,700[2]	77[2]	3.8[2,4]	2100[2,4]	74[2,4]
Clonidine	207[2], 3.16[6]	3,602[6]	54[2], 27[6]	980[2]	40,000[2]	56[2]
Idazoxan	78[2], 7.32[6]	411[6]	83[2], 68[6]			
Bromoxidine	113[2]	63,000[2]	60[2]	96[2]	7,000[2]	74[2]
Guanabenz	14.9[6]	768[6]	77[6]			
Amiloride	21.8[6]	1231[6]	45[6]			
UK-14,304	35.2[6]	3,157[6]	63[6]			
LSL 60101	879[2]	3,200[2]	38[2]	350[2,4]	116,000[2,4]	79[2,4]
LSL 60125				151[4]	17,000[4]	60[4]
Yohimbine				32.39[3]	>100 μM[3]	
Clorgyline	11.7[6]	6,767[6]	46[6]	0.04[5]	10600[5]	38[5]
Deprenyl				1,400[1]	55,000[1]	76[1]
Pargyline				150[1]	35,000[1]	35[1]
Tranylcypromine				357[5]	771,000[5]	85[5]
Phenelzine				5,900[5]	38,400[5]	78[5]

*Medetomidine, unspecified isomer of medetomidine. Sources: [1] Olmos et al (1993), [2] Alemany et al (1997), [3] Mallard et al (1992), [4] Alemany et al (1995b), [5] Alemany et al (1995a), [6] Lione et al (1998).

1.1.3.3). I_2 Species Selectivity

In the Rabbit brain [^{3}H]-2-BFI labelled I_2 sites show a similar order of potency to the rat; (K_i < 5 nM), BU224 < BU239 < cirazoline << (K_i < 100 nM), idazoxan < guanabenz < naphazoline < debrisoquin < amiloride < clorgyline << (K_i < 10,000 nM), clonidine < deprenyl < pargyline << (K_i < 100,000nM), Ro 41-1049 << Ro 41-6491 (Lione et al, 1996). Hence all

profiles are roughly similar between the rat and rabbit. Low concentrations (K_i <5 nM) of cirazoline, BU224, BU239 are needed to displace tritiated ligand binding and slightly higher concentrations (K_i >100 nM) are needed for idazoxan, guanabenz and amiloride. However, in human brain more marked species differences are apparent. [^{3}H]idazoxan labelled I_2 sites show an identical displacement profile to that of [^{3}H]clonidine labelled I_1 sites. This being cirazoline (nM) < 2-BFI < BU224 < BU239 < (+)idazoxan < (-) ethoxy-idazoxan (mM) < (+) methoxy-idazoxan < (+) ethoxy-idazoxan < (-) efaroxan < (+) efaroxan < (-) methoxy-idazoxan (Flamez et al, 1996).

Table 1.3). ***Inhibitory concentrations (K_i) for [^{3}H]2-BFI and [^{3}H]idazoxan labelled sites in human and rabbit brain for I_2 ligands that exhibit biphasic displacement curves***

	Rabbit^H [^{3}H]2-BFI (nM)	Rabbit^L [^{3}H]2-BFI (nM)	Rabbit$^{\%H}$ [^{3}H]2-BFI	Human^H [^{3}H]IDX (nM)	Human^L [^{3}H]IDX (nM)	Human$^{\%H}$ [^{3}H]IDX
BU 224	0.018[2]	1.54[2]	20[2]			
Cirazoline	1 site[2]			2.4[1]	1,900[1]	88[1]
Clonidine	122[2]	45,380[2]	36[2]	435[1]	56,500[1]	40[1]
Idazoxan	1.02[2]	20.4[2]	48[2]	1 site[1]		
Naphazoline	7.2[2]	2,320[2]	33[2]	22[1]	2,500[1]	65[1]
Debrisoquin	0.96[2]	284[2]	36[2]			
UK-14,304				532[1]	14,000[1]	69[1]
Clorgyline	210[2]	>10,000[2]	38[2]			
Deprenyl	178[2]	29,660[2]	36[2]			
Pargyline	263[2]	>10,000[2]	29[2]			
Ro 41-1049						
Ro 16-6491	419[2]	>10,000[2]	49[2]			

Source: [1] Miralles et al (1993), [2] Lione et al (1996)

1.1.3.4). I_2 Subtypes I_{2A} and I_{2B}

Further division has been suggested based on the sensitivity of I_2 sites to amiloride. Nanomolar concentrations of amiloride are need to displace I_2 radioligand binding from the (I_{2A} amiloride-sensitive) I_2 site whilst micromolar concentrations are needed to displace radioligand binding from the insensitive I_{2B} site (see table 1.4.; Diamant et al, 1992).

Table 1.4). ***Amiloride sensitivities at various tissues***

	Amiloride (K_i) [^{3}H]idazoxan (nM)	Amiloride (K_i) [^{3}H]2-BFI (nM)
Rat Brain	5500[3]	9000[3]
Rat Astrocyte	20[7]	
Rat Liver	4,808[12]	
Rabbit Brain	148[1]	66.8[2], 66.3[4]
Rabbit Kidney	30[1]	76.2[8]
Rabbit Liver	160[11], 158[1]	
Guinea Pig Kidney	>10,000[9]	
Frog Brain		122.6[6]
Human Brain	2,500[15]	
Human Kidney	369[10]	
Human Liver	>10,000[11]	
Human placenta	72[13]	
Guinea Pig Ileum	2850[13]	
Rabbit Fat cells	48.6[14]	
Chicken Brain		1403[16]

Sources: [1] Tesson et al (1992), [2] Lione, (1994), [3] Alemany et al, (1997), [4] Lione et al (1996), [5] Lione et al (1998), [6] Hudson et al (1996), [7] Reis et al (1992), [8] Hosseini et al (1997), [9] Wikberg et al (1992), [10] Lachaud-Pettiti et al (1991), [11] Tesson et al (1990), [12] Zonnenschein et al (1990), [13] Diamant et al (1992), [14] Langin et al (1989), [15] Miralles et al (1993), [16] Danbury et al, (1999).

I_{2A} sites have been recorded within frog brain (Hudson et al, 1996), rat cultured astrocytes (Reis et al 1992), rabbit brain (Teeson et al, 1992: Lione et al, 1994; Lione et al, 1996; Olmos et al, 1999), human placenta (Diamant et al, 1992), rabbit kidney (Parini et al, 1989; Lachaud-Pettiti et al, 1991) and rabbit liver (Tesson et al, 1990). The amiloride-insensitive I_{2B} site is found within the human kidney (Michel & Insel, 1989; Lachaud-Pettiti et al, 1991), guinea pig ileum (Diamant et al, 1992), guinea pig kidney (Wikberg et al, 1992), rat liver (Zonnenschein et al, 1990), human liver (Tesson et al, 1990) and chicken brain (Danbury et al, 1999). Studies within the rat brain have indicated it expresses the amiloride insensitive I_{2B} site. Radiochemical binding assays with amiloride and a number of its analogues indicate micromolar concentration of these compounds are needed to displace [^{3}H]2-BFI from the rat cerebral cortex (Alemeny et al, 1997; Olmos et al, 1999).

Based on the affinity of clorgyline - an irreversible MAO inhibitor – a further distinction has been drawn between I_2 sites. Preincubation of cerebral cortex membranes with clorgyline reduces [^{3}H]2-BFI and [^{3}H]idazoxan binding in the rat but not the rabbit (Olmos et al, 1997; Olmos et al, 1999). Suggesting that clorgyline irreversibly binds to the I_{2B} site not the I_{2A} site predominately found within the rabbit (Olmos et al, 1997; Olmos et al, 1999).

1.1.3.5). Endogenous Ligands

A number of substances have been proposed to be endogenous ligands for imidazoline binding sites. Such compounds have been collectively known as "clonidine displacing substance" (CDS) for their abilities to displace tritiated clonidine. However evidence is beginning to accumulate suggesting that CDS purified from various species may contain a number of different, but potentially active compounds (Parker et al, 1999; Parker et al, 2000). Currently, compounds that have been purified from tissue have been separated into four groups. Classical CDS, immunoreactive CDS, agmatine and recently harmane (Atlas & Burstein, 1984; Wang et al, 1997; Reis and Regunathan, 2000; Hudson et al, 1999a)

1.1.3.5.1). Classic Clonidine-Displacing-Substance (cCDS)

CDS or a substance that displaces clonidine was initially reported by Atlas and Burstein in 1984. This molecule has an apparent molecular mass of 587 Da (Daltons) and is devoid of an NH_2 group (Atlas and Burstein, 1984; Atlas et al, 1987). Classic CDS dose dependently displaces [^{3}H]clonidine, but not [^{3}H]prazosin an α_1-adrenoceptor ligand or the β-adrenoceptor and 5-$HT_{1A/1B}$ ligand [^{3}H]cyanopindolol. Atlas et al (1987) thus proposed the molecule, clonidine displacing substance, as an endogenous α_2-adrenoceptor ligand. It was only after this period that it was widely accepted that clonidine binds to sites other than α_2-adrenoceptor, namely imidazoline binding sites (Ernsberger et al, 1987). Further binding assays, in which cCDS was pre-incubated with 10 μM noradrenaline indicated that cCDS binds, in addition to

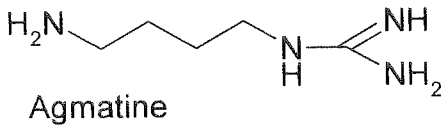
α_2-adrenoceptor, [^{3}H]clonidine I_1 sites and [^{3}H]idazoxan I_2 sites (Meeley et al, 1986; Regunathan et al, 1991b).

Classic CDS has been extracted from a number of tissues including bovine brain, rat brain, human serum and human cerebrospinal fluid (Atlas and Burnstien 1984; Grigg et al, 1998; Chan et al, 1997; Dontenwill et al, 1993; Goldberg-Stern et al, 1993). Classical CDS induces, an increase in insulin secretion from the pancreas, the release of catecholamines from bovine adrenal chromaffin cells, alters blood pressure when injected into the ventrolateral medulla of rats and inhibits the vas deferens twitch response (Diamant & Atlas, 1986; Meeley et al, 1986; Bousquet et al, 1987; Regunathan et al, 1991b; Chan et al, 1997). However, many of these effects have been attributed to CDS's affinity for α_2-adrenoceptors, rather than imidazoline binding sites (Diamant et al, 1987; Atlas et al, 1987).

1.1.3.5.2). Agmatine

Agmatine, see figure 1.3, was first purified by Li et al (1994), has an apparent molecular mass of 130 Daltons, and is present in the brain (0.2-0.4ng/g) in similar quantities to that of noradrenaline (0.4ng/g) and dopamine (0.4ng/g). Previous to this agmatine and its synthetic enzyme arginine decarboxylase had only been associated with plants and bacteria (Wu et al, 1973; Buch et al, 1990; Moore et al, 1990). Mammalian arginine decarboxylase is a mitochondrial membrane bound enzyme that catalyses the conversion of L-arginine to agmatine (Regunathan et al, 2000). Degradation occurs through two pathways; oxidation by diamine oxidase or hydrolysis catalysed by agmatinease (Holt & Baker, 1995; Satishchandran et al, 1986).

Agmatine release is dependent on depolarisation and is calcium dependent (Reis and Regunathan, 1998). Rat brain slices pre-incubated with [^{14}C]-agmatine and exposed to depolarising concentrations of KCl show a significant Ca^{2+} increase after depolarisation and before [^{14}C]-agmatine efflux (Reis and Regunathan, 1998).



Agmatine

Figure 1.3). *Chemical structure agmatine*

Agmatine has been purified from bovine brain via HPLC (high pressure liquid chromatography) and like cCDS displaces [^{3}H]clonidine in a pure α_2-adrenoceptor assay and in the presence of saturating concentrations of adrenaline, i.e., in the I_1 assay (Li et al, 1994; Feng et al, 1997). Under similar masking conditions agmatine also displaces [^{3}H]idazoxan, thus indicating agmatine binds I_2 sites. Agmatine binds to these sites with moderate potency, i.e., the concentrations needed to displace 50% of radioligand binding range from 4 to 16 μM for α_2-adrenoceptors, 1μM for I_2 sites and 33nM to 0.7μM for I_1 sites (Li et al, 1994; Piletz et al, 1995). However, agmatine's *in vitro* potency to the I_2 site seems to be tissue and species specific. Initial studies in bovine brain indicated that agmatine has a moderate to high affinity for I_2 sites (Li et al, 1994). Recent studies have indicated that agmatine has a very low affinity (>100 μM) for [^{3}H]2-BFI defined rabbit and rat brain I_2 sites (Lione et al, 1996; Lione et al 1998). However, in rat vascular smooth muscle agmatine has a high affinity for the [^{3}H]idazoxan I_2 site (240nM) and at frog brain [^{3}H]2-BFI labelled I_2 sites it is equipotent to idazoxan (6200 nM Vs 6700 nM respectively; Hudson et al, 1996; Regunathan et al, 1996).

In the rat brain agmatine distribution varies regionally (Feng et al, 1997). Immunogobulins raised against agmatine have been used to map its distribution in the rat CNS (Wang et al, 1995). In contrast to arginine decarboxylase, which is found almost exclusively in glia, immunoreactive agmatine is primarily found neuronally, within the cytosol and throughout the cell body and its process (Regunathan et al, 1995; Otake et al 1998; Reis et al 1998). In the rat brain - cell bodies that contain immunoreactive agmatine can be found within the cortex; particularly the retrosplenial, cingulate and

somatosensory cortices; and the hippocampus (Otake et al 1998; Reis et al 1998). Telencephalic areas such as the amygdala, septum, bed nucleus of the stria terminals, show high levels of immunoreactivity. The highest levels of immunoreactivity were found within the paraventricular thalamus, hypothalamus, locus coeruleus, dorsal raphe and peri-aqueductal gray. The brain stem showed moderate levels of immunoreactivity within the solitary tract and pontine parabracial complex (Otake et al 1998). Subcellularly, and similarly to sites labelled by immunoreactive proteins raised against imidazoline binding sites, agmatine immunoreactive proteins can be found within axons and axon terminals (Ruggiero et al, 1995; Reis et al 1998). These areas of binding were heavily associated with, small, circular synaptic vesicles. In contrast agmatine activity within the perikarya was heavily associated with mitochondria and tubular vesicles (Reis et al 1998).

In the pancreas agmatine has the ability to dose-dependently release insulin from glucose exposed islet β-cells and blocks glutamate-induced neurotoxicity in cerebrellar granule cells (Sener et al, 1989; Olmos, 1999). Within the rat brain agmatine releases luteinizing hormone releasing hormone from the hypothalamus (Kalra et al, 1995). In the rat moderate doses of agmatine, 10 mg/kg i.p. increases carbohydrate intake in the 4 hrs after administration (Prasad and Prasad, 1996). *In vivo* agmatine also has a reported ability to potentiate morphine induced analgesia assessed by the rat and mouse tail-flick assay (Kolesnikov et al, 1996; Li et al, 1999). However, agmatine does have an affinity for α_2-adenoceptors so it is unclear whether I_2 sites purely mediate these effects.

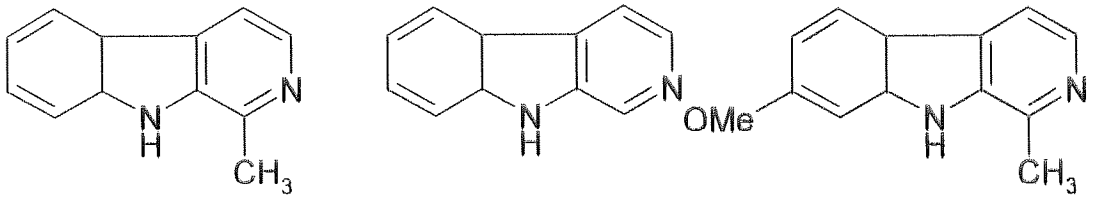
1.1.3.5.3). Immunoreactive Clonidine-Displacing Substance (iCDS)

A further molecule, of an unknown weight has been isolated by polyclonal antibodies raised against an idazoxan-albumin antigen. Immunoreactive CDS, similarly to cCDS dose dependently inhibits [^{3}H]idazoxan binding in bovine brain homogenates. However, distinct to cCDS which identifies imidazoline sites within brain tissue, iCDS identifies a molecule within human and rat serum. This same molecule was identified in human

cerebrospinal fluid of eight patients with serious neurological disorders, i.e., subarachnoid haemorrhage or glioblastomas, but not controls (Wang et al, 1997).

1.1.3.5.4). Harmane

A fourth potential candidate has recently been proposed in harmane (Hudson et al, 1999a). Harmane has been purified from human brain and radiochemical binding studies have indicated it has a high affinity for both I_1 (30.8 nM) and I_2 (49.4nM) sites, but a low affinity for α_2-adrenoceptors (17.6μM) within the rat brain (Moncrieff 1989; Hudson et al, 1999a; Alan Hudson, personal communication).



Harmane Norharmane Harmaline

Figure 1.4). *Chemical structure of harmane, norharmane and harmaline*

Harmane belongs to a large group of compounds that are collectively known as the β-carbolines, see figure 1.4. In addition to harmane and its metabolite norharmane, the family includes - harmine, tetrahydro β-carboline (noreleagnine), and the hallucinogen harmaline (Grella et al, 1998; Naranjo 1967, Naranjo 1969). Some of these compounds, in addition to being found present within the human brain, can be derived from the Asian and Middle Eastern plant *peganum harmala*, and have been used for centuries as psychoactive compounds (Naranjo 1967; Callaway et al, 1999). In addition to harmane, norharmane, noreleagnine, harmane, harmine and harmaline also show a high (nM) affinity for the I_2 site (Hudson et al, 1999a). Recent work has indicated that microinjection of harmane into the rat rostral ventrolateral medulla significantly reduces blood pressure with a potency similar to clonidine (Musgrave and Badoer, 2000). This effect was antagonised by administration of I_1 ligand and α_2-adrenoceptor antagonist

efaroxan (Musgrave and Badoer, 2000). However, as harmane has a high micromolar affinity for α_2-adrenoceptors it has been suggested by the authors that the hypotension reported here is mediated through I_1-sites (Hudson et al, 1999a; Musgrave and Badoer, 2000).

1.2). Distribution

1.2.1). Molecular Distribution

1.2.1.1). Cellular and Subcellular Location

I_2 binding sites have been localised to both plasma and intracellular membranes in a number of tissues (Piletz et al, 1991, Diamant et al, 1992). A great deal of evidence has indicated that the intracellular membrane bound I_2 site may be on monoamine oxidase, the enzyme associated with the oxidative deamination of catecholamines and 5-HT (5-hydroxytryptamine; Von Korff, 1979; Raddatz et al, 1999). Radiolabelled I_2 sites with similar molecular weights to MAO have been immunoprecipitated with MAO specific monoclonal antibodies (Tesson et al 1995). However, a range of other proteins have been isolated and their function and cellular location has yet to be clarified (Escriba et al, 1999). I_1, I_2 binding sites have been localised to both plasma and intracellular membranes on human platelets (Piletz et al, 1991). Internal platelet membranes showed a higher proportion of I_2 [^{3}H]idazoxan binding (B_{max} = 192 fmol/mg) when compared with plasma membranes (B_{max} = 141 fmol/mg protein), whilst [^{125}I]PIC ([^{125}I]p-iodoclonidine) I_1 binding was almost exclusively found on plasma membranes. Inhibition curves against [^{3}H]idazoxan using internal membranes indicated that cirazoline, guanabenz, naphazoline, clorgyline and clonidine, exhibit single site binding profiles. These drugs have previously been reported to exhibit biphasic inhibition curves against [^{3}H]idazoxan I_2 sites (Olmos et al 1992; Olmos et al, 1993; Alemany et al, 1997). [^{3}H]idazoxan assays have also identified a number of high density binding regions (B_{max} = 1800-3642 fmol/mg protein) on placental tissue,

these binding sites were reportedly expressed on the cell surface (Diamant et al, 1992).

1.2.1.2). Molecular Binding Proteins

In an attempt to clarify the molecular nature of the imidazoline binding site researchers have utilised three methods, anti-imidazoline antibodies, anti-idiotypic (anti-anti-idazoxan) antibodies and iodinated cirazoline derivatives. Initial studies by Wang and co-workers involved the isolation of 66 kDa [^{3}H]idazoxan binding protein from bovine chromaffin cells (Wang et al, 1992). Antibodies raised against this protein have been subsequently used to identify other imidazoline binding proteins in a number of species (Wang et al, 1992; Wang et al, 1993). In rat and human brain, proteins with a molecular weight of 29, 35, 45, and 66 kDA, have been isolated (Garcia-Sevilla et al, 1996; Escriba et al, 1999). The 29, 35, and 45 kDa proteins have been detected within the rat frontal cortex, parietooccipital cortex, caudate nucleus, hypothalamus, hippocampus, medulla and cerebellum. In addition the optical densities of these SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) bands significantly correlated with [^{3}H]idazoxan binding densities (B_{max}).

In human brain only the 29 kDa was consistently present within all the afore mentioned brain areas. The 45 kDa protein could only be detected in human frontal cortex, hypothalamus, hippocampus and medulla. In addition the human medulla has been shown to contain additional immunoreactive proteins with molecular weights of 42, 43 and 50 kDa (Escriba et al, 1994; Greney et al, 1994). In rat peripheral organs, such as the liver, immunoreactivity is only present for the 29 kDa protein. However, the human liver shows banding for both the 29 and 66 kDa proteins (Escriba et al, 1994).

Competition experiments against [^{3}H]idazoxan using the rat 45 kDa protein have indicated this protein shows the characteristic, biphasic, inhibitory profile of the I_2 site, i.e., (nM) cirazoline < guanabenz < amiloride << (μM) efaroxan < agmatine (Escriba et al, 1995; Escriba et al, 1999).

Radiochemical binding studies against the 66 kDa protein show a similar pattern i.e., [^{3}H]idazoxan binding is inhibited by nanomolar concentrations of cirazoline and guanabenz but micromolar concentrations of amiloride, efaroxan and agmatine are needed for the same effect. Hence, these data indicate the 45 kDa protein is amiloride sensitive, whilst the 66 kDa protein is amiloride insensitive (Olmos et al, 1994).

Seven day chronic treatment with I_2 selective ligands significantly increases the immunoreactivity in some of these proteins and the [^{3}H]idazoxan binding maximum in all. High doses of idazoxan, i.e., 10mg/kg, significantly increased levels of the 29/30 and 66 kDa protein. Low doses of LSL 60101 (10mg/kg), only significantly increased levels of the 66 kDa protein and cirazoline (1mg/kg) significantly increased the 45 kDa protein. A similar dosing scheme using the specific α_2-adrenoceptor ligands yohimbine and efaroxan failed to alter immunoreactivity or the [^{3}H]idazoxan I_2 B_{max} (Escriba et al, 1996).

Anti-idiotypic antibodies, i.e., polyclonal antibodies raised in rabbits against purified polyclonal anti-idazoxan antibodies, have also been used to identify potential imidazoline binding proteins. These, anti-idiotypic antibodies inhibit [^{3}H]idazoxan binding to anti-idazoxan antibodies, but fail to alter [^{3}H]rauwolscine α_2-adrenoceptor binding in the rat brain – suggesting these anti-idiotypic antibodies do not bind to α_2-adrenoceptors (Bennai et al, 1996). Western blots conducted in human brain membrane preparations show these anti-idiotypic antibodies identify two proteins with apparent molecular weights of 43 and 85 kDa (Bennai et al, 1996; Ivanov et al, 1998b). The 85 kDa has also been isolated in rat brain (Ivanov et al, 1998b). It has been proposed that the 85KDa protein represents the complete imidazoline receptor. The authors suggest the smaller proteins that have been previously isolated are products of proteolytic breakdown during processing (Ivanov et al, 1998b). However, whether this molecule represents an I_1 or an I_2 receptor remains to be clarified.

Further proteins have been isolated with the use of the iodinated cirazoline derivatives ^{125}I-AMIPI (2-[3-amino-4-{125I}iodophenoxy]methyl imidazoline)

and ^{125}I-AZIPI (2-[3-azido-4-{125I}iodophenoxy]methyl imidazoline; Lanier et al, 1993; Ivkovic et al, 1994; Lanier et al, 1994). In rat liver, purification, labelling with ^{125}I-AZIPI, and gel electrophoresis has isolated three proteins with apparent molecular weights of 25-27, 55 and 61 kDa. However, only the 55 and 61 kDa proteins were present in rat brain (Lanier et al, 1995). Further ^{125}I-AZIPI studies in rat and human liver have suggested that the 55 and 61 kDa proteins correspond to MAO-A (monoamine oxidase A) and MAO-B (monoamine oxidase B) respectively - the enzymes associated with the oxidative deamination of dopamine, 5-HT (5-hydroxytryptamine), and noradrenaline (Von Korff, 1979; Raddatz et 1997; Raddatz et al, 1999).

1.2.1.3). Monoamine Oxidase

I_2 sites have been repeatedly associated with the enzyme monoamine oxidase [MAO; amine: oxygen oxidoreductase (deaminating, flavin containing); EC 1.4.3.4] (Tesson et al, 1991; Tesson et al, 1992; Regunathan et al, 1993; Alemany et al, 1995; Gargalidis-Moundanos et al, 1997; Raddatz et al 1999). MAO catalyses the oxidative deamination of a number of indolamine and catecholamine neurotransmitters, i.e., 5-HT (5-hydroxytryptamine or serotonin), dopamine (DA) noradrenaline (NA), and dietary amines (Von Korff, 1979). MAO is a mitochondrial membrane bound enzyme and contains a flavin (FAD) cofactor (Greenawalt & Schnaitman, 1970; Kalgutkar et al, 1995). MAO has been separated into two distinct isoforms - MAO-A and MAO-B – on substrate specificity, pharmacological profile, autoradiographical distribution, deduced amino acid sequence, mRNA distribution and DNA sequence (Johnson, 1968; Knoll & Magyar, 1972; Saura et al 1992; Bach et al, 1988; Luque et al, 1995).

In vitro MAO-A preferentially oxidises 5-HT (5-hydroxytryptamine, serotonin) and is inactivated irreversibly by clorgyline (Johnson, 1968) - whereas MAO-B shows a preference to, benzylamine, β-phenylethylamine and is sensitive to inhibition by l-deprenyl (Knoll & Magyar, 1972). Dopamine and noradrenaline have equal selectivity to the catalytic activity of MAO-A and -B (Rivett et al, 1982). MAO-A and -B have been separated into distinct entities

on their deduced amino acid structure and cDNA base sequence. The A isoform has a molecular weight of 59,700 Da and the B isoform 58,800 Da, with the isoforms showing 70% homology (Bach et al, 1988).

In situ hybridisation, receptor autoradiographical and immunocytochemical techniques have indicated that MAO-A is found predominately within noradrenaline and 5-HT neurons (Westlund et al, 1988; Fagervall & Ross, 1986; Butcher et al, 1986; Luque et al 1995). However, a small but significant quantity of MAO-A can be found within dopamine neurons (Westlund et al, 1988; Fagervall & Ross, 1988; Luque et al 1995). MAO-B is found primarily on glial cells (Konradi et al, 1989) and within only 5-HT cell bodies in the dorsal raphe (Westlund et al, 1988; Konradi et al, 1988). *In vivo* studies have indicated that dopamine is degraded by MAO-A not -B. However, under conditions of MAO-A inhibition metabolism of the afore mentioned neurotransmitters is mediated by MAO-B (Fagervall & Ross, 1986; Butcher et al, 1990). Hence, which substrate MAO-A or -B degrades is dependent on whether 5-HT, noradrenaline or dopamine is available.

MAO Inhibitors were initially used to treat depression (Crane. 1957). But due to their ability to irreversibly inhibit MAO, potentiate existing plasma tyramine levels, and induce a hypertensive crisis their use has been restricted. Distinction of the isoforms indicated this side effect was associated with the irreversible inhibition of MAO-A (Youdim, 1995). However, the recent development of reversible inhibitors of the enzyme has led to resurgence in interest. These reversible inhibitors do not react with dietary tyramine and drugs such as Moclobemide are clinically effective in the treatment of depression (Sambunaris et al, 1997) and have reportedly low side effects (Youdim, 1995; Anderson et al, 2000). Selegline (deprenyl), an irreversible inhibitor of the B isoform has been used extensively in the treatment of Parkinson's disease, during the initial onset of the disease and as an adjunct to levodopa in the later stages (The Parkinson Study group, 1989 & 1993a; Quinn et al, 1986). Recently, Lazabemide (RO19-6327), a well-tolerated reversible inhibitor of MAO-B has also been shown to be clinically effective in the treatment of the disease (The Parkinson Study group, 1993b).

Although research irrefutably links I_2 sites with MAO, the relationship they have to each other is far from clear. Such a relationship is characterised by a number of experimental findings.

Firstly, a number of studies have indicated that I_2 ligands inhibit MAO in a reversible manner (Brown et al, 1995; Carpene et al, 1995, Ozaita et al, 1997; Gargalidis-Moudanous et al, 1997: Lalies et al, 1999). As table 1.5 illustrates *in vitro* the specific I_2 ligand 2-BFI and its analogues BU224, BU226, BU239 and BU216 show a preference for MAO-A over -B and inhibit MAO with a similar potency to the reversible MAO-A inhibitor moclobemide (Lalies et al, 1999). The imidazole moiety containing compounds LSL 60101 and LSL 61122 show a slight preference for MAO-B over MAO-A and inhibit with moderate potency when compared with deprenyl or Ro 16-6491 (Ozaita et al, 1997). In addition to the ability of I_2 ligands to inhibit MAO they also have a moderate ability to inhibit other amine oxidases, notably bovine plasma amine oxidase (Carpene et al, 1995).

Secondly, *In vivo* the chronic treatment with the MAO inhibitors, clorgyline, phenelzine and pargyline results in the down regulation of rodent [^{3}H]-idazoxan I_2 binding sites (Olmos et al, 1993; Alemany et al, 1995). *In vitro*, only clorgyline and pargyline irreversibly reduce [^{3}H]-idazoxan binding. Phenelzine fails to alter [^{3}H]idazoxan binding, but does decrease the levels of [^{3}H] Ro 41-1049 (a reversible MAO-A inhibitor) binding (Alemany et al, 1995). Chronic treatment with non specific doses of the MAO inhibitors, phenelzine and clorgyline, significantly reduces the levels of the three imidazoline immunoreactive proteins mentioned above and their [^{3}H]-idazoxan I_2 B_{max} (Escriba et al, 1996).

Thirdly, autoradiographical studies of [^{3}H]2-BFI I_2 distribution within the rat CNS indicate high levels of binding within the arcuate nucleus (Arc), area postrema (Ap), pineal gland (Pi), lateral mammillary nucleus (Lm), interpeduncular nuclei (Ip) and ependyma/lateral ventricles (Lione et al, 1998), This distribution closely matches the autoradiographical localisation of MAO-B, but not MAO-A (Saura et al, 1992; Luque et al, 1995) see table 1.6.

Table 1.5). ***Potency of Various ligands to Inhibit MAO***

Drugs	[^{14}] 5-HT[1,2] *MAO-A* IC_{50} μM	[^{14}] PEA[1,2] *MAO-B* IC_{50} μM	[^{14}C]Tyramine[3] *MAO* pIC_{50} μM
2-BFI	11	23	48
BU226	2.9	16.9	
Guanabenz	4	40	
BU224	4.8	44.8	
Cirazoline	11	31	20
BU216	36.1	88.9	
BU239	33.5	102.8	
LSL 60101	58	16	
LSL 61122	100	32	
RX 821209	185.7	229.7	
(+)-Idazoxan	280	624	<1 mM
Clonidine	700	6,000	
Agmatine	>10,000	>10,000	
Moclobemide (MAO-A specific)	36	>1,000	
Ro 41-1049 (MAO-A specific)	0.13	31	
Clorgyline (MAO-A specific)	0.03	8	12
Deprenyl (MAO-B specific)	1.5	0.019	
Ro 16-6491 (MAO-B specific)	85	0.12	
Pargyline (MAO-B specific)			1.5
isatin	18	2	

Sources: [1] Ozaita et al (1997), [2] Lalies et al (1999), [3] Carpene et al (1995).

Photo-labelled I_2 binding proteins can be immunoprecipitated by monoclonal antibodies specific to MAO-A and MAO-B (Raddatz et al, 1995). Antibodies specific for MAO-A immunoprecipitated an ^{125}I-AZIPI labelled protein with an apparent molecular weight of `63,000 and MAO-B antibodies immunoprecipitated a ^{125}I-AZIPI labelled protein with a molecular weight of 59,000 (Raddatz et al, 1995). These weights are similar to those published for MAO-A (59,700 Da) and B (58,800) (Bach et al, 1988).

Finally, human MAO-A and MAO-B expressed in yeast cells exhibits spontaneous [^{3}H]idazoxan binding in comparison to controls or wild types. This binding was of high affinity, saturable, reversible and inhibited by cirazoline and guanabenz. These gene products retained their ability to

oxidise [^{14}C]tyramine. Pre-incubation with clorgyline, deprenyl, pargyline, Ro 41-1049, Ro 19-6327, all failed to inhibit [^{3}H]idazoxan binding (Tesson et al, 1995). MAO-B protein fragments, which fail to bind the specific MAO-B inhibitor [^{3}H]RO-19-6327, have been labelled with ^{125}I-AZIPI and immunoprecipitated with antibodies raised against MAO-B (Raddatz et al, 1997). These authors take this as evidence to suggest that in whole mitochondria idazoxan is binding to a site on MAO-A and -B that is distinct to the catalytic region (Raddatz et al, 1999).

Table 1.6). *Pharmacological and molecular binding parameters of [^{3}H]2-BFI I_2 sites, MAO-A and -B mRNA*

Region	Autoradiography			In Situ Hybridisation	
	MAO-A[1] fmol/mg protein	*MAO-B[1] fmol/mg protein*	*I_2[2] fmol/mg protein*	*MAO-A mRNA[*3]*	*MAO-B mRNA[*3]*
Arcuate nucleus	4,730	9,510	112.1		
Subfornical organ	3,700	16,290		0.0	7.0
Area postrema	3,820	7,350	96.7	0.5	7.5
Interpeduncular nuc.	9,170	6,920	88.7	4	10
Ependyma/lateral ventricles	4,150	7,960		0.5	7.0
Pineal gland	1,750	10,510	63.9	0.0	2.0
Lateral mammillary nuc.	4,560	5,820	54.3	0.5	7.5
Tubero-mammillary	5,030	6,670			
Dorsal Raphe	5,910	7,800	32.6		

[1] Saura et al, (1992), [2]Lione et al (1998), [3]Luque et al (1995) * data derived from relative optical density measurements, 0-1 background, 10 maximum.

1.2.1.4). Glial Fibrillary Acidic Protein (GFAP)

GFAP (glial fibrillary acidic protein) is the main component of astrocyte intermediate filaments (Eng et al, 1971) and as such is used as a marker in a variety of neurological disorders that exhibit astrocyte hypertrophy i.e., scrapie, Creutzfeldt-Jakob disease and dementia (Herrera & Cuello, 1992; Wallin et al, 1996; Kordek et al, 1997; Tatzelt et al, 1996).

Previous work has indicated that cultured astrocytes treated with idazoxan exhibit a dose dependent increase in GFAP mRNA (Regunathan et al, 1993). Other binding experiments in the rat cerebral cortex have indicated an increase in glial fibrillary acidic protein (GFAP) immunoreactivity after *in vivo* chronic treatment with cirazoline, idazoxan (Olmos et al, 1994) and LSL 60101 (Alemany et al, 1995). The 49kDa protein identified via [^{3}H]idazoxan binding and SDS-PAGE was within the range, 47-51 kDa, previously reported for GFAP. However, this treatment did not reduce the level of MAO-B sites, as labelled by [^{3}H]-Ro 19-6327, another binding site found on astrocytes (Olmos et al, 1994). Parallel experiments indicated that imidazoline treatment not only increased GFAP levels, but also increased [^{3}H]idazoxan I_2 site binding. However, this increase was not associated with changes in 2-methoxy idazoxan, an analogue of idazoxan that has a low affinity for I_2 sites but high affinity for α_2-adrenoceptors (Olmos et al, 1994). Alemany et al (1995) replicated these findings using the imidazole compound LSL 60101, but not with its 6-methoxy analogue LSL 60125. This led the authors to suggest that LSL 60101 is a specific ligand for I_2 GFAP binding sites (Alemany et al, 1995).

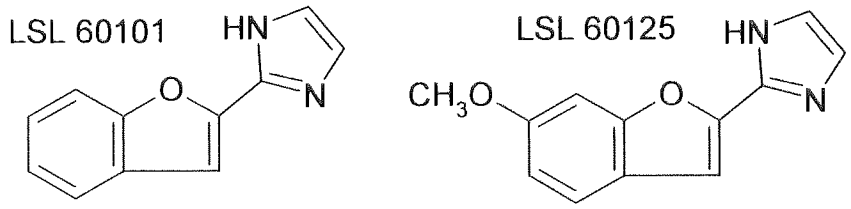


Figure 1.5). *Chemical structure LSL 60125 and LSL 60101*

Recently rat hippocampal GFAP levels have been assessed in response to global forebrain ischemia. As previously mentioned, an increased level of GFAP is associated with brain injury, particularly vascular injury (Herrera & Cuello, 1992). Rodent models of global forebrain ischaemia report an increase in GFAP levels and upregulation of benzodiazepine receptors, but no changes were apparent in I_2 levels as assessed with [^{3}H]2-BFI (Conway et al, 1998).

1.2.1.5). Ion Channels

The results of a number of studies have suggested that Imidazoline ligands alter ion channel function in a variety of receptor systems, i.e., K^+ channel, nicotinic acetylcholine, 5-HT_3, and NMDA (N-methyl-D-aspartate) receptors (Dunne, 1991; Musgrave et al, 1995; Molderings et al, 1995a; Olmos et al, 1996).

An abundance of K^+ (potassium) and K^+ channel blockers has been reported to reduce [^{3}H]idazoxan I_2 binding in rat liver, human kidney and placenta (Zonnenschein et al, 1990; Lachaud-Pettiti et al, 1991; Diamant et al, 1992). In cultured rat insulin secreting cells the application of imidazoline ligands, i.e., efaroxan and phentolamine, inhibit K^+_{ATP} channels (Dunne, 1991; Chan et al, 1994). However, as previously mentioned the insulin associated imidazoline site exhibits pharmacology distinct to that of the I_1 or I_2 site. Although idazoxan does bind to K_{ATP} channels it does not stimulate insulin secretion from the perfused rat pancreas (Chan et al, 1995; Dunne 1990). On the basis of these findings and those mentioned previously, it has been suggested that the pancreatic K^+_{ATP} site represents a third imidazoline site (Chan et al, 1994; Dunne et al, 1995). Potassium channel blockers also reduce the firing reported in the locus coeruleus after application of 2-BFI to rat brain slices (Ugedo et al, 1998).

In cultured PC-12 cells (Pheochromocytoma cells), the nicotine induced intracellular accumulation of Ca^{++} can be blocked in a dose dependent manner by clonidine and cirazoline. This effect is reported to be independent of α_2 adrenoceptor interaction as PC 12 cells are reported not to express them (Musgrave et al, 1995). In addition clonidine, oxymetazoline, phentolamine also block nicotinic induced catecholamine secretion from adrenal medullary cells (Imaizumi & Kumakura, 1992).

The mouse N1E-115 neuroblastoma cell line heavily expresses 5-HT_3 receptors and [^{14}C]guanidinium influx represents a model of ion channel activation. Imidazoline ligands such as, idazoxan, agmatine and naphazoline inhibit, albeit a little weakly, 5-HT induced [^{14}C]guanidinium influx

(Molderings et al, 1995a). This effect was dose dependent and also observed with the application of sigma ligands, but not adrenaline - thereby excluding the direct mediation of these effects through adrenaline (Molderings 1996).

Glutamate is the major excitatory neurotransmitter in the brain and acts as an agonist at the NMDA receptor ion channel complex (Watkins & Olverman, 1987). Radiochemical studies have indicated that at high micromolar concentrations imidazoline ligands, i.e., naphazoline, clonidine, idazoxan, displace the NMDA specific antagonist [^{3}H] MK801 (Olmos et al, 1996). *In vitro*, glutamate exposure to cultured cerebellar granule cells leads to neurotoxicity (Choi, 1988). This mechanism is thought to be mediated through the over stimulation of NMDA (N-methyl-D-aspartate) receptors (Schramm et al, 1990). Concurrent incubation of glutamate and high micromolar concentrations of imidazoline ligands, i.e., agmatine or idazoxan, dose dependently prevents glutamate neurotoxicity (Olmos et al, 1996; Sener et al, 1989; Olmos, 1999; Milhaud et al, 2000) and it has been proposed that imidazoline ligands exhibit this effect by direct interaction with the ligand-gated ion channel (Musgrave & Hughes, 1999; Olmos et al, 1999; Milhaud et al, 2000).

1.2.2). Gross Distribution

1.2.2.1). Autoradiography

Autoradiographical studies have localised I_2 sites within the frog, rabbit and rat brain (Tyacke et al, 1999; Lione et al, 1997; Mallard et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). These studies have used three I_2 specific ligands, [^{3}H]idazoxan, [^{3}H]-2-BFI and [^{3}H]-RS-45041-190. All have consistently bound to the same discrete brain nuclei. [^{3}H]-RS-45041-190, [^{3}H]idazoxan and [^{3}H]2-BFI show high levels of specific binding within the area postrema (215.6 Vs 208.7 Vs 96.73 fmol/mg tissue respectively), arcuate nucleus (247.3 Vs 102.2 Vs 112.09 fmol/mg tissue) and interpeduncular nucleus (217.4 Vs 123.4 Vs 88.75 fmol/mg tissue; Mallard et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). [^{3}H]-RS-45041-190 and

[^{3}H]idazoxan label with relatively high density the medial habenular nucleus (159.2 Vs 45.4 fmol/mg tissue), caudate putamen (52.1 Vs 29.7 fmol/mg tissue), solitary tract (100.6 Vs 22.5 fmol/mg tissue), and entorhinal cortex (85.1 Vs 18.6 fmol/mg tissue; Mallard et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). Autoradiographical studies in the frog brain show a similar pattern of binding, i.e., high levels are found within the interpeduncular nucleus. Lower levels of binding were reported within the optic tracts, central grey, anterodorsal tegmental nuclei, oculomotor nucleus, perioptic recess and periventricular perioptic nucleus (Tyacke et al, 1999).

1.2.2.2). Immunocytochemistry

The location of I_2 binding sites within the CNS has been further attempted with the use of immunoreactive imidazoline antibodies. The distribution of sites broadly matches those consistently identified by [^{3}H]idazoxan, [^{3}H]2-BFI and [^{3}H]-RS-45041-190 autoradiography. Namely, the medial habenular nucleus, interpeduncular nucleus, solitary tract, inferior olives, subfornical organ, arcuate nucleus. These areas show moderate to high levels of immunoreactivity and radiochemical ligand binding (Ruggiero et al, 1995; MacKinnon et al, 1995; Lione et al, 1998). However, little immunoreactivity was observed within the locus coeruleus, area postrema and pineal gland and none within the ependyma, areas which exhibit [^{3}H]-RS-45041-190 and [^{3}H]2-BFI binding (Ruggiero et al, 1995; MacKinnon et al, 1995; Lione et al, 1998). The authors note the heavy immunolabelling of the nucleus ambiguus, in a manner that suggested the immunoreactive proteins could be localised to terminal boutons which lie close to motorneurons (Ruggiero et al, 1995). Ultrastructural analysis on a section of the area postrema suggested these binding sites could be localised to astroglial mitochondria (Ruggiero et al, 1995). However, immunoreactivity was also detected within unmyelinated axons containing microtubules and axon terminals which formed synapses with small dendrites, suggesting a potential non MAO/mitochondrial bound I_2 site (Ruggiero et al, 1995).

1.2.2.3). Relationship of I_2 Binding to Potential Neural Circuits

As previously mentioned, in rat brain [^{3}H]2-BFI autoradiographic binding correlates with MAO-B but not MAO-A distribution (Saura, et al, 1992; Lione et al, 1998). Of the regions outlined above, the area postrema, arcuate nucleus and interpeduncular nucleus, all showed high levels of MAO-B (< 6.9 pmol mg/protein) and moderate levels of MAO-A (< 3.8 pmol mg/protein; Saura et al, 1992). Some of those sites labelled densely by [^{3}H]-RS-45041-190, i.e., the solitary tract and dorsal raphe, but not [^{3}H]2-BFI also show high MAO-A and MAO-B activity (Saura et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). However, areas in which high levels of MAO binding are apparent, i.e., ventromedial hypothalamus, show only low levels of [^{3}H2-BFI binding. Such data suggests that an I_2 site can be localised to or around MAO, but it remains to be clarified how this distribution relates to that of MAO-A and MAO-B (Saura et al, 1992). The MAO-B association is further supported by the immunochemical data which, as previously indicated, suggests immunoreactive proteins can be localised to astroglia - a CNS cell type expressing high levels MAO-B (Konradi et al, 1989; Ruggiero et al, 1995).

The striking thing about autoradiographical and immunocytochemical studies of this type is the number of ventricular and circumventricular areas that show high quantities of I_2 ligand binding, i.e., the subfornical organ, arcuate nucleus, area postrema, pineal gland and ependyma/lateral ventricles (Saura et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). The circumventricular organs have been associated with the detection of blood borne proteins and the secretion of hormones that exert their effects in the periphery i.e., glucagon like peptide (GLP), gonadotrophin releasing hormone, and melatonin (Goke et al, 1995; Simonneauex 1995; Borjigin et al, 1999). Recent autoradiographical studies of glucagon like peptide (GLP) binding sites indicate they have a similar autoradiographical distribution to that of I_2 sites (Goke et al, 1995; Lione et al, 1998). Hence, high levels of GLP binding were found within the area postrema, subfornical organ,

interpeduncular nucleus, thalamus, hypothalamus, inferior olives and solitary tract (Goke et al, 1995).

Two neurotransmitter pathways have been associated with innervating the pineal gland: the noradrenergic sympathetic pathway which originates from the superior cervical ganglion and the cholinergic parasympathetic pathway that originates from the peripheral parasympathetic ganglia (Phansuwan-Pujito et al, 1999). However, pathways that innervate the gland, and the gland itself also contain a number of neuropeptides, i.e., neuropeptide Y, vasoactive intestinal peptide and the peptide histidine isoleucine (Moller 1997). But it is the pineal gland's primary association with melatonin that it is best known (Borjigin et al, 1999). It has been proposed that, along with noradrenaline, these neuropeptides regulate the synthesis and secretion of melatonin from the pineal gland. It has been long established that circulating levels of melatonin alter on a diurnal and seasonal basis. Melatonin levels are always high throughout the night but upon exposure to light these levels rapidly fall. Hence, it has been suggested that the pineal gland and its ability to secrete melatonin represents the hormonal signal for the onset of day time within vertebrates (Klein et al, 1997).

The area postrema is located towards the caudal end of the fourth ventricle and as such is the only circumventricular organ within the hindbrain. Due to the increased permeability of the blood brain barrier around the area postrema it is anatomically well situated to detect toxins within the CSF (cerebrospinal fluid) and blood which initiate emesis or vomiting – hence, the area postrema acts as a chemoreceptor (Miller and Leslie, 1994). Along with the solitary tract, another area which exhibits I_2 binding, the area postrema and dorsal motor nucleus form the dorsal vagal complex (Miller and Leslie, 1994). Although experimental evidence suggests that the area postrema is not essential for emesis it is thought that the dorsal vagal complex represents the beginning of the final common pathway that different emetic factors use to instigate vomiting (Miller and Leslie, 1994). Experimental evidence has also suggested that the area postrema regulates cardiovascular function as the hypertensive response elicited by angiotensin II is cancelled by lesioning the area postrema (Bishop and Hay, 1993).

Angiotensin II has also been heavily associated with regulating blood pressure and body fluid balance. It has been previously shown that intracranial injection of angiotensin II into the subfornical organ increases blood pressure and drinking behaviour (Mangiapane and Simpson, 1980). As mentioned above autoradiographical studies using [^{3}H]-RS-45041-190 localise high levels of binding within the subfornical organ (MacKinnon et al, 1995). Recent studies have indicated that injection of rilmendine or clonidine into the hypothalamic paraventricular nuclei significantly antagonises the angiotensin II induced hypertension reported after injection of anginotension II into the subfornical organ (Arrais et al, 1997; Saad et al, 1998; Araujo-Almeida et al, 1999). However, co-administration of idazoxan with rilmendine or clonidine into the paraventricular hypothalamus and angiotensin II into the SFO, leads to blood pressure rising back up to levels comparable to that after angiotensin II had been administered to the SFO alone (Saad et al, 1998). Hence these data suggest that idazoxan antagonises clonidine or rilmendine's effects in the paraventricular thalamus. Saad et al (1998) suggest that imidazoline binding sites within both the hypothalamic paraventricular nuclei and subfornical organ control blood pressure. Bearing in mind the high levels of I_2 binding found within the SFO and moderate areas within the hypothalamus it is tempting to agree with this conclusion. However, Saad et al (1998) fail to show these effects are specific to imidazoline ligands, i.e., if this response is mediated by imidazoline binding sites then non-imidazoline α_2-adrenergic agonists and antagonists should have no effect on the hypertensive effects of angiotension II injection into the SFO. Unfortunately these compounds were not tested.

The hypothalamic arcuate nucleus surrounds the 3rd ventricle and represents the largest circumventricular organ within the body. Due to the position of the arcuate nucleus within the brain, on the hypothalamic-pituitary axis, it is heavily associated with relaying hormonal responses to the periphery.

To date a number of hormone associated binding sites have been identified. These include progesterone, growth hormone and thyroid hormone associated sites (Scott et al, 2000; Willesen et al, 1999; Fekete et al, 2000). The arcuate nucleus acts as a central relay point in the thyroid axis. Thus in response to fasting the arcuate nucleus releases thyroid releasing hormone into the adenohypophysis, which in turn releases thyroid stimulating hormone onto the thyroid gland. Conversely, high levels of T3 and T4 (the primary hormones released from the thyroid) within the arcuate nucleus reduce gene expression of thyroid releasing hormone, thereby regulating T3 and T4 activity by a negative feedback loop (Fekete et al, 2000). The arcuate nucleus is also associated with other hypophyseal hormones notably growth hormone. In a manner similar to that of the thyroid axis, the arcuate nucleus releases growth hormone secretagogues into the neurohypophysis which through receptor mediated interaction releases growth hormone into the periphery (Willesen et al, 1999).

1.3). *In Vivo* Studies.

1.3.1). Bioavailability

In the rat, a single dose of 7mg/kg 2-BFI, given intraperitonially leads to an estimated peak brain concentration of 1000nM (Jordan et al, 1996). Similar CNS concentrations are apparent after administration of the 2-BFI analogue BU224 (Hudson et al, 1999b). Given i.p. a dose of 1mg/kg BU224 gives rise to a CNS concentration of 124 nM. Uptake across the blood brain barrier is dose dependent as 5mg/kg gives rise to a CNS concentration of 220 nM and at 10 mg/kg this increases to 1,280 nM (Hudson et al, 1999b). This dose dependent increase is also apparent when BU224 is administered intravenously (i.v.). A dose of 1mg/kg give i.v., gives rise to a brain concentration of 190 nM. This increases to 650 nM at 5mg/kg and 2100 nM (2.1μM) at 10 mg/kg (Hudson et al, 1999b).

Despite their low affinity for α_2 adrenoceptors *in vivo*, I_2 specific ligands such as 2 BFI, BU224 and 2 BDI (2-(1,3-benzodioxanyl)-2-imidazoline) increase extraneuronal levels of NA (Jordan, 1993; Lalies and Nutt, 1993; Hudson et

al, 1999b). Using the technique of *in vivo* microdialysis it has been shown that i.p. administration of 2-BFI, 2-BDI, BU224 dose dependently increases NA overflow within the rat frontal cortex and striatum (Jordan, 1993; Lalies and Nutt, 1993; Hudson et al, 1999b). In addition to NA, dialysis studies in the rat striatum indicate i.p. administration of 2-BDI significantly increases dopamine overflow; decreases levels of DOPAC (3, 4-dihydroxyphenylacetic acid) – one of the primary metabolites of dopamine, and 5HIAA the primary metabolite of 5-HT (Lalies and Nutt, 1993). Similarly to 2-BDI the administration of 2-BFI, increases dopamine overflow, reduces levels of DOPAC, but in contrast increases levels of 5 HIAA in the rat striatum. Levels of striatal HVA (homovanillic acid), the other primary metabolite of dopamine, and frontal cortex 5-HIAA (5-hydroxyindoleacetic acid) were shown to be unaltered (Lalies and Nutt, 1993; Hudson et al, 1999b). As is the case with 2-BFI and 2-BDI, i.p. administration of BU224 increases striatal overflow of NA, dopamine and reduces DOPAC, HVA, 5-HIAA and cortical 5-HIAA levels (Hudson et al, 1999b). This profile is consistent with the inhibition of MAO, i.e., increases in catecholamines and 5-HT through inhibition of the enzyme and a decrease in metabolites (DOPAC, HVA, 5-HIAA) as they are cleared from the brain (Kato et al, 1986; Butcher et al, 1990).

1.3.2). Behavioural and Physiological Drug Screening

A wide variety of behavioural and physiological states have been assessed in 2-BFI treated rats. These include, head twitches, tail pinch response, piloerection, vasodilation and tremor (Jordan, 1993). However, only one of the thirty seven measures, outlined by Irwin (1968) were affected. At a dose of 7 mg/kg 2-BFI significantly ($p<0.05$) reduced grooming behaviour. This effect was apparent at all doses, 1, 3, 7, 10 mg/kg but only significant at the 7mg/kg dose and only after 65 minutes (Jordan 1993). In contrast to these results I_2 ligands that also have an affinity for other receptors i.e. idazoxan significantly ($p<0.05$) altered, in addition to grooming behaviour; locomotor activity (increase), spontaneous head twitches (increase) and an increase in

the magnitude of the pinna reflex (Jordan 1993). As Jordan (1993) notes, these physiological effects have been largely associated with the blockade of adrenoceptors, rather than the activation of imidazoline sites.

A similar lack of effect is seen with the specific I_2 ligands RS-45041-190. Administration of moderate doses, intravenously, intraperitonially, and subcutaneously (s.c.) has no significant effect on blood pressure, heart rate, core and tail skin temperature, sleeping time and rotarod performance (Brown et al, 1995).

1.3.3). Imidazoline Ligands and Depression

The Porsolt forced swim test represents an animal model of depression in which the therapeutic efficiency of antidepressants can be assessed (Porsolt et al, 1978). A rat placed in a tank of water makes an initial frenzied attempt at escape. This is followed by an immobile posture which has been termed 'behavioural despair' and suggested to represent an inability or reluctance to maintain the effort needed for escape. The test covers two swims. The first swim represents a control value, in which the time taken between placement in the tank and onset of the immobile posture is measured. Twenty-four hours later, and after sub chronic treatment of the animal with antidepressants, i.e. 2 or 3 injections, the test is repeated. Previous research has indicated that pre-treatment with antidepressants, e.g. desipramine reduces the immobility time evident in swim two (Finnegan et al, 1987). Thereby, theoretically reducing the levels of behavioural despair experienced by the animal.

The I_2 specific compound 2-BFI has been previously reported to reduce total immobility time at doses of 3 and 10 mg/kg. This effect has been shown to be significantly different to controls over three different time periods. Immobility time was significantly reduced between 0 and 5 minutes, 3 and 8 minutes, and 0 to 8 minutes (Jordan 1993). The results from the 2-BFI condition were similar to those exhibited by the clinically effective antidepressant desipramine (Checkley et al, 1981), thereby indicating that 2-BFI may have antidepressant properties. Other less specific I_2 compounds,

i.e. compounds such as idazoxan and ethoxy-idazoxan, that have an antagonistic affinity for α_2 adrenoceptors, also reduced immobility time. However, these reductions were not significant (Jordan, 1993).

1.3.4). Imidazoline Ligands and Opioid analgesia.

A number of studies have indicated that imidazoline ligands modulate opiate analgesia and opioid withdrawal signs. The animal model of opioid dependency involves implanting a morphine pellet then inducing withdrawal by administering the opioid antagonist naltrexone. In the rat withdrawal is characterised by weight loss, ejaculation, wet dog shakes, ptosis, and mouth movements (Maldonado and Koob, 1993). In rats i.p. injection of clonidine (0.4 mg/kg) significantly, prevents weight loss; reduces, mouth movements, wet dog shakes, ptosis and ejaculation when compared with animals undergoing withdrawal after injection of vehicle. Administration of 10mg/kg i.p. BU224, significantly surpress' mouth movements and prevented weight loss but did not significantly alter the other variables (Hudson et al, 1999b).
The proposed endogenous ligand agmatine also has a reported ability to increase or potentiate morphine analgesia (Kolesnikov et al, 1996). In this instance analgesia is measured by the tail flick response i.e., the latency of animal tail flick after placement on a warm plate. Administration of agmatine alone fails to alter tail flick latency. But, administration to rats of 10 mg/kg agmatine and 5 mg/kg morphine, significantly ($p<0.05$) increases the latency by 40% over the control group in which morphine is given alone (Kolesnikov et al, 1996). Given alone morphine's initial analgesic effects are lost over five days of dosing. After such a time co administration with agmatine (0.1 mg/kg or 10 mg/kg) potentiates morphine's effects, i.e., increases tail flick latency back up to the same levels reported at the outset of morphine dosing. Interestingly the 0.1 mg/kg dose does not significantly increase morphine's analgesic affect but does potentiate the ongoing control level of morphine analgesia (Kolesnikov et al, 1996). These results have been replicated in the mouse, again using the tail flick assay (Li et al 1999; Sanchez-Blazquez et al, 2000). Administration of 2-BFI, LSL 60101, LSL

601122, agmatine, but not idazoxan or BU224, before morphine significantly increases tail flick latency (Li et al 1999; Sanchez-Blazquez et al, 2000). Co-administration of 2-BFI, LSL 60101, LSL 601122, agmatine with either BU224 or idazoxan significantly antagonised I_2 ligand potentiated morphine analgesia. Based on this evidence the authors propose idazoxan and BU224 are antagonists at the I_2 site (Sanchez-Blazquez et al, 2000).

1.3.5). Food and Water Intake

Previous rodent work with I_2 ligands has indicated that RS-45041-190, idazoxan, LSL 60101, benazoline and metrazoline significantly increase food consumption in the hours after dosing whereas idazoxan in addition to initiating hyperphagia increases water intake (Jackson et al, 1991; Menargues et al 1994; Menargues et al 1995; Brown et al, 1995; Polidori et al, 2000). Agmatine, a proposed endogenous ligand at imidazoline binding sites has also been shown to increase food intake, particularly carbohydrates (Prasad and Prasad, 1996). However, these effects on food intake are only apparent in the 4 hours after injection and such ligands have no effect on food intake if the experiment is conducted during the dark period of the rats 24 hour cycle (Menargues et al, 1994). None of the reported doses: idazoxan (10 mg/kg), RX 821002 (3 mg/kg) yohimbine (10 mg/kg) and LSL 60101 (3-30 mg/kg) had any effect on food intake after 24 hours (Menargues et al 1994; Brown et al, 1995). After alkylation of the rats α_2-adrenoceptors with EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) the hyperphagia induced by yohimbine, RX 821002 and idazoxan was attenuated, but not that induced by LSL60101 (Menargues et al 1994). The ability of imidazoline ligands to increase insulin and decrease glucagon secretion may go some way in explaining these effects (Rustenbeck et al, 1999; Morgan et al 1999).

1.3.6). Drug Discrimination

The Drug discrimination paradigm represents a powerful behavioural technique for assessing the psychopharmacological consequences of drug

administration (Sanger 1987; Stolerman et al 1989; Jordan et al 1993, 1995 & 1996). It is based on the ability of a ligand to generate a cue, i.e., administration of specific ligands alters brain or body neurochemistry to the extent an animal can perceive it. Animals are trained to associate one lever of a two lever Skinner box with a drug and the other with a saline vehicle using a positive reward system. When such animals are responding with 100% accuracy, i.e., on a day in which they are trained with the drug they only respond to the drug lever they are entered into a test day. During a test session the proportion of lever presses on the drug lever, when compared with the total for both levers, represents the ability of a ligand to substitute for the training drug. For example if drug X leads to the rat pressing the drug lever ten times this reflects a 100% substitution for the training drug. Conversely, if drug Y gives rise to 10 presses on the drug associated lever and 9 on the saline, this would give rise to approximately 50% substitution for the training drug.

Testing can take a number of forms. Firstly the potency of other ligands can be compared with that of the training drug. Alternately, by using specific agonists, i.e., dobutamine (β_1) or salbutamol (β_2), the pharmacology of the training drug can be characterised. Alternately, attempts can be made to antagonise the cue, i.e., administer a potential antagonist before the training drug. Hence, if the potential antagonist does antagonise the training drugs effects then drug lever appropriate responding should be reduced when compared with control. The relevant control day should give high levels (<90%) because vehicle is administered prior to the training drug.

Rats trained to recognise, the I_1 ligand and α_2 adrenoceptor agonist, clonidine show high levels of clonidine appropriate lever pressing after administration of the α_2 adrenoceptor agonists, guanabenz, UK 14,304 (bromoxonidine) rilmendine and clonidine itself (Jordan et al, 1993). Moderate levels of clonidine appropriate responding were induced after administration of the I_2 specific ligand 2-BFI (Jordan et al, 1993). The non-imidazoline α_2-adrenoceptor antagonist fluparoxan showed low levels of substitution when administered alone and when administered prior to that of

clonidine itself fluparoxan successfully antagonised the cue, suggesting the clonidine cue is mediated through α_2-adrenoceptors (Jordan, 1993). Ethoxy idazoxan, inspite of its imidazoline moiety possesses a low affinity for I_2 sites and high affinity for α_2-adrenoceptors as an antagonist (Mallard et al, 1992). Consistent with the ethoxy idazoxan cue being mediated through α_2-adrenoceptors, idazoxan, fluparoxan and 2-ethoxy-idazoxan itself induce high levels of ethoxy-idazoxan appropriate lever pressing (Jordan et al, 1995). Low levels of ethoxy-idazoxan responding were elicited by clonidine and the I_1, I_2 ligand, adrenoceptor agonist cirazoline. The I_2 specific ligand 2-BFI only shows moderate levels of substitution when administered to rats trained on the α_2-adrenoceptor antagonist ethoxy-idazoxan (Jordan et al, 1995). In contrast animals trained with 2-BFI fully recognise, fluparoxan, idazoxan and ethoxy-idazoxan (Jordan et al, 1996). *In vivo* microdialysis studies have indicated administration of ethoxy-idazoxan increases extraneuronal levels of noradrenaline via antagonism of the α_2-adrenoceptor autoreceptor (Starke, 1977; Thomas & Holman, 1991). However, 2-BFI also increases extraneuronal levels of NA, but independent of any direct α_2-adrenoceptor interaction as it has a very low affinity for this site (Jordan 1993; Hudson et al, 1999b; Nutt et al, 1995). Thus it is likely that the 2-BFI and ethoxy idazoxan cues, at least in part, are associated with an increase in extraneuronal levels of NA. As I_2 ligands such as 2-BFI inhibit MAO it is plausible to suggest that this could account for the increase in NA after 2-BFI administration. Consistent with this theory high doses of the MAO-B specific inhibitor pargyline and the reversible MAO-A inhibitor specific moclobemide showed high levels of 2-BFI appropriate responding (Jordan et al, 1996).

1.4). Relationship to Health and Disease

1.4.1). Depression

A number of lines of evidence suggest that I_2 sites are associated with depression. It has also been previously reported that idazoxan has an antidepressant effect in humans (Osman et al, 1989). In animals models of

the disorder 2-BFI has been shown to be as effective as the clinically effective antidepressant desipramine (Jordan 1993: Nutt et al, 1995; Checkley et al, 1981). Use of further animal models of depression, namely the rat olfactory bulbectomy, have indicated chronic treatment with the clinically effective antidepressant imipramine significantly, decreases [^{3}H]clonidine I_1 binding, but increases [^{3}H]idazoxan I_2 binding in rat brain (Zhu et al, 1997a; Zhu et al, 1997b). On a molecular level chronic imipramine treatment significantly decreases the immunoreactivity of the anti-imidazoline antibody identified 45 kDa protein, but leaves unaltered levels of a 35 kDa protein (Garcia-Sevilla et al 1996).

It is, however, unclear how these antidepressant effects are mediated. The link between I_2 sites and MAO tentatively suggests that I_2 ligands may represent a new class of reversible inhibitors of the enzyme. As previously mentioned 2-BFI, BU224, BU226, BU239 and BU216, inhibit MAO-A with a similar potency to the clinically effective MAO-A inhibitor moclobemide (Lalies et al, 1999; Anderson et al, 2000).

Previously depression and suicide have been associated with an increase in α_2-adrenoceptor density (Ferrier et al, 1986; Meana and Garcia-Sevilla, 1987; Ordway et al, 1994). However, some studies suggest a decrease or observe no change in binding (Crow et al, 1984; Gross-Isseroff et al, 2000). It has recently been suggested that the choice of radioligand and therefore the presence of imidazoline binding sites within the assay can account for these discrepancies (Sastre and Garcia-Sevilla, 1997). Hence, the possibility exists that depression is associated with an increase in I_2 sites rather than α_2-adrenoceptors. Consistent with this theory the brains of depressed unipolar suicide victims, some who have experienced drug treatment and others who hadn't, show significantly lower levels of the imidazoline associated 29/30 kDa and 35 kDa proteins, and increased levels of the 45 kDa protein (Piletz et al, 1996; Garcia-Sevilla et al, 1996; Sastre and Garcia-Sevilla, 1997). The decrease of the 29/30 and 35 kDa protein significantly correlated with a reduction in [^{3}H]idazoxan I_2 and [^{3}H]clonidine I_1 binding (Piletz et al, 1996; Sastre and Garcia-Sevilla, 1997). However, no

changes were observed with the specific α_2-adrenoceptor antagonist [^{3}H]RX821002 or the specific MAO-B inhibitor [^{3}H]Ro 19-6327 (Sastre and Garcia-Sevilla, 1997). In addition levels of a 45 and 35 kDa imidazoline immunoreactive protein, present in platelet membranes of clinically depressed patients, has been shown to be significantly increased when compared with controls (Garcia-Sevilla et al, 1996a; Garcia-Sevilla et al, 1996b). A subset of these depressed patients underwent a short course of antidepressant treatment, i.e., 2-12 weeks with clomipramine, citalopram, and imipramine, and levels of the 45 kDa, but not the 35 protein, were significantly increased after treatment. (Garcia-Sevilla et al, 1996). Platelet membrane levels of the 45 kDa, but not the 35 kDa protein, also significantly correlate with levels of two major G proteins - $G\alpha_{q/11}$ and $G\alpha_{i/s}$. $G\alpha_{q/11}$ (mediating the phospolipase pathway) and $G\alpha_{i/s}$ (mediating the adenylyl cyclase pathway) are major G proteins involved with extracellular signalling. However, it is unlikely that the 45 kDa protein is directly attached to G proteins that utilise the phospolipase or adenylyl cyclase pathway because levels of these enzymes are unaltered after imidazoline ligand administration (Regunathan et al, 1991a). Similar studies have indicated these effects may be specific to unipolar depressives. No alterations in the 35 or 45 kDa platelet imidazoline binding protein, or [^{3}H]idazoxan I_2 density, occur between, bipolar depressives, lithium treated bipolar depressives or controls (Garcia-Sevilla et al, 1998).

1.4.2). Alzheimer's and Ageing

A number of studies have indicated that [^{3}H]idazoxan I_2 sites and I_2 associated protein immunoreactivity increase with age and are upregulated in the brains of Alzheimer's suffers (Ruiz et al, 1993; Garcia-Sevilla et al, 1995; Sastre and Garcia-Sevilla, 1997; Garcia-Sevilla et al, 1998). Alzheimer's disease is a progressive neurodegenerative disease characterised by acute astrocyte hypertrophy, the accumulation of senile plaques and neurofibrillary tangles (Delacourte, 1990; Prince et al, 1993). As previously mentioned some I_2 ligands, i.e., cirazoline, idazoxan and LSL

60101 increase GFAP immunoreactivity (Olmos et al, 1994b; Alemany et al 1995). GFAP is found primarily on astrocytes and its increase is associated with the acute astrocyte hypertrophy present in Alzheimer's type dementia (Wallin et al, 1996). Molecular studies have indicated significant age dependent increases in the human brain imidazoline immunoreactive 29/30 kDa protein (Garcia-Sevilla et al, 1995). In addition to levels of this protein, immunoreactivity levels of the 45 kDa imidazoline associated protein and GFAP were significantly increased in the brains of Alzheimer's suffers when compared with controls (Garcia-Sevilla et al, 1998). Although a significant increase in imidazoline site density is apparent in the brains of Alzheimer's sufferers, it is not present in platelets (Soto et al, 1999). In addition to GFAP, an increase in human brain MAO-B, is associated with the onset of age and Alzheimer's associated astrocyte hypertrophy (Jossan et a, 1991; Sastre and Garcia-Sevilla, 1997). However, although I_2 sites and MAO-B both increase with age and in the presence of Alzheimer's, these increases do not correlate with each other – suggesting these binding sites may be separate entities (Sastre and Garcia-Sevilla, 1997; Ballesteros et al, 2000). However, whether I_2 ligand modulation of GFAP has any physiological outcome has yet to be clarified. Mice in whom the GFAP gene had been disrupted at the embryonic stem cell level have shown normal development with no obvious brain abnormalities (Gomi et al 1995). GFAP increase is associated with a number of other neurological disorders, these include, multiple sclerosis, and the transmissible spongiform encephalopathies - CJD (Creutz feldt Jacob Disease) and Scrapie (Rosengren et al, 1995; Kordek et al, 1997; Tatzelt et al, 1996). It has been convincingly established that in Scrapie and CJD its accumulation is as a result of 'reactive glyosis' (Tatzelt et al, 1996), i.e., astrocyte proliferation occurs as a result of brain injury, rather than it actually being the cause.

1.4.3). Parkinson's and Huntington's Disease

As previously mentioned I_2 ligands inhibit MAO and other inhibitors of the enzyme, i.e, deprenyl, are used clinically in the treatment of Parkonsinism

(Carpene et al, 1995; Lalies et al, 1999; The Parkinson Study group, 1989 & 1993a; Quinn et al, 1986). However, despite this association no clear alterations in I_2 binding have been established in the brain tissue of Parkinson suffers. To date two studies have indicated that there are no changes in [^{3}H]2-BFI or [^{3}H]idazoxan binding in the putamen of Parkinson's brain (Reynolds et al, 1996; Gargalidis-Moundanos et al, 1997). However, post-mortem brain studies have indicated that [^{3}H]2-BFI binding sites are significantly reduced in the putamen of Huntington's disease sufferers (Reynolds et al, 1996).

1.4.4). Drug Addiction

As previously mentioned a number of behavioural studies have indicated that I_2 site ligands modulate morphine induced analgesia (Kolesnikov et al, 1996; Li et al 1999; Hudson et al, 1999b; Sanchez-Blazquez et al, 2000). It has been long established that, in humans, clonidine attenuates the symptoms that characterise opiate withdrawal (Gold et al, 1978). Immunodetection of heroin addicts brain at post-mortem has indicated that the 29/30 kDa, but not the 45 kDa, protein previously mentioned and [^{3}H]idazoxan I_2 binding are significantly reduced. A parallel study conducted in rats indicated that chronic treatment with heroin resulted in the same effects, i.e., a significant reduction in levels of the 29/30 kDa protein and [^{3}H]idazoxan I_2 binding (Sastre et al, 1996).

1.5). Aim of the study.

A great deal of *in vitro* information is available on the I_2 site. However, very little is known about the behavioural pharmacology of I_2 ligand administration. Hence, the technique of drug discrimination will initially be used to probe the psychopharmacology of I_2 ligand administration. This will involve a number of stages.

1). Firstly the ability of the I_2 specific ligand 2-BFI to generate a cue in drug discrimination will be assessed. Namely what is the minimal dose of 2-BFI needed for rats to successfully discriminate it from saline?

2). A number of specific I_2 ligands have recently become available, i.e., BU224, BU216 and LSL 60101. Hence, the *in vivo* potency of these compounds will be compared with the training drug (2-BFI).

3). Previous studies have indicated 2-BFI administration leads to a significant release of NA within the rat brain. This mechanism is thought to be independent of direct α_2-adrenoceptor interaction as 2-BFI has a very low affinity for this receptor. Hence, thirdly the psychopharmacological consequences of this increase in NA will be assessed.

4). I_2 sites have also been heavily associated with the enzyme MAO and so the ability of various irreversible and reversible MAO inhibitors to substitute for 2-BFI will be studied.

5). Harmane, a proposed endogenous ligand for I_2 sites has been shown to have a high affinity for I_2 sites *in vitro*. Hence later studies will initially verify the potency of harmane and other related beta carbolines to substitute for the 2-BFI cue in drug discrimination.

Recently a tritiated version of 2-BFI has become commercially available and autoradiographical studies have indicated [^{3}H]2-BFI binds to discrete brain nuclei (Lione et al, 1998). Hence, autoradiographical experiments will explore any alterations in [^{3}H]2-BFI binding that may become apparent after the repeated administration of various I_2 associated ligands. This will form a number of stages.

6). The initial autoradiographical studies will localise regions of [^{3}H]2-BFI binding and then further studies will look at any alterations that have occurred within discrete nuclei of rats trained for drug discrimination.

7). A second series of experiments will look at the ability of certain irreversible MAO inhibitors to reduce [^{3}H]2-BFI binding in drug naive rats.

8). Recently a line of transgenic mice that over express MAO-B 4 to 6 fold have become available (Richards et al, 1998). Hence the final experiments will initially localise [^{3}H]2-BFI binding within the mouse brain and secondly compare these levels of binding to their transgenic litter mates.

Chapter 2

Methods

1). Animals

Seven groups (G1, G2, G3, G4. G5, G6, G7) each containing eight drug naive male Hooded Lister rats (Charles River, UK), starting weight of 100g, were trained for drug discrimination (DD) experiments. A further group (G8) of ten drug naive male Hooded Lister rats (Charles River, UK) were used for a chronic 2-BFI autoradiographical study. These rats followed the DD injection schedule (see below) but did not receive Skinner box training, i.e, they experienced 'pseudo training sessions', see Chapter 3 for details.

Autoradiography experiments used Hooded Lister rats (starting weight of 100g; Charles River, UK), male Wistar rats (270-320g) and C/57B mice (25-30g; Charles River, UK; Dr Grayson Richards, Hoffman La Roche, Basel Switzerland). See individual chapters for details.

All animals were housed in a specialised animal unit at an ambient temperature of 21 degrees centigrade, humidity of 45%, on a dark/light 12 hour cycle, lights on at 08.00 hours. All animals were housed in pairs, given free access to food (Lillico, UK) and tap water ad libitum. DD animals were housed in one room and transferred to an adjacent one for testing.

2). Injections

All injections were through the intraperitoneal (i.p) route with a 25G needle and attached 1ml syringe (Fischer, UK). Drugs and vehicle were made up to appropriate volumes in 20ml multi dose bottles (BDH). All drugs were passed through a microfilter, 0.2μM pore size (Schleicher & Schuell, Germany), before injection into sterilised capped multi dose vial (Fischer, UK). See individual chapters for vehicles and suspension details. All doses were administered at volume of 1ml/kg.

3). Drug Discrimination

3.1). Equipment

The DD procedure was conducted in standard two lever operant conditioning units (Campden Instruments, UK). Data was collected on an Apple IIe computer using 'Operant Program for the Neurosciences' (Emmett-Oglesby et al, 1982). Each of the 8 conditioning units contained a delivery hatch, equidistant from each of the two levers. Upon completion of the various fixed ratio (FR) schedules a dipper presented a reward of sweetened condensed milk (approximately 0.1ml; Nestle, UK). Food deprivation was not required as non-deprived rats show a high level of lever pressing for a condensed milk reward (Jordan, 1993; Jordan et al, 1993, 1995 & 1996). All sessions began with the presentation of a free reward and were conducted between 10:00 am and 2pm each day.

3.2). Preliminary training

Animals were placed in the conditioning box for one 15 minute session per day. Initially each lever press was rewarded with condensed milk this constituted a fixed ratio of 1 (FR 1) schedule. Both levers were operable. This ratio of reinforcement was increased through FR2, FR4, FR6, FR8 until FR10, at which point the animal had to press a lever 10 times before receiving a reward. When all rats were responding consistently to the FR10

schedule, and they exhibited no lever bias, they were admitted to the next stage - drug conditioning.

3.3). Drug conditioning

After animals were responding consistently to the FR 10 schedule and they showed no lever bias they entered 2-BFI training. Rats were initially injected with either 2-BFI (the training drug) or a saline vehicle 20 minutes prior to entry into the conditioning box. Each session lasted 15 minutes and animals received one session per day. A reward was delivered after 10 presses on the correct lever. The correct lever was defined as the lever that delivered the condensed milk reward. Depending on the particular animal and conditioning box the correct lever could be either left or right, i.e., rats were randomly divided into two groups, thus for four rats the 2-BFI lever was the left and the saline vehicle the right, whilst the opposite was true for the other four rats. Animals were always placed in the same box. Training occurred in a cycle, SDDSSDSSDD (S = saline day, D = drug day) which consisted of 10 sessions, 5 days in which the animals were administered drug and 5 in which they were administered saline. This was spread over a two week period in which weekdays were used only (Sanger, 1989; Jordan, 1993).
Two criteria existed that had to be fulfilled before animals completed an error free training session. These were; 1, 90% or over of total responses, over a session must have been on the correct lever and; 2, the first 7 of the first 10 responses emitted by the animal when placed in the box must have been on the correct lever. When 8 animals had completed 10 consecutive error free sessions they entered a testing session.

3.4). Testing Sessions

Testing sessions or days were added into the normal 10 session cycle in the following way STDTSDTSTD (S = saline, T = test day, D = drug day), weekdays only. An actual testing session ended after an animal made 10 responses to either the saline or 2-BFI lever or when 30 minutes elapsed (whichever was sooner).

No reward was administered in these test sessions. Substitution drugs were administered 20 minutes prior to test session, whilst drugs injected that aimed to antagonise the 2-BFI cue were injected 20 minutes before the test drug (i.e., 2-BFI or methoxamine). The number of responses by each rat to the 2-BFI associated lever was expressed as a percentage of the total responses to both levers. The mean of these percentages represented the ability of a drug to substitute for the conditioned 2-BFI cue. The response per minute (RPM) represents the number of lever presses per minute. This was derived from the latency of the 10th response, over both levers, from the first response and then divided by the total number of lever presses. This gave rise to the number of responses per minute or RPM. Control data was obtained by administering saline in place of a test dose. All doses of a single drug and associated saline day were given in a pseudo random order.

4). Cresyl Violet Staining and Autoradiography

Brain sections for both autoradiography and cresyl violet staining were obtained and mounted through the following method.

4.1). Brain preparation and Cryosectioning

Male Wistar rats (270-320g) were anaesthetised with halothane and perfused (intra-cardiac) with 50ml ice-cold phosphate (0.01 M) buffered saline (pH 7.4). Briefly, 1.41g of Na_2HPO_4 (Di sodium hydrogen orthophosphate) and 1.56g of NaH_2PO_4 (Sodium dihydrogen orthophosphate) were each dissolved in 50 mls 0.9% saline. 40.5 ml of the resultant 0.2 M $Na_2 HPO_4$ and 9.5 ml of the resultant 0.2 M $NaH_2 PO_4$ were than added to 50mls saline and cooled in a bath of wet ice. Brains were rapidly removed and placed in a Kopf Brain Blocker (Kopf Instruments, USA). Brains for transverse sections were blocked at approximately 0mm Bregma thereby giving a flat face for flush mounting of the brain onto the cryostat chuck, whilst brains for parasagittal sections were sectioned using the Kopf PA-001 Brain Blocker parasagittal cutting guide. After blocking, brains were placed in isopentane (Fisher, UK) cooled to –40°C in a bath

containing a mixture of propan-2-ol (Fisher, UK) and dry ice for 5 minutes. Brains were then placed in a Bright OTF cryostat (Bright Instruments, UK) for 1 hour to enable cryostat and specimen temperature to equilibrate.

Transgenic MAO-B drug naive male SPF (specific pathogen free) C/57B mice and control litter mates were anaesthetised with fluothane. These animals were killed by decapitation and their brains rapidly removed and placed in a mouse brain blocker. All brains were blocked at approximately 0.8mm from the Bregma line, placed on cork disks, then frozen by gently sprinkling crushed dry ice around them.

During this equilibrium time rat brains were mounted onto cork disks (Bright Instruments, UK) and subsequently the cryostat chuck with embedding fluid (Bright Instruments, UK). The chuck and attached brain section were then mounted in the cryostat (specimen temperature of -14 to -16 degrees centigrade and chamber temperature of -18 to -20 degrees centigrade). Cryosectioning then commenced, with an initial section being cut at 30μm for cresyl violet staining then a 12 μm section for total binding, and an adjacent 12μm section for NSB (non-specific binding). All slides containing sections were stored in slide racks (Fisher, UK) at –70 degrees centigrade until use. All slide-mounted sections were stored for no longer than 14 days before radiochemical assay.

4.1.1). Slide Preparation

All slides used were cleaned and coated, whether supplied as washed and cleaned or not. Superfrost slides (BDH, UK) were initially left in a 5% solution of Decon 90 overnight, left under a running tap for seven hours, rinsed in de-mineralised water and rinsed again, briefly in acid-alcohol (Absolute alcohol at pH 4.5), then left to dry. All slides were then coated using the Chrom-alum gelatin coating technique (Wharton & Polack, 1993). Briefly, 2g of gelatin (BDH, UK) and 0.2g of chromic potassium sulphate (BDH, UK) were added to 400ml of distilled water and mixed using a magnetic stirrer with gentle heating. The resultant solution was then filtered through Whatman Qualitative Circles (UK) and allowed to cool. Using a slide

rack (Fisher, UK) slides were dipped in the solution allowed to drain, and then dried at 37 degrees centigrade. Slides were then stored in their original packing until needed.

4.2). Cresyl Violet Stain

Cresyl violet staining followed the procedure outlined in (Wharton & Polack, 1993; Paxinos & Watson, 1998). 500ml cresyl violet stain was made up according to the following method. 2.5g of cresyl violet (BDH, Gurr Microscope stains, UK) was dissolved in; 300 ml distilled water, 30 ml of 1.0M sodium acetate (Fisher, UK) and 170ml of 1.0M acetic acid (Fisher, UK). This solution was then stirred for seven days using a magnetic stirrer and filtered through Whatman Qualitative Circles (Whatman, UK). Slides were thawed for 45 minutes; placed in cresyl violet stain for 15 minutes; allowed to differentiate in distilled water for 2-5 minutes; dehydrated through, 50%, 70%, 95%, 100% alcohol and then immersed in xylene (BDH, UK) for 2-3 minutes. Coverslips (Fischer, UK) were mounted to stained sections by the addition of DPX mountant (BDH, UK).

4.3). Rat Autoradiography studies

All rat autoradiographical studies followed the assay developed by Lione et al, (1998). [^{3}H]2-BFI ([5,7-(n)-^{3}H]2-(2-Benzofuranyl)-2-imidazoline; specific activity 70Ci/mmol; radioactive concentration 200μCi/ml) was obtained from Amersham, UK and serial dilutions established relevant concentrations. See individual chapters to see details of actual concentration of [^{3}H]2-BFI used. Sections were initially thawed and pre-washed for 30 minutes at room temperature (21-25°C) in 50mM Tris HCl buffer (pH7.4) containing 1 mM $MgCl_2$ (magnesium chloride). Briefly, 50 ml 0.1M tris (Tris [hydroxymethyl] methylamine or $[OH.CH_2]_3C.NH_2$) was added to 42 ml 0.1M HCl (Hydrochloric acid) and made up to 100ml with distilled water. $MgCl_2$ (1M solution, Sigma) was added at a volume appropriate to the quantity of Tris-HCl buffer made. Sections were laid out flat on tissue paper and dried under a stream of cool air. After drying slide mounted sections were placed in a

tray lined with wet tissue so as to minimise evaporation of the buffer containing the ligand. Sections were incubated with 200μl assay buffer containing [^{3}H]2-BFI or alternately 200μl assay buffer containing [^{3}H]2-BFI and 10μM BU224 (total and non-specific [NSB] binding conditions respectively). Incubation lasted 40 minutes at room temperature and was stopped by aspiration of buffer and ligand from the section with a vacuum pump. Sections were then washed twice for 20 seconds in ice cold buffer, dipped once in ice cold distilled water and dried rapidly under a stream of cool air.

These sections and activated ^{3}H-microscale standards (Amersham, UK; activation established through 15 minutes floating on the surface of a waterbath heated to 55°C and baked on slides until dry) were then mounted on cardboard attached to the bottom of autoradiography cases (Genetic Research Instrumentation, Braintree, UK). Sections and standards where then apposed to tritium sensitive film ([^{3}H]-Hyperfilm, Amersham, UK) for six weeks at room temperature.

4.3.1). Radioactive ligand stability.

A number of previous studies have utilised [^{3}H]2-BFI in homogenate binding studies (Alemany et al, 1997; Wiest and Steinberg, 1997; Steinberg et al, 1999) and autoradiography (Lione et al, 1994; Lione et al, 1996; Hosseini et al, 1998; Lione et al, 1997; Lione et al, 1998; Conway et al, 1998; King et al, 1998). However, a question has arisen over the metabolic stability of the ligand whilst, within CNS tissue, apposed to tritium sensitive film for six weeks. The suppliers (Tony Roberts, Amersham, Cardiff, UK), have not tested the stability of the compound within these parameters. However, Alan Hudson (Bristol University, UK, personal communication), who has used the ligand extensively in rat and rabbit brain sections is confident that [^{3}H]2-BFI is stable under these conditions.

4.4). Mouse Autoradiographical studies

Mouse autoradiographical experiments were conducted according to the above protocol except post wash times were altered from two washes for 20 seconds to two washes of 10 seconds. These wash times were defined as optimal for giving the largest difference between the total and NSB components of ligand binding.

4.5) Film Development

Dark room safelight conditions were established with a GBX-2 filter (Sigma, UK) and 15 watt bulb (BHS, UK). Autoradiography films were developed for 90 seconds in Ilford High Contrast developer (1 part developer to 9 parts distilled water), immersed in acetic acid (0.01%) for 20 seconds and fixed with Ilford Hypam fixer (1 part fixer to 4 parts distilled water) plus Ilford rapid hardener (1 part hardener to 50 parts distilled water). Films were then washed in distilled water containing wetting agent (1 part wetting agent to 200 parts distilled water) to prevent spotting and allowed to air dry. Final volumes were always one litre.

4.6) Data Analysis

The films were then quantified by computer-assisted densitometry (MCID version 4, Microcomputer Image Device, Imaging Research, St Catherines, OT, Canada) and values were converted to fmol ^{3}H-ligand/mg wet tissue using ^{3}H-microscale standards (Amersham, UK). Data used for individual means was derived in different ways depending on the experimental design, see Individual chapters.

5). Statistical Analysis

All data was assessed for deviations from gaussian distribution. Providing parametric assumptions were met significant differences were established by comparing experimental groups to relevant control groups, using one way analysis of variance (ANOVA with Dunnets or Newmans-Keuls post hoc test). Statistical analysis was completed on either, GraphPAD Prism (version 3), StatsDirect (version 1.608) or SPSS (Statistical Package for the Social Sciences) version 6.13.

6). Drugs

Pseudonym	Chemical Name	Supplier
[^{3}H]2-BFI	[5,7-(n)-^{3}H]2-(2-benzofuranyl)-2-imidazoline	Amersham, UK
2-BFI HCl	2-(-2-benzofuranyl)-2-imidazoline hydrochloride	Pierre Fabre, France
BU216 HCl	3-(4,5-dihydroimidaz-2-yl)-quinoline hydrochloride	A Hudson, Bristol
BU224 HCl	2-(4,5-dihydroimidaz-2-yl)-quinoline hydrochloride	A Hudson, Bristol
BU226 HCl	2-(4,5-dihydroimidaz-2-yl)-isoquinoline hydrochloride	A Hudson, Bristol
LSL 60101 HCl	2-(2-benzofuranyl)-2-imidazole hydrochloride	J Garcia-Sevilla, Spain
LSL 60125 HCl	2-(6-Methoxybenzofuran-2-yl)imidazole hydrochloride	J Garcia-Sevilla, Spain
Agmatine Sulfate	(4-aminobutyl)guanidine sulfate	Sigma, UK
Amiloride HCl	3,5-diamino-N-(aminoiminomethyl)-6-chloropyrazine-carboxamide hydrochloride	Sigma, UK
Methoxamine HCl	methoxamine hydrochloride	Sigma, UK
Phenylephrine HCl	R-(-)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol hydrochloride	Sigma, UK
ST587	2-(2-chloro-5-trifluromethylphenylimino)imidazolidine	Boehringer Ingelheim
D-amphetamine Sulfate	S(+)-α-methylphenylethylamine sulfate	Sigma, UK
Dobutamine HCl	(±)-4-[2-[[3-(4-hydroxyphenyl0-1-methylpropyl]amino]ethyl]-1,2-benzenediol hydrochloride	Sigma, UK
Salbutamol Hemisulfate	α'-[[(1,1-dimethylethyl)amino]methyl]-4-hydroxy-1,3-benzenedimethanol hemisulfate	Sigma, UK
Clonidine HCl	2-(2,6-dichlorophenyl)-2-imino imidazoline hydrochloride	Sigma, UK
Cirazoline HCl	2-[(2-cyclopropylphenoxy)methyl]-4,5-dihydro-1H-imidazole hydrochloride	Synthelabo Recherche
WB4101 HCl	2-([2,6-dimethoxyphenoxyethyl] aminomethyl)-1,4-benzodioxane hydrochloride	Sigma, UK
Prazosin HCl	1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanyl-carbonyl)piperazine hydrochloride	Sigma, UK
Tranylcypromine HCl	trans-2-phenylcyclopropylamine HCl	Sigma, UK
Desipramine HCl	10,11-diydro-N-methyl-5H-dibenz[b,d]azepine hydrochloride	Sigma, UK
Reboxetine Methanesulfonate	(2RS, 3RS)-2-(α-(2-ethoxyphenoxy)benzyl)morpholine Methanesulfonate	Pharmacia & Upjohn
L-659,066	(2R,12bs)-*N*-[1,3,4,6,7,12b-hexahydro-2'-oxospiro[2H-benzofuro[2,3,-a]quinolizine-2,4'-imidazolidin-3'yl)ethyl]methane sulphonamide	Merck Sharp & Dohme

Deprenyl HCl	R(-)-N-α-Dimethyl-N-2-propynyl-benzeneethanamine hydrochloride.	Sigma, UK

Pseudonym	**Chemical Name**	**Supplier**
Clorgyline HCl	N-methyl-N-propargyl-3-(2,4-dichlorophenoxy)-propylamine hydrochloride	Sigma, UK
Pargyline HCl	N-methyl-N-(2-propynyl)benzenemethanamine hydrochloride	Sigma, UK
Ro 41-1049 HCl	N-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazolecarboxamide hydrochloride	Sigma, UK
RO16-6491 HCl	N-(2-aminoethyl)-4-chlorobenzamide hydrochloride	Sigma, UK
Moclobemide HCl	P-chloro-N-(2-morpholinoethyl)benzamide	Roche, Switzerland
Lazabemide HCl	N-(2-aminoethyl)-5-chloro-2-pyridinecarboxamide hydrochloride	Roche, Switzerland
Harmane HCl	1-methyl-9H-pyrido(3,4-b)indole hydrochloride	Sigma, UK
Norharmane HCl	9H-pyrido(3,4-b)indole hydrochloride	Sigma, UK
Ibogaine HCl	Ibogaine hydrochloride	Sigma, UK
Harmaline HCl	1-methyl-7-methoxy-3,4-dihydro-beta-carboline	Sigma, UK
Citalopram hydrobromide	1-(3-(Dimethylamino)propyl]-1-(P-fluro-phenyl)-5-phthalancarbonitrile, monohydrobromide	Lunbeck Limited
Clomipramine HCl	3-Chloro-10,11-dihydro-N,N-dimethyl-5H-dibenz[b,f]axepie-5-propanamine hydrochloride	Sigma, UK
Paroxetine HCl	(3S-trans)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluporophenyl)piperidine hydrochloride	SmithKline Beecham, UK
Fenfluramine HCl	S(+)-N-ethyl-α-methyl-3-(trifluromethyl)-benzeethanamine hydrochloride	Sigma, UK
Way 100,635 maleate	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-N-2-pyridinyl-cyclohexane-carboxamide maleate	Sigma, UK
SKF10,047	2'hydroxy-5,9-dimethyl-2-allyl-6,7-benzomorphans	SmithKline Beecham, UK

7). General Regents and Equipment

Reagent	Supplier
Isopentane	Fisher, UK
Propan-2-ol	Fisher, UK
BDX mounting fluid	BDH, UK
Di sodium hydrogen orthophosphate ($Na_2 HPO_4$)	Fisher, UK
Sodium dihydrogen orthophosphate ($NaH_2 PO_4$)	Fisher, UK
Cresyl violet	BDH, UK
Sodium acetate	Fisher, UK
Acetic acid	Fisher, UK
Tris (hydroxymethyl) methylamine ($[OH.CH_2]_3C.NH_2$)	Fisher, UK
Magnesium chloride ($MgCl_2$)	Fisher, UK
Hydrochloric acid	Fisher, UK
Ilford PQ universal developer	Jessops, UK
Ilford wetting agent	Jessops, UK
Ilford hypam fixer	Jessops, UK
Ilford rapid hardener	Jessops, UK
Gelatine	Fisher, UK
Chromic potassium sulphate	Fisher, UK
Scintillation fluid (Optiphase Hisafe 3)	Wallac, UK
Tissue solubilizer (NCS II)	Amersham, USA
Tween 80	Sigma, UK

Hardware	Supplier
Bright OTF cryostat	Bright Instruments, UK
cork discs	Bright Instruments, UK
Kopf Brain Blocker	Kopf Instruments, USA
Embedding fluid	Bright Instruments, UK
Whatman Qualitative Circles	Whatman, UK
Superfrost slides	BDH, UK
Slide Cover slips	Fischer, UK
Microfilter (0.2μM pore size)	Schleicher & Schuell, Germany
20ml multidose vials and caps	Fischer, UK
Autoradiography cases	Genetic Research Instrumentation, UK
Autoradiographic [^{3}H]Microscale	Amersham, UK
[^{3}H]Hyperfilm	Amersham, UK
GBX-2 safelight filter	Sigma, UK
15 watt bulb	BHS, UK
Packman 1900 scintillation counter	Packman, UK
MCID version 4	Microcomputer Image Device, Imaging Research, Canada

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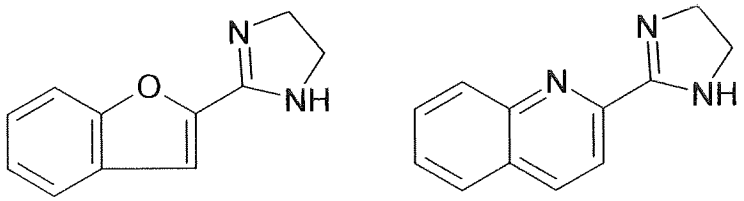
Chapter 3

Development of the 2-BFI cue, its relationship to weight gain and lever pressing behaviour

1). Introduction

2-BFI is a specific I_2 ligand that has a high affinity for I_2 sites (1.71nM) and low affinity for α_2-adrenoceptors (1.86 μM; see figure 3.1; Lione et al, 1998; Alemany et al, 1997).



2-BFI BU224

Figure 3.1). ***Chemical structure of 2-BFI and BU224***

In spite of its high affinity *in vitro*, previous *in vivo* assessments have indicated 2-BFI and other I_2 specific ligands have very little overt behavioural pharmacology (Jordan 1993; Brown et al, 1995). As previously mentioned 2-BFI is equipotent to desipramine in the forced swim model of depression and I_2 ligands modulate opioid analgesia and food intake (Nutt et al, 1995; Hudson et al, 1999b; Sanchez-Blazquez et al, 2000; Jackson et al, 1991; Menargues et al 1994; Menargues et al 1995; Brown et al, 1995; Prasad and Prasad, 1996; Polidori et al, 2000).

However, these techniques tell us little of the pharmacology associated with imidazoline ligand administration. Using the technique of *in vivo* microdialysis it has been shown that the i.p. administration of 2-BFI increases extraneuronal levels of NA (Nutt et al, 1995; Hudson et al, 1999). This mechanism is independent of antagonism of the α_2-noradrenergic autoreceptor because 2-BFI, as previously mentioned, has a low affinity for this site (Alemany et al, 1997).

Administration of a single dose (7mg/kg) of 2-BFI, with a dosing time of 20 minutes, given i.p. leads to an estimated brain concentration of 1000nM (Jordan et al, 1996). Similarly, i.p. administration of 10 mg/kg BU224, an analogue of 2-BFI, with a dosing time of 30 minutes gives rise to an actual concentration of 1,280 nM. This effect is dose dependent. Administration of a low dose of BU224, i.e. 1mg/kg, gives rise to CNS concentrations of 124 nM, whilst 5mg/kg gives rise to a CNS concentration of 220 nM (Hudson et al, 1999b).

Due to the lack of overt pharmacology previous work has focused on the technique of drug discrimination in a bid to clarify the pharmacology behind the I_2 site (Jordan et al, 1996). Drug discrimination is dependent on the principle that an animal can learn a discriminable cue. A number of species have been previously trained for drug discrimination studies, these include the, rat, gerbil pigeon and monkey (Actor and Griffiths, 1985; Hiltunen et al, 1986; Jordan et al, 1996; Kleven and Koek, 1998).

The driving force behind drug discrimination is the reinforcer, i.e., that thing that makes the likelihood of an event occurring again. Drug discrimination studies involve training animals to associate the administration of a compound with pressing a particular lever. On the whole drug discrimination studies have used positive reinforcers of two types. The first type involves limiting food in the animal's home cage and only giving them access to it during a training session (Sanger, 1989; Kleven and Koek, 1998). Hence, the lever pressing driving force is starvation. However, as rats show high levels of lever pressing when exposed to condensed milk reward (Flip et al, 1993), recent studies have given rats free access to food and used a condensed milk reward to

encourage lever pressing (Flip et al, 1993; Jordan et al, 1993, Jordan et al, 1995, Jordan et al, 1996).

Animals have been trained to discriminate a number of different compounds from saline, e.g., amphetamine, idazoxan, clonidine, ethoxy idazoxan, 2-BFI, ibogaine, clenbuterol, imipramine, clozapine, cocaine, 8-OH-DPAT (Porsolt et al, 1984; Sanger, 1989; Jordan et al, 1993, Jordan et al, 1995, Jordan et al, 1996; Helsley et al, 1997; McElroy and O'Donnell, 1988; Schechter, 1983; Kelly and Porter, 1997; Kleven and Koek, 1999; Sanger and Schoemaker, 1992).

Previous work, by Jordan et al, (1996) has indicated that rats can be successfully trained to discriminate 7 mg/kg 2-BFI from saline, in two lever skinner boxes, with condensed milk reward. This process took an average of 44 training sessions before animals had responded with over 70% accuracy for 10 consecutive sessions. When 2-BFI was administered as a test drug it showed dose dependent substitution. Low doses, 1mg/kg elicited 4.9% 2-BFI appropriate lever pressing. Moderate doses (3 mg/kg) elicited slightly higher percentages (52%) and when the training dose was administered all rats responded with 100% accuracy to the 2-BFI associated lever. Moderate doses of 2-BFI (3 mg/kg) were enough to elicit levels of 2-BFI appropriate responding that were significantly different from saline.

In the drug discrimination studies reported here animals have free access to food/water and condensed milk is used as a positive reinforcer of correct lever pressing. As I_2 ligands have a reported ability to increase food intake in the hours after dosing, the first group were to be trained on a number of different doses of 2-BFI, and in the presence of an appetite driven variable (i.e., the number of reinforcements an animal receives in a 15 minute session), it was felt important to examine lever pressing behaviour between drug days and saline days.

Hence, the initial part of the study was concerned with two factors. Firstly, finding the minimal dose needed to elicit a discriminable cue. Secondly, because of the relationship between I_2 ligands and food intake lever pressing behaviour, condensed milk intake, and weight gain were examined. This was done through two methods. Firstly, using rats that had been trained on a

number of doses of 2-BFI, the relationship between reinforcements received on saline days and those received on particular drug/dose days was examined. Secondly, this dose regime was also examined for changes that may occur in bodyweight between DD and saline dosed animals.

2). Method

2.1). Animals

Drug discrimination training followed the method outlined in the methods Chapter. Six groups of eight rats were trained in age cohorts (group 1, 2, 3, 4, 5, 6 and 7). One further group of ten rats, group 8, were administered 2-BFI following the drug discrimination schedule but underwent no drug discrimination training. Groups 1 and 2 were used to establish the minimum dose of 2-BFI that would elicit a discriminable cue. All animals had free access to food and water at all times except when entered into training or test sessions.

2.2). Statistics

2.2.1). Effects of 2-BFI and lever pressing behaviour

The ARR or average reinforcement rate represents the average number of reinforcements an animal receives, in response to pressing the correct lever, over the fifteen minute training session. The data for individual doses was derived from ten drug days of a particular dose and ten associated saline days. Thus the ARR emitted at doses of 3.5 mg/kg, 4 mg/kg, 5 mg/kg, and 7 mg/kg, were compared with their associated saline days, i.e., the saline days that were between drug days of that particular dose, see table 3.1. The data points for each of the drug/dose associated day or saline associated days represented the average ARR emitted by the group on that day. The last 10 3.5 mg/kg days were used so as to avoid any practice effects that may be apparent at the beginning of training, see table 3.1. For 4 and 5 mg/kg only data from 20 (10 drug days and 10 saline) sessions were available - this data was used, see table 3.1. For 7 mg/kg the data from the first 20 sessions were used (after this point training session times were reduced from 15 to 10 minutes) again 10 drug and 10 saline sessions were used. Hence, statistical analysis was between the average group ARR on 10 drug/dose days and the ARR on the 10 associated saline days.

Table 3.1). *Dosing Schedule of 2-BFI treated rats*

Dose	3.5	d 3.5	d 3.5	s 3.5	s 3.5	d 3.5	d 3.5	s 3.5	s 3.5	d 3.5	s 3.5	d 3.5	d 3.5
Date	9/2	10/2	11/2	12/2	13/2	16/2	17/2	18/2	19/2	20/2	23/2	24/2	25/2
Dose/Day	s 3.5	d 3.5	d 3.5	s 3.5	s 3.5	d 3.5	d 3.5	s 3.5	s 3.5	d 3.5	d 3.5	s 3.5	d 3.5
Date	26/2	27/2	2/3	3/3	4/3	5/3	6/3	9/3	10/3	11/3	12/3	13/3	16/3
Dose/Day	s 3.5	s 3.5	d 3.5	d 3.5	s 3.5	d 3.5	d 3.5	s 3.5	s 3.5	d 3.5	s 3.5	**s 3.5**	**d 3.5**
Date	17/3	18/3	19/3	20/3	23/3	24/3	25/3	26/3	27/3	30/3	31/3	1/4	2/4
Dose/Day	**d 3.5**	**s 3.5**	**s 3.5**	**d 3.5**	**s 3.5**	**s 3.5**	**s 3.5**	**d 3.5**	**d 3.5**	**s 3.5**	**d 3.5**	**s 3.5**	**d 3.5**
Date	3/4	6/4	7/4	8/4	10/4	14/4	15/4	16/4	17/4	20/4	22/4	23/4	24/4
Dose/Day	**d 3.5**	**s 3.5**	**s 3.5**	**d 3.5**	**d 3.5**	**s 4**	**d 4**	**d 4**	**s 4**	**s 4**	**d 4**	**s 4**	**s 4**
Date	27/4	28/4	29/4	30/4	1/5	4/5	5/5	6/5	7/5	8/5	11/5	12/5	13/5
Dose/Day	**d 4**	**d 4**	**s 4**	**d 4**	**d 4**	**s 4**	**s 4**	**d 4**	**s 4**	**s 4**	**d 4**	**d 4**	**s 5**
Date	14/5	15/5	18/5	19/5	20/5	21/5	22/5	25/5	26/5	27/5	28/5	29/5	1/6
Dose/Day	**d 5**	**d 5**	**s 5**	**s 5**	**d 5**	**s 5**	**s 5**	**d 5**	**d 5**	**s 5**	**d 5**	**d 5**	**s 5**
Date	2/6	3/6	4/7	5/7	8/6	9/6	10/6	11/6	12/6	15/6	16/6	17/6	18/6
Dose/Day	**s 5**	**d 5**	**s 5**	**s 5**	**d 5**	**d 5**	**s 7**	**d 7**	**d 7**	**s 7**	**s 7**	**d 7**	**s 7**
Date	19/6	22/6	23/6	24/6	25/6	26/6	29/6	30/6	1/7	2/7	3/7	6/7	7/7
Dose/Day	**s 7**	**d 7**	**d 7**	**s 7**	**d 7**	**d 7**	**s 7**	**s 7**	**d 7**	**s 7**	**s 7**	**d 7**	**d 7**
Date	8/7	9/7	10/7	13/7	14/7	15/7	16/7	17/7	20/7	21/7	22/7	23/7	24/7

Data in bold used to derive dose/RPM data. d, drug day. s, saline day. Number denotes dose (mg/kg) of 2-BFI administered or dose of 2-BFI associated with saline day. Data derived from G1.

2.2.2). Effects of 2-BFI and weight gain.

Rat group 8 were defined as a DD shadowing group. Upon receiving the animals they were split into two groups of five. Group 8A followed the same dosing schedule as groups, 3, 4, 5, 6 and 7, i.e., they were injected with either 7mg/kg 2-BFI or saline following the SDDSSDSSDD schedule but did not enter the Skinner box for training. Conversely the second group of rats received only saline injections (group 8B). This group functioned as a control group.

Group 8A received 96 'pseudo training sessions', 46 saline and 50 2-BFI (7mg/kg). Whilst group 8B received 96 'pseudo training sessions' – all of these being saline This occurred over 163 days (as with actual DD experiments these animals were not injected over weekends or holidays).

Based on the data obtained from groups one to six we felt 96 pseudo sessions would be sufficient to mimic any alterations that were occurring within the brains of DD trained animals. The reason for the shadowing were two fold. Firstly, we were concerned with the effects such a dosing scheme may have on 2-BFI binding site density (see Chapter 8). Secondly, as I_2 ligands are associated with an increase in eating behaviour we wanted to look at any weight gain that may be apparent with such a dosing regime. Hence, every 15 days the weight of each rat within rat group 8A and group 8B were taken.

2.2.3). Statistical Testing

As mentioned in the method (Chapter 2) all data was initially tested deviations from gaussian principles. Providing parametric assumptions were met significant differences were established by comparing experimental groups to relevant control groups, using one way analysis of variance (ANOVA with Newmans-Keuls post hoc test). All other details are as outlined in Chapter 2.

3). Results

3.1). Establishment of the 2-BFI cue.

The first group, group 1 (G1) was initially started on a dose of 3.5mg/kg. After 57 training sessions it became apparent that although the total number of responses was over 90% on the correct lever, the first 7 responses were not consistently on the correct lever - suggesting that the animals were failing to discriminate between the saline vehicle and 2-BFI. Essentially all 8 rats had developed a strategy in which they initially responded to both levers until they obtained a reward. They then continued pressing the lever on which they obtained reward. Thus after completing 57 sessions on 3.5 mg/kg, all animals training 2-BFI dose was elevated to 4 mg/kg. After 20 sessions it became apparent that this dose was not sufficient for all animals to discriminate between the saline and 2-BFI levers. We increased the dose to 5 mg/kg, and again after 20 sessions of training, eight animals falied to fulfil both criteria. However, at 5mg/kg, all animals were approaching the criteria for entry into a test day. Three rats had completed over 10 consecutive error free sessions, but had dropped a session at the end of these error free 'runs' thus nullifying their potential for entry into a test session. The remaining 5 rats all showed error free runs, but none of these runs constituted over 6 consecutive error free sessions. It was finally decided to increase the dose to that used in Jordan's (1996) original studies of 7mg/kg 2-BFI. It took a further 36 sessions before all animals reached criteria for entry into a test session. Thus, group one rats had to complete an average 213 training sessions before test criteria were reached, see table 3.2.

From this data it was decided to begin group 2 on 5 mg/kg. However after 50 sessions it was decided to increase group two's dosage 7 mg/kg. Thus animals further completed 49 sessions, at 7mg/kg 2-BFI, before successfully being able to discriminate between 2-BFI and saline. Hence, in total 99 training sessions were needed before group 2 developed the 2-BFI cue. The average number of training sessions needed for discrimination thus being 99.

Groups, 3, 4, 5 6 and 7 were all started on 7mg/kg 2-BFI and received 52.2, 60.3, 38.2, 41.3 and 54.3 sessions respectively before reaching criteria and therefore eligibility for entry onto a test session (see table 3.2)

Table 3.2). *Average number of training sessions experienced by rats before completion of test session criteria*

Group	Number of session training before entry to test session	
	Mean	*SD*
1	213.7	5.7
2	99.7	15.5
3	52.2	21.7
4	60.3	15.1
5	38.2	21.4
6	41.3	7.2
7	54.3	13.1

SD. Standard Deviation.

3.2). Effects of 2-BFI on lever pressing behaviour

Due to the ability of I_2 ligands to increase food intake the potential exists for a dose relationship to occur between reinforcements obtained and by association the amount of condensed milk received over a 15 minute training session. Thus the average reinforcement rates for each dose of 2-BFI and their associated saline days were calculated, see table 3.3.

Table 3.3*). Average Reinforcement Rates for Group 1 Rats in DD Training*

Dose of 2-BFI	Associated Drug ARR	Associated Saline ARR
	Mean ± SD.	*Mean ± SD.*
3.5 mg/kg 2-BFI	38.60 ± 1.03	37.20 ± 1.46
4 mg/kg 2-BFI	43.50 ± 1.62	42.50 ± 1.86
5 mg/kg 2-BFI	50.70 ± 0.97	52.80 ± 1.21
7 mg/kg 2-BFI	50.50 ± 1.97	46.50 ± 2.95

SD. Standard Deviation.

Table 3.3 and figure 3.2 indicate that there was a dose dependent increase in the 2-BFI average reinforcement rate (ARR). The average reinforcement rate at the outset of the study, when animals were dosed with 3.5 mg/kg 2-BFI, was 38.6 over the 15 minute training session. This increased to 50.5 after administration of the highest dose (7 mg/kg) 2-BFI to the same group. These differences were significant (see table 3.4). The average ARR emitted at 3.5 mg/kg 2-BFI (mean 38.60) was significantly lower than the ARR at doses of 5 mg/kg ($p<0.01$) and 7 mg/kg 2-BFI ($p<0.01$; mean, 43.50 and 50.70 respectively). However, these dose dependent differences were also apparent between 2-BFI associated saline days. The saline ARR associated with the 3.5 mg/kg 2-BFI was significantly lower than the saline ARR's associated with 5 ($p<0.01$) and 7 mg/kg 2-BFI ($p<0.01$).

Table 3.4). *Statistical differences between average reinforcement rates for group one rats in DD training*

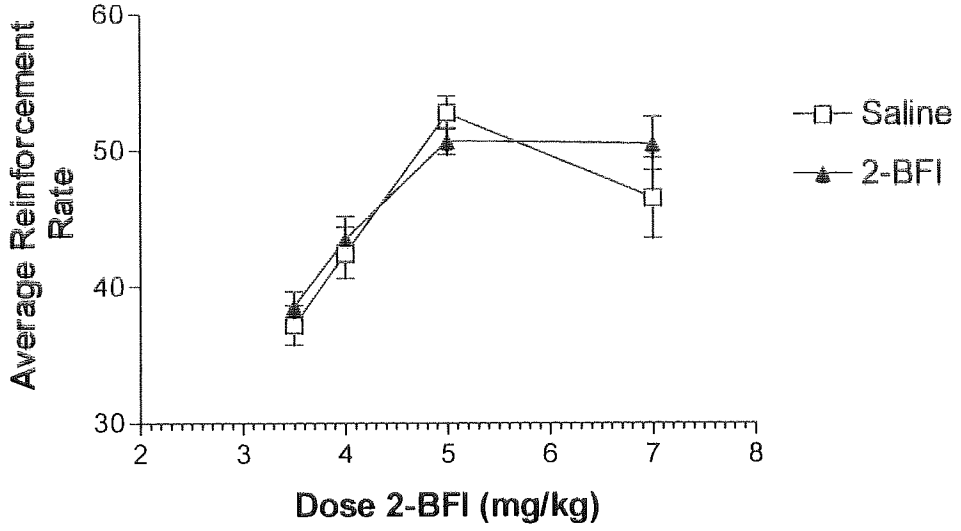
Variable	Saline 3.5 mg/kg 2-BFI	Drug 3.5 mg/kg 2-BFI	Saline 4 mg/kg 2-BFI	Drug 4 mg/kg 2-BFI	Saline 5 mg/kg 2-BFI	Drug 5 mg/kg 2-BFI	Saline 7 mg/kg 2-BFI	Drug 7 mg/kg 2-BFI
Saline 3.5 mg/kg 2-BFI	*	ns	ns	ns	p<0.01	p<0.01	p<0.05	p<0.01
Drug 3.5 mg/kg 2-BFI		*	ns	ns	p<0.01	p<0.01	ns	p<0.01
Saline 4 mg/kg 2-BFI			*	ns	p<0.05	ns	ns	ns
Drug 4 mg/kg 2-BFI				*	p<0.05	ns	ns	ns
Saline 5 mg/kg 2-BFI					*	ns	ns	ns
Drug 5 mg/kg 2-BFI						*	ns	ns
Saline 7 mg/kg 2-BFI							*	ns
Drug 7 mg/kg 2-BFI								*

Ns. non-significant result. $p<0.05$ and $p<0.01$ repeated measures one way ANOVA with post hoc Newman-Keuls.

Significant differences were also observed between the 3.5 mg/kg saline ARR (mean 37.20) and the ARR's at 5 mg/kg ($p<0.01$) and 7 mg/kg 2-BFI ($p<0.01$;

mean, 50.70 and 50.50 respectively). However, interestingly 3.5 mg/kg 2-BFI was significantly higher than the ARR on the 5 mg/kg 2-BFI associated saline days, but not on the saline 7 mg/kg 2-BFI associated saline days. Two other significant differences were observed. The ARR emitted on the 5 mg/kg 2-BFI associated saline days (mean, 52.80) was significantly lower than that at 4 mg/kg 2-BFI (mean 43.50, $p<0.05$) and 4 mg/kg 2-BFI saline associated days (mean 42.50, $p<0.05$).

Figure 3.2). ***Average Reinforcement Rate (ARR) at various doses of 2-BFI***

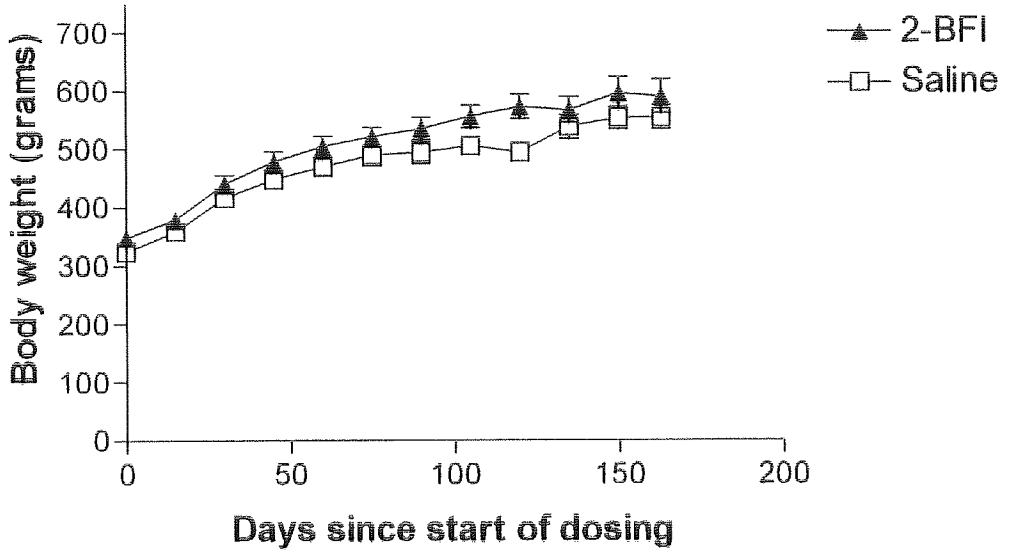


3.3). Effects of 2-BFI on weight gain

Group 8 followed the drug discrimination schedule, i.e., they were injected with either 7mg/kg 2-BFI or saline following the SDDSSDSSDD schedule, for 96 sessions. The weight gain, for group 8A and 8B is shown in figure 3.3. As figure 3.3 clearly illustrates both groups of animals gained weight over the 163 days that comprised the experiment. Group 8A, the 2-BFI dosed group, started the session with a slightly higher average weight than the saline dosed group 8B rats. The difference between these two groups stayed consistent through out the study. There was no significant main ($p>0.05$; repeated measures one way ANOVA) effect between the body weights of rats who had

experienced the DD dosing schedule versus those who had been admnistered saline vehicle.

Figure 3.3). ***Weight gain in response to drug discrimination dosing schedule***



4). Discussion

The present study had three aims. Firstly we sought to find the minimal dose of 2-BFI needed to elicit a discriminable cue in eight rats. Secondly, we sought to assess any increases in lever pressing behaviour and therefore condensed milk intake that may be associated with administration of a particular dose of 2-BFI. Finally we compared the weights of animals that were undergoing DD dosing with those that obtained only saline to see if alterations in animal weight were present.

The results of this study indicate that the minimal dose of 2-BFI needed to enable animals to discriminate 2-BFI from saline is 7 mg/kg. This would equate to a CNS concentration of 1000nM (Jordan et al, 1996). Based on the previous significant substitution of 3 mg/kg 2-BFI in 7 mg/kg 2-BFI trained animals (Jordan et al, 1996), it was felt that it would be possible to train rats to discriminate saline from 3.5 mg/kg. However, this was not possible as these animals developed a strategy, i.e., they pressed both levers until they found the lever which gave them condensed milk. The dose was increased to 4mg/kg 2-BFI, and then through 5, and finally at 7mg/kg all eight rats successfully learnt to discriminate 2-BFI from saline.

Five subsequent groups were trained on only 7 mg/kg 2-BFI. The fastest group of rats to successfully discriminate between the two levers was group 5 who reached the criteria for entry into a test session in an average of 38.2 sessions. The slowest group was group 4 who took an average of 60.3 sessions. These data are consistent with Jordan et al's (1996) original study which indicated an average of 44 sessions were needed before the criteria needed for entry into test sessions were fulfilled.

Previous work has suggested that I_2 ligands increase food intake in the hours after dosing. Hence it is not surprising that a significant dose dependent increase in the ARR was apparent between the lowest dose of 2-BFI and the two higher ones (5 and 7 mg/kg). Theoretically, an increase in the ARR represents an increase in condensed milk, and thus an increase in food intake. This agrees with previous findings, which employ a similar model - i.e., a repeated measures design, that found agmatine significantly increases

carbohydrate intake (Prasad and Prasad, 1996). However, a significant dose dependent increase was also observed between the saline days associated with each dose of 2-BFI. Hence, the ARR exhibited on the saline days associated with 5 and 7 mg/kg 2-BFI were significantly higher those associated with 3.5 mg/kg 2-BFI saline days. Hence, it is possible to suggest that practice effects could account for an increase in the 2-BFI ARR.

However, this may not be the case. The ARR exhibited on the 4 mg/kg 2-BFI associated saline day was significantly lower than the ARR for the 5 mg/kg 2-BFI associated saline day. It could be suggested that the significant increase between saline associated 2-BFI days is as a result of drug associated carry over effects from the previous drug day, rather than practice effects from the previous months of training. It is noteworthy that the ARR does not increase over the dose of 5 mg/kg 2-BFI, i.e., the ARR associated with 5 mg/kg 2-BFI is virtually identical to that associated with 7 mg/kg 2-BFI. However, the same is not true for the corresponding saline days. The ARR on the saline days associated with 7mg/kg 2-BFI are significantly lower than those exhibited on the 5 mg/kg saline days. It is suggestive of a threshold being reached, at which point lever pressing behaviour reaches a drug-induced maximum. The animal habituates to this maximum and becomes more discriminating against any residual traces from a previous sessions drug dosing. This theory would account for the large but non-significant drop in the mean ARR on saline days associated with 7mg/kg 2-BFI and those associated with the 5mg/kg saline days, see figure 3.2. The proposition that - the animals have reached the maximal number of times they can physically press the lever and receive reinforcement over the 15 minute session cannot simply explain the plateau effect that occurs at 5mg/kg and 7mg/kg for two reasons. Although the ARR on the two highest doses of 2-BFI stabilised, the ARR on the 7mg/kg 2-BFI associated saline day actually dropped. Secondly, and probably more importantly, an ARR of 50 equates to 500 lever presses over the 15 minute session. We have recorded in excess of 1000 lever presses or an ARR of 100 in some animals over the same time period. Hence, these rats are not working at their physical maximum.

On the basis of these results it was surprising that there was no significant difference between the bodyweight of the animals who had followed the DD dosing schedule compared with those that experienced the saline dosing schedule. However, this result is not really surprising for two reasons. Firstly, the drug discrimination schedule consists of 50% 2-BFI and 50% saline. Hence, any hyperphagic effects and associated body weight increase induced after I_2 dosing may be reduced by the surrounding saline days. Secondly, I_2 ligands increase food intake in the hours after dosing. The animals used to derive ARR data were actually trained within Skinner boxes and thus received condensed milk reward, after dosing. Whereas group 8, in which bodyweight was compared, received no training within the Skinner box and thus only received access to normal rat chow after dosing. Sweetened condensed milk is extremely high in calories. It may well be the case that group 8 DD dosed animals might have significantly increased their body weight if they had been given the opportunity to consume condensed milk in the hours after dosing rather than only rat chow.

The results from this study indicate the imidazoline I_2 site ligand can generate a cue for drug discrimination studies. The minimum dose in which eight animals can be trained to discriminate 2-BFI from saline is 7mg/kg. Administration of 2-BFI dose dependently increases lever pressing behaviour and subsequent milk intake. However, this process did not result in a significant increase in bodyweight compared with saline controls.

Chapter 4). Drug Discrimination: I_2 specific ligands 87

Chapter 4

Drug Discrimination: I_2 specific ligands

1). Introduction

The initial studies carried out by Jordan et al, (1996) indicated that the specific I_2 ligand 2-BFI can generate a discriminable cue in rats. Since the publication of this work a number of structurally related analogues to 2-BFI have become available. These include the quinoline and isoquinoline analogues of 2-BFI; BU216, BU224, BU226, and the imidazole moiety containing LSL60101 and LSL 60125 (see figure 4.1). Hence, the current series of studies sought to explore the similarities of various I_2 specific ligands to 2-BFI.

In rat brain, BU 224, BU 216 and BU 226, have a high affinity (K_i) for the I_2 site (2.06, 6.4 and 2.7 nM respectively) and low affinity for α_2-adrenoceptors (12,100, 22,900 and 12,600 nM respectively; Lione et al, 1998; Hudson et al 1994; Hudson et al, 1999b). Further compounds have been synthesised using a similar carbon ring structure except replacing the imidazoline moiety for an imidazole moiety, namely LSL60101 and LSL 60125. Again these compounds have a high affinity for the I_2 site (879 and 151 nM, respectively) but low affinity for α_2-adrenoceptors (100,148 and 56,150 nM respectively; Alemany et al, 1997; Alemany et al 1995b).

Agmatine has been proposed as a potential endogenous I_2 ([^{3}H]idazoxan labelled), I_1 & α-adrenoceptor ligand and is present in the brain in similar

quantities to that of noradrenaline and dopamine (Li et al, 1994). The affinity of agmatine to I_2 sites seems to be species and tissue specific. Initial studies in bovine brain indicated that agmatine has a moderate affinity for I_2 sites (Li et al, 1994). However, recent studies have indicated that agmatine has a very low affinity (>100 μM) for [^{3}H]2-BFI defined rabbit and rat brain I_2 sites (Lione et al, 1996; Lione et al 1998). However, in rat vascular smooth muscle agmatine has a high affinity for the [^{3}H]idazoxan I_2 site (240 nM; Regunathan et al, 1996). A question has also arisen over agmatine's bioactivity (Eglen et al, 1998). Although these studies have established that agmatine binds to these sites they do not indicate if their is any intracellular consequence after binding (Pinthong et al, 1995; Eglen et al, 1998). Studies in isolated tissue have given conflicting results. The application of agmatine singularly, or in conjunction with the α_2-adrenoceptor agonists clonidine or UK 14304, to the guinea pig isolated ileum or the rat vas deferens fails to alter noradrenaline release. However, a recent study has indicated that agmatine whilst in the presence of the non-imidazoline α_2-adrenoceptor antagonist rauwolscine inhibits noradrenergic induced [^{3}H]noradrenaline overflow (Molderings et al, 2000). Thereby suggesting agmatine is an antagonist at α_2-adrenoceptors.

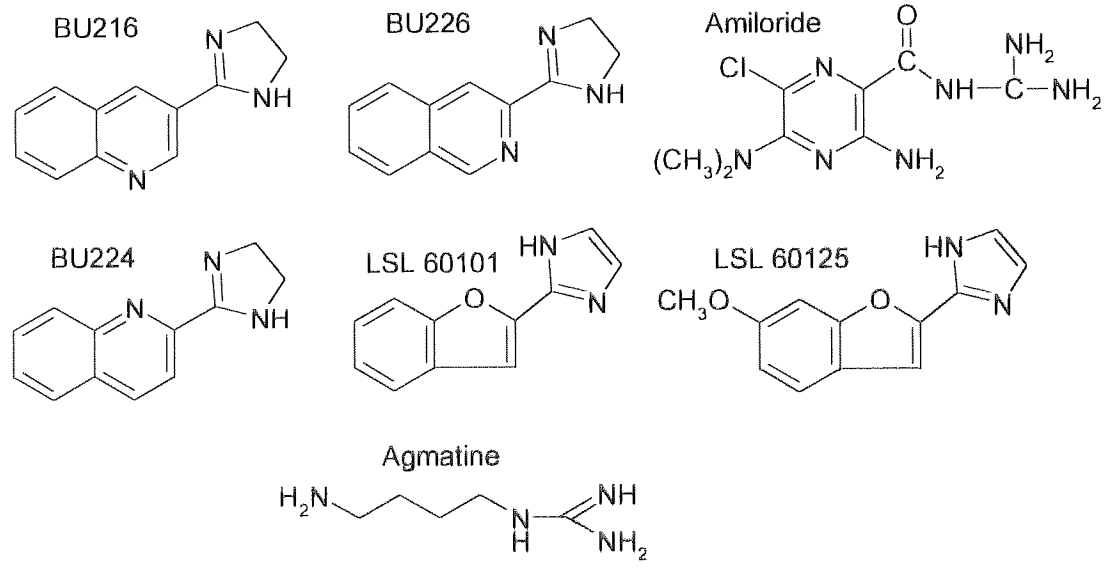


Figure 4.1). Chemical structure of I_2 specific ligands

Amiloride primarily gained prominence as a potent Na^+ (sodium) channel blocker (Hamill & McBride, 1996). However, amiloride has also been proposed as a ligand that can distinguish between two I_2 proposed subtypes – the amiloride sensitive I_{2A} site and the amiloride insensitive I_{2B} site (Diamant et al, 1992). The rat brain, similarly to the human brain, expresses the I_{2B} (amiloride insensitive) site (Alemany et al, 1997; Miralles et al, 1993). This is in contrast to the rabbit brain and frog brain that express the amiloride sensitive I_{2A} site and hence amiloride has a low affinity for the rat brain I_2 site (K_I = 9000nM; Alemany et al, 1997; Lione et al 1994; Lione et al 1996).

As mentioned in the introduction the administration of I_2 specific compounds does give rise to little overt behavioural pharmacology. However, it has been firmly established that I_2 ligands increase food intake in the hours after dosing; are equipotent to desipramine in animal models of depression; and the administration of the imidazole compound LSL 60101 alters levels of GFAP within the brain (Jackson et al, 1991; Menargues et al 1994; Menargues et al 1995; Brown et al, 1995; Polidori et al, 2000; Nutt et al, 1995; Jordan, 1993; Olmos et al, 1994; Alemany et al, 1995).

In addition to these behavioural effects previous *in vivo* work has indicated administration of 10mg/kg BU224 reduces some of the side effects associated with behavioural models of opiate withdrawal (Hudson et al, 1999b). Other I_2 ligands notably 2-BFI, LSL 60101, LSL 601122 and agmatine potentiate morphine-induced analgesia (Kolesnikov et al, 1996: Sanchez-Blazquez et al, 2000). However, idazoxan and BU224 do not share this ability to increase rodent tail flick latency – a behavioural model of opioid analgesia. Co-administration of idazoxan or BU224 intracerebroventricularly (10μM, i.c.v) with morphine (1μM, i.c.v.) does not increase morphine's ability to increase tail flick latency (Sanchez-Blazquez et al, 2000). However, BU224 and idazoxan (10μM, i.c.v) does appear to antagonise 2-BFI's (10μM, i.c.v) ability to potentiate morphine analgesia as co-administration of these compounds with 2-BFI reduces tail flick latency to levels reported after morphine injection only (Sanchez-Blazquez et al, 2000).

This data suggests that idazoxan and BU224 act as antagonists at the I_2 site (Sanchez-Blazquez et al, 2000).

In 2-BFI trained rats previous drug discrimination work has indicated that the administration of idazoxan elicits significant levels of 2-BFI appropriate responding, i.e., the psychopharmacological consequences of idazoxan administration closely match that of 2-BFI (Jordan et al, 1996). This is in contrast to the opioid studies mentioned above which indicate 2-BFI and idazoxan do different things, i.e., one appears to be agonist whilst the other is an antagonist, or *vice versa* (Sanchez-Blazquez et al, 2000). Hence if BU224 or idazoxan are antagonists these compounds should show only low levels of 2-BFI appropriate responding as antagonists show high affinity but low efficacy. *In vivo* microdialysis studies also indicate that the administration of BU224 and 2-BFI have similar consequences, i.e., both increase extraneuronal levels of NA (Hudson et al 1999b). These studies, in contrast to the results of Sanchez-Blazquez et al (2000), indicate that 2-BFI and BU224 have the same pharmacological consequences after administration.

The aim of this study was to compare the potency *in vivo* of a number of recently synthesised I_2 specific ligands and the proposed endogenous imidazoline ligand agmatine.

2). Method

2.1). Animals

All animals were trained following the drug discrimination schedule outlined in the method (Chapter 2). Rats from drug discrimination group one and two (see Chapter 2 for training details) were aggregated for experiments that involved testing 2-BFI. All other compounds, i.e., BU224, BU216, BU226, LSL 60101, LSL60125, agmatine and amiloride were tested on group two. All drugs were dissolved in 0.9% physiological saline. All doses of a single drug and associated saline day were given in a pseudo random order.

2.1). Statistics

Statistical analysis was as outlined in the method (Chapter 2). Significant differences, using one way ANOVA (Dunnets post hoc), were established by comparison of drug percentage substitution levels for drug/dose days to drug percentage substitution days in which saline had been administered.

3). Results

3.1). Levels of Substitution

Table 4.2 shows the ability of a number of I_2 specific compounds to substitute for 2-BFI. The administration of 7 mg/kg 2-BFI, the training dose, gave rise to 100 % substitution for the 2-BFI cue. This was significantly higher than test sessions in which vehicle was administered ($p<0.001$). The administration of lower doses of 2-BFI indicated this effect was dose dependent as 4.8 mg/kg ($p<0.001$) and 3.2 mg/kg ($p<0.001$) also significantly substituted for 2-BFI, see figure 4.2. Significant dose dependent levels of substitution were also apparent after administration of 4.8 ($p<0.001$) and 7 mg/kg ($p<0.001$) BU224 but not at the lower doses of 1.6 and 3.2 mg/kg, see table 4.1. However, the other quinoline derivative of 2-BFI BU216 showed lower potency *in vivo*. Significant levels of substitution only occurred at the highest dose tested – 7mg/kg, see table 4.1 ($p<0.001$). BU226, an isoquinoline analogue of 2-BFI, proved to be the most potent of the three. Significant levels of 2-BFI appropriate responding occurred at 3.2mg/kg ($p<0.01$), 4.8 mg/kg ($p<0.001$) and 7mg/kg i.p. ($p<0.001$) , see table 4.1.

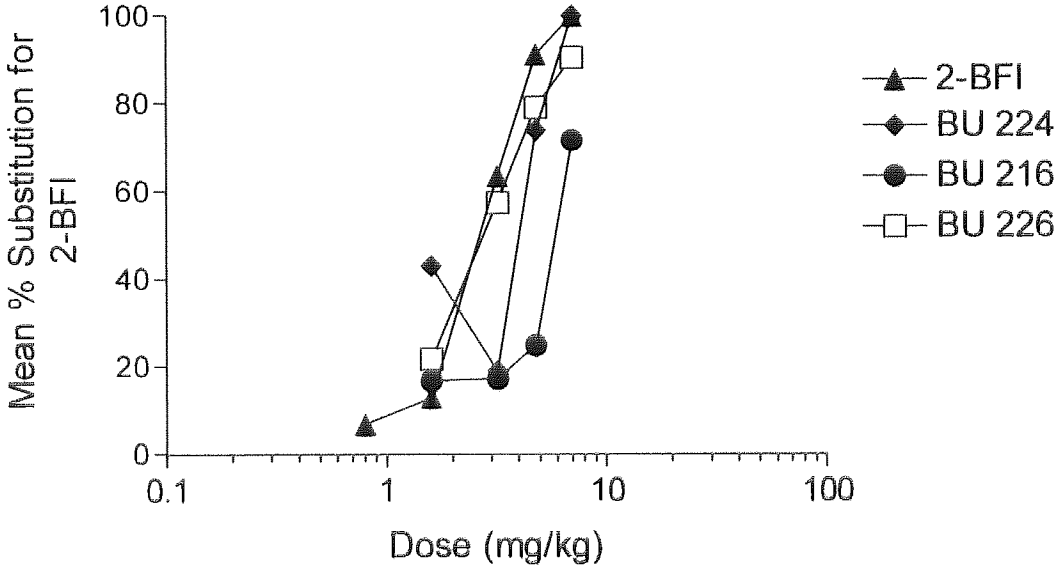


Figure 4.2). *Ability of drugs to substitute in 2-BFI trained rats (7 mg/kg)*

The imidazole compounds LSL 60101 and LSL 60125 proved to be less potent *in vivo*, see figure 4.3. LSL 60101 only elicited significant levels of 2-

BFI lever appropriate responding at 12.25 ($p<0.05$) and 14 mg/kg ($p<0.05$), see table 4.1. LSL 60125 significantly substituted for 2-BFI at 10.5 ($p<0.001$) and 12.25 mg/kg ($p<0.001$) but not at 14 ($p>0.05$) or 7 mg/kg ($p>0.05$), see table 4.1. At high doses, 30 mg/kg ($p<0.05$), agmatine significantly substituted for the 2-BFI. However, agmatine failed to significantly substitute for 2-BFI at the highest (50mg/kg; $p>0.05$) or the lowest dose (10mg/kg; $p>0.05$). Amiloride was administered at a single dose of 7mg/kg, showed low levels of substitution, and thus failed to significantly substitute for the 2-BFI cue, see table 4.1.

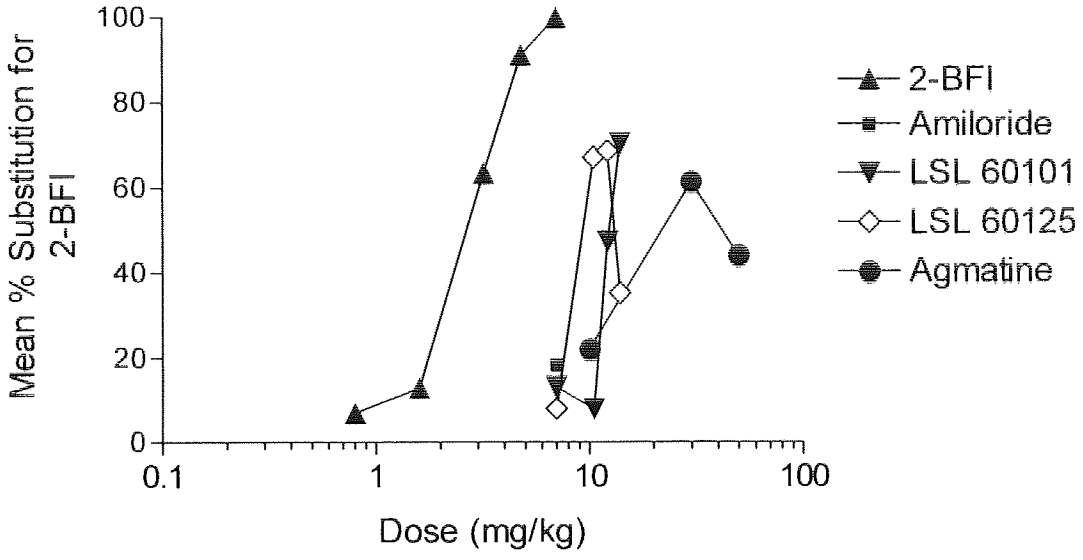


Figure 4.3). ***Ability of drugs to substitute in 2-BFI trained rats (7 mg/kg)***

3.2). Responses per minute (RPM)

The administration of 2-BFI at all doses tested (0.8, 1.6, 3.2, 4.8 mg/kg) and the quinolines BU216, BU226, at all doses tested (1.6, 3.2, 4.8 and 7mg/kg) failed to significantly alter the number of responses per minute (RPM) elicited by 2-BFI trained rats, see table 4.1. However, the highest dose of the isoquinoline BU224 (7mg/kg), but not the three lower doses, did significantly reduce the RPM ($p<0.001$). The imidazole compounds LSL 60101 and LSL 60125 at all doses tested failed to significantly alter the RPM, see table 4.1. The same was true after the administration of agmatine

and amiloride, i.e., non significant decreases in the number of responses elicited per minute by 2-BFI trained rats, see table 4.1.

Table 4.1). ***Ability of various compounds to substitute for 2-BFI***

Test Drug	Dose	% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
2-BFI	0.8	6.8 ± 2.6	ns.	11/11	14.0 ± 3.6	ns.
2-BFI	1.6	12.8 ± 4.9	ns.	11/11	11.9 ± 3.8	ns.
2-BFI	3.2	63.5 ± 11.6	p<0.001	11/11	10.1 ± 2.6	ns.
2-BFI	4.8	91.1 ± 3.0	p<0.001	11/11	8.7 ± 1.3	ns.
2-BFI	7.0	100	p<0.001	11/11	9.2 ± 1.5	ns.
Saline		1.6 ± 1.0		11/11	12.7 ± 3.0	
BU224	1.6	42.8 ± 14.0	ns.	8/8	11.1 ± 2.2	ns.
BU224	3.2	18.8 ± 7.9	ns.	8/8	16.2 ± 8.5	ns.
BU224	4.8	73.8 ± 16.1	p<0.001	8/8	8.6 ± 2.1	ns.
BU224	7.0	100	p<0.001	8/8	13.0 ± 4.0	ns.
BU216	1.6	16.8 ± 9.9	ns.	8/8	9.6 ± 1.9	ns.
BU216	3.2	17.3 ± 11.5	ns.	8/8	8.3 ± 1.1	ns.
BU216	4.8	24.9 ± 6.6	ns.	8/8	8.9 ± 1.9	ns.
BU216	7.0	71.6 ± 14.0	p<0.001	8/8	8.8 ± 2.2	ns.
BU226	1.6	21.6 ± 10.7	ns.	8/8	5.5 ± 1.2	ns.
BU226	3.2	57.4 ± 16.4	p<0.01	8/8	7.6 ± 1.5	ns.
BU226	4.8	79.1 ± 12.8	p<0.001	8/8	8.7 ± 1.3	ns.
BU226	7.0	90.4 ± 9.6	p<0.001	8/8	1.5 ± 0.6	p<0.001
Saline		3.3 ± 1.6		8/8	10.8 ± 3.6	
LSL 60101	7	13.1 ± 8.7	ns.	8/8	6.8 ± 1.0	ns.
LSL 60101	10.5	7.8 ± 6.5	ns.	8/8	7.5 ± 0.6	ns.
LSL 60101	12.25	47.4 ± 13.0	p<0.05	8/8	5.7 ± 0.9	ns.
LSL 60101	14	70.4 ± 11.9	p<0.05	8/8	2.0 ± 0.8	ns.
LSL 60125	7	7.9 ± 5.8	ns.	8/8	8.3 ± 1.9	ns.
LSL 60125	10.5	67.1 ± 13.3	p<0.001	8/8	11.6 ± 3.0	ns.
LSL 60125	12.25	68.6 ± 11.4	p<0.001	8/8	11.2 ± 5.3	ns.
LSL 60125	14	35.1 ± 15.4	ns.	8/8	9.7 ± 3.4	ns.
Saline		5.7 ± 3.7		8/8	12.3 ± 3.7	
Agmatine	10	21.9 ± 12.2	ns.	8/8	9.6 ± 2.1	ns.
Agmatine	30	61.3 ± 17.1	p<0.05	8/8	8.9 ± 1.9	ns.
Agmatine	50	44.0 ± 16.6	ns.	8/8	7.2 ± 1.8	ns.
Saline		0		8/8	11.7 ± 3.2	ns.
Amiloride	7.0	18.4 ± 12.4	ns.	8/8	7.4 ± 1.6	ns.
Saline		9.0 ± 3.7			10.3 ± 3.3	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug responses per minute.

4). Discussion

The results from this study indicate that BU216, BU224 and BU216, the quinoline and isoquinoline derivatives of 2-BFI, dose dependently substitute in animals trained to discriminate 2-BFI from saline. The same is true, albeit at a lower potency for the imidazoles LSL60101 and LSL60125. At high doses agmatine, a proposed endogenous ligand for imidazoline sites, significantly substituted for 2-BFI. However, amiloride failed to induce 2-BFI appropriate levels of 2-BFI appropriate responding, this may have been for a number of reasons, see below. None of the ligands at any of the doses tested significantly altered the RPM (responses per minute), except for the highest dose of BU224 which significantly decreased it.

These results indicate BU224, BU226 and BU216 have a high potency *in vivo*. This confirms previous findings which similarly indicate these compounds have a high affinity for the I_2 site *in vitro* (Lione et al, 1998). All compounds showed significant dose dependent levels of substitution for the 2-BFI cue. Interestingly the quinoline BU 216, which shows the lowest affinity of the three *in vitro* also shows the lowest potency *in vivo*. Hence, BU2226 and BU224 showed full substitution for the 2-BFI, i.e., 80% or over of the animals responses were to the 2-BFI lever, whereas at the highest dose tested (7mg/kg) BU216 only exhibited partial substitution (between 20 and 80% of the animals lever presses were to the 2-BFI lever). A similar case is also true for the imidazoles LSL60101 and LSL 601125. *In vivo* these compounds have a lower potency than BU224, BU226 and BU216 and correspondingly these compounds show a much lower affinity *in vitro* (Alemany et al, 1997; Alemany et al 1995b). Hence at the highest doses administered LSL 60101 and LSL 60125 only gave rise to significant partial substitution. We were unable to administer higher doses of LSL60101 because of its ability to markedly reduce the RPM from 13.3 to 2 lever presses per minute. However, this was not a significant difference, probably due to the large variance between RPM's exhibited after administration of the highest dose of LSL 60101. The administration of LSL 60125 reduced the RPM but not to the same extent as LSL 60101. However, at the highest

dose administered, 14mg/kg LSL 60101, animals showed lower levels of 2-BFI appropriate responding than those elicited at 12.25mg/kg. Hence this higher dose was non-specific, i.e., the concentration of LSL 60101 was so high that it was likely to be activating other neurochemical transmitter mechanisms which masked the I_2 specific effects. Only one of the drugs administered, BU226 at 7mg/kg, reduced the RPM significantly. There is no apparent reason for this. *In vitro* BU226 and BU224 have a similar affinity for I_2 sites and α_2-adrenoceptors and as BU224 does not alter the RPM it is unlikely that this effect is modulated by either I_2 sites or α_2-adrenoceptors. However, of the three 2-BFI derivatives tested; BU226, BU224 and BU216; the isoquinoline BU226 does have the highest affinity *in vitro* for the I_1 site (Hudson at al, 1999b).

The results from this study indicate that agmatine significantly substitutes for 2-BFI at high doses. This is surprising as agmatine has a low affinity for the I_2 site *in vitro* (>100 μM; Lione et al, 1996 & 1998). The dose administered, 30 mg/kg is far larger than those previously cited in the literature. Previous studies that have administered agmatine peripherally, i.e., via the s.c. or i.p. route, show significant differences between agmatine and vehicle at much lower doses. At 10mg/kg agmatine significantly potentiates morphine analgesia and increases carbohydrate consumption in the rat (Kolesnikov et al, 1996: Prasad and Prasad, 1996). As previously mentioned agmatine has a higher affinity for α_2-adrenoceptors and I_1 sites when compared with I_2 sites (Li et al, 1994: Lione et al, 1996 & 1998). Recently, it has been suggested that agmatine is an antagonist at α_2-adrenoceptors (Molderings et al, 2000). Hence, bearing in mind the high doses of agmatine needed to elicit significant levels of 2-BFI appropriate responding it is possible that 2-BFI trained rats recognise the increase in NA release associated with antagonism of the α_2-adrenoceptor rather than the non-adrenergic NA increase associated with the administration of I_2 ligands (Hudson et al, 1999b). Consistent with this theory previous drug discrimination studies have indicated 2-BFI trained rats recognise non-imidazoline α_2-adrenoceptor antagonists, i.e., fluparoxan (Jordan et al, 1996). Hence, it is likely these

animals recognise the increase in NA rather than any I_2 specific effects. Amiloride failed to significantly substitute for 2-BFI. There may be a number of reasons for this. Firstly we were unable to find data on psychopharmacologically active doses. Secondly, we are unsure of amiloride's ability to cross the blood brain barrier. Hence the choice of dose was based on two factors, a previous report which studied peripheral effects but showed a dose of 10mg/kg was safe (Tatsuta et al, 1996) and the maximum quantity (7mg/ml) that could be dissolved in saline. Hence, firm conclusions about amiloride's ability to substitute for 2-BFI cannot be drawn from this data.

Recently Sanchez-Blazquez et al (2000) showed that BU224 antagonises 2-BFI induced potentiated morphine analgesia. If BU224 does behave as an antagonist it should show only low levels of substitution in drug discrimination, as antagonists have high affinity but low efficacy. However, BU224 induces high levels of 2-BFI appropriate behaviour (see table 4.1). In their original study Sanchez-Blazquez et al, (2000) show agmatine and 2-BFI, at the same dose (10 μg/kg, i.c.v.) are equipotent at potentiating morphine analgesia. Our results show that agmatine and 2-BFI are far from equipotent at inducing 2-BFI appropriate lever pressing. As shown in figure 4.3 approximately ten times the amount of agmatine (30mg/kg) are needed to elicit the same levels of 2-BFI appropriate responding reported at 3.2 mg/kg 2-BFI. However, it is possible that this difference in potency could be accounted for by a difference in the ability of agmatine and 2-BFI to penetrate the brain. Sanchez-Blazquez et al (2000) administered all ligands directly into the ventricles (i.c.v.) and thus circumvent the blood brain barrier whereas we administer intraperitonially (i.p.). Nevertheless, based on these results; the results from Jordan et al (1996) who show idazoxan substitutes for 2-BFI; and the *in vivo* microdialysis studies which indicate both BU224 and 2-BFI are associated with the release in NA; it may be the case that drug discrimination and tail flick latency measure two different pharmacological phenomena.

So to conclude, the quinoline and isoquinoline derivatives of 2-BFI elicited significant dose dependent levels of 2-BFI appropriate lever pressing in a manner similar to 2-BFI itself. These data suggest these compounds may be of a similar type, i.e., they are all either agonists or antagonists. The imidazole compounds LSL 60101 and LSL 60125 also showed significant levels of substitution for 2-BFI but at a lower potency. The proposed endogenous ligand agmatine also showed significant substitution. This was however, at very high doses and may be associated with its ability to antagonise α_2-adrenoceptors.

Chapter 5). Drug Discrimination: Noradrenergic Ligands 100

Chapter 5

Drug Discrimination: Noradrenergic Ligands

1). Introduction

One of the most notable features of I_2 ligand administration is their ability to increase extraneuronal levels of noradrenaline and dopamine (Jordan, 1993; Lalies and Nutt, 1993; Hudson et al, 1999b). This mechanism is independent of direct antagonism of the α_2-adrenoceptor as I_2 specific ligands have a very low affinity for this site (Nutt et al, 1995; Hudson et al, 1999b). However, the question remains to what, if any noradrenergic receptor does this released NA bind to. This in essence could contribute to the cue or psychopharmacological consequences of I_2 ligand administration *in vivo*. The current set of studies therefore set out to test the ability of various specific noradrenergic ligands to the substitute for 2-BFI.

It has been well established that extraneuronal levels of NA increase after the administration of α_2-adrenoceptor antagonists such as fluparoxan and idazoxan (Dennis et al, 1987; Halliday et al, 1991; Lachaud-Pettiti et al, 1991; Thomas & Holman, 1991). Presynaptic α_2-adrenoceptors act as autoreceptors and control the release of NA via a negative feedback mechanism using NA itself (Langer, 1974; Starke 1977). Hence, increases in NA will activate presynaptic α_2-adrenoceptors which, through an adenyl cyclase G protein mediated pathway, inhibit further NA release from the terminal (Byland, 1988). Hence, antagonists such as idazoxan, fluparoxan and yohimbine block this pathway (Dennis et al, 1987; Halliday et al, 1991;

Raiteri et al, 1983; Thomas & Holman, 1991). Conversely, agonists such as clonidine and guanabenz mimic NA's action at presynaptic α_2-adrenoceptors and thus reduce NA release from the terminal (Langer, 1981).

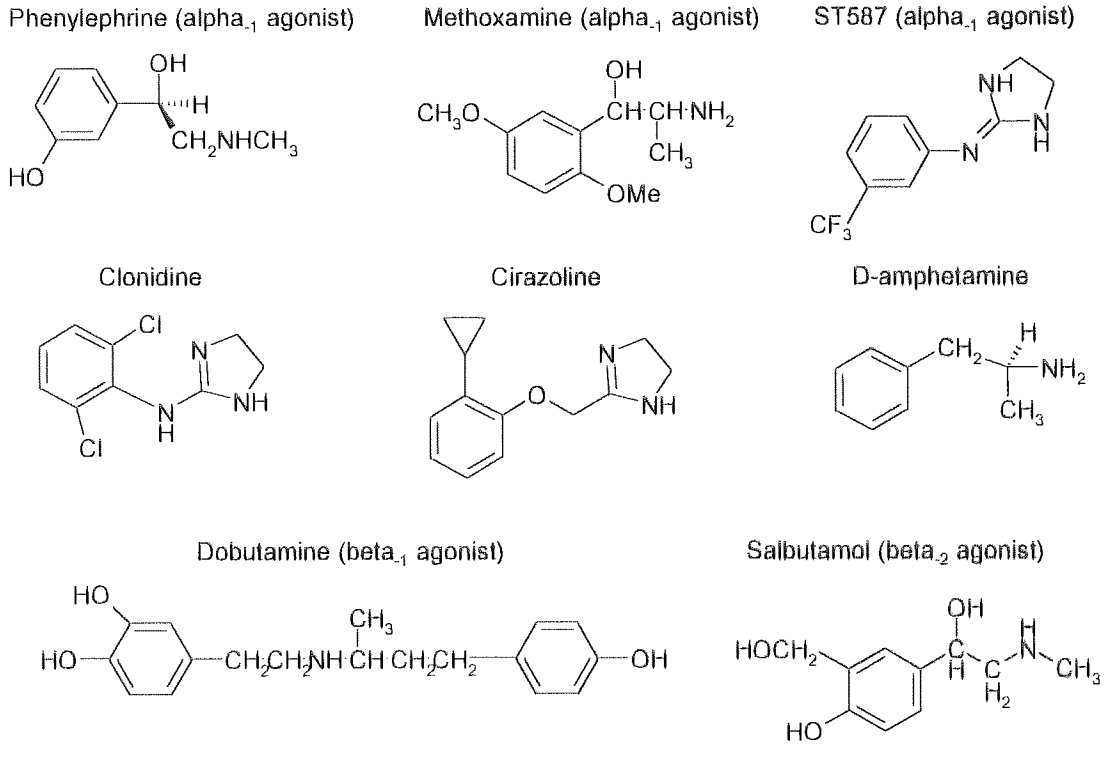


Figure 5.1). ***Chemical structure of noradrenaline associated ligands***

Using the technique of *in vivo* microdialysis it has been shown that the administration of I_2 specific ligands such as 2-BFI, 2-BDI and BU224 significantly increases extraneuronal levels of NA (Jordan, 1993; Lalies and Nutt, 1993; Hudson et al, 1999b). These ligands have low affinity for the α_2-adrenoceptor and hence this increase does not seem to be mediated though the antagonism of the pre-synaptic α_2-autoreceptor (Nutt et al, 1995; Alemany et al, 1997; Hudson et al, 1999b).

Bearing this in mind it is not surprising that drug discrimination studies have shown 2-BFI trained rats recognise the α_2-adrenoceptor antagonists, idazoxan, ethoxy-idazoxan and fluparoxan (Jordan et al, 1996). Hence, in the case of ethoxy-idazoxan and fluparoxan, these animals recognise the increase in NA associated purely with antagonism of the α_2-adrenoceptor.

Whilst, after the administration of idazoxan or 2-BFI these animals recognise the increase in NA resulting from I_2 ligand binding. Conversely previous studies have indicated that 2-BFI trained rats fail to recognise the α_2-adrenoceptor agonist clonidine. As previously mentioned the α_2-adrenoceptor acts as a presynaptic autoreceptor and clonidine binding will reduce NA release (Langer, 1981).

The drug discrimination studies mentioned above indicate that 2-BFI trained rats recognise drugs that increase extraneuronal levels of NA. However, it does not tell us what, if any, subtype of adrenoceptor this NA is released onto. We can be certain that NA release onto the α_2 subtype does not contribute to the cue as the α_2-adrenoceptor agonist clonidine fails to substitute for 2-BFI (Jordan et al, 1996).

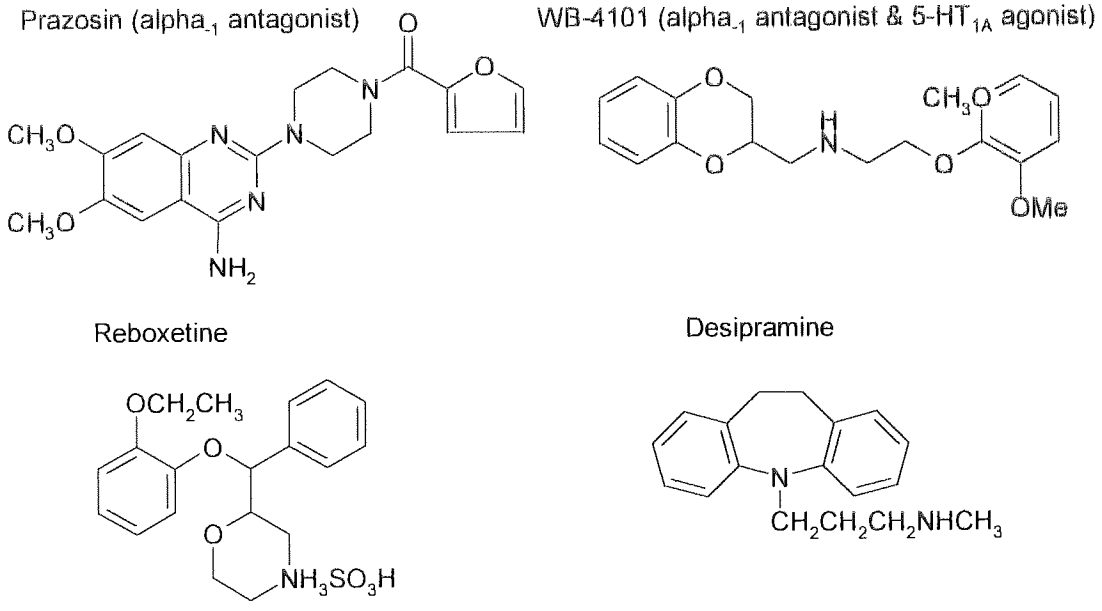


Figure 5.2). *Chemical structure of α_1-adrenoceptor antagonists and re-uptake inhibitors*

Hence the current set of experiments sought to administer adrenergic ligands and explore their ability to elicit 2-BFI appropriate responding. The first series of experiments administered a number of noradrenaline reuptake inhibitors and the sympathomimetic amphetamine, so as to verify the noradrenergic nature of the 2-BFI cue. In the second series of experiments specific α_1, β_1 and β_2 agonists (see figure 5.1) were administered to see if

they elicit 2-BFI appropriate responding. Depending on these results a final series of experiments attempted to antagonise the 2-BFI cue with noradrenergic antagonists, see figure 5.2.

2). Method

2.1). Animals

All animals were trained following the drug discrimination schedule outlined in the method (Chapter 2).

Cirazoline was tested in drug discrimination (DD) group two (see Chapter 2 for training details). Salbutamol, dobutamine, phenylephrine and clonidine were tested in DD group three. WB4101 and prazosin were tested in group four. ST587 and Methoxamine were tested in DD group five. D-amphetamine, desipramine, L659,066 and reboxetine were tested in group six. All drugs were dissolved in 0.9% physiological saline except for reboxetine and WB4101, which were dissolved in deionised water (so as to enable larger quantities of the drug to be dissolved). All doses of a single drug and associated saline day were given in a pseudo random order.

3). Results

3.1). Levels of Substitution

Table 5.1 and figure 5.3 shows the mean percentage substitution rates elicited by 2-BFI trained rats after the administration of various noradrenaline associated ligands.

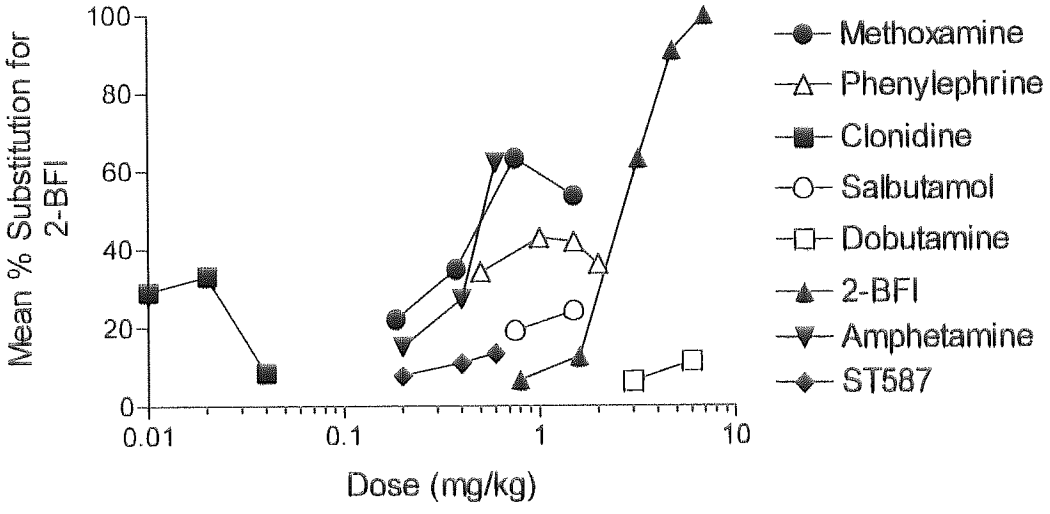
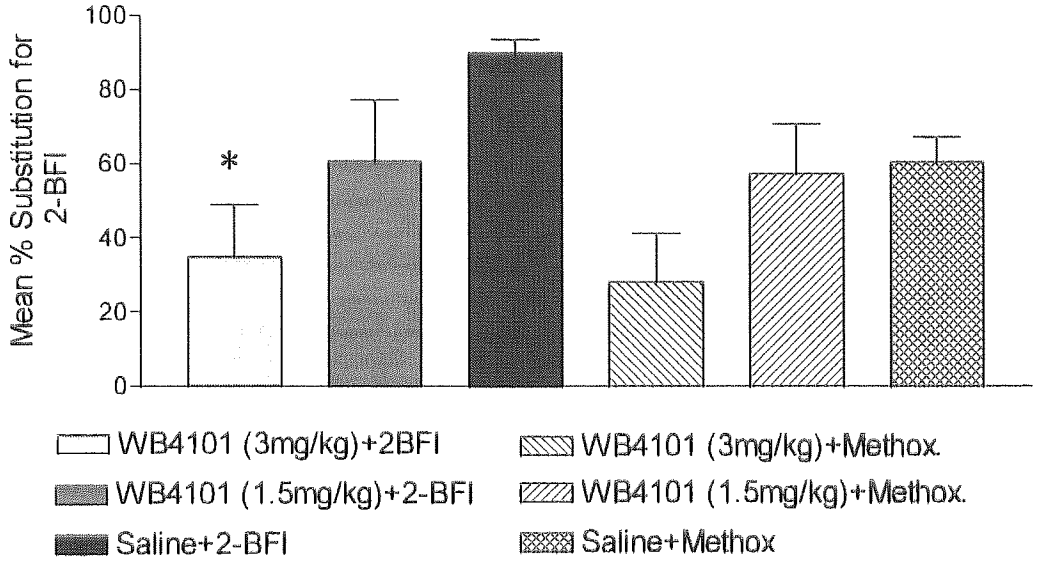


Figure 5.3). ***Ability of various noradrenergic ligands to substitute for 2-BFI***

The administration of the α_1-adrenoceptor agonist methoxamine at one dose gave rise to significantly ($p<0.05$) elevated levels of 2-BFI appropriate responding in comparison to test days in which saline had been administered (see table 5.1). All other doses of methoxamine (0.185, 0.375, 1.5 mg/kg) and phenylephrine (0.5, 1, 1.5, 2mg/kg) failed to significantly substitute for 2-BFI, see figure 5.3 and table 5.1. The α_1-adrenoceptor agonist and imidazoline ST 587 failed to substitute for 2-BFI, see figure 5.3. We administered the I_1 ligand and α_2-adrenoceptor agonist clonidine which similarly failed to elicit significant levels of 2-BFI appropriate responding (see table 5.1). However, the I_1, I_2 and α_2-adrenoceptor agonist cirazoline, at the only dose tested, showed significant levels of 2-BFI appropriate responding ($p<0.05$; see table 5.1). Administration of the sympathomimetic d-amphetamine gave rise to significant levels of substitution at the highest dose tested (0.6mg/kg; $p<0.05$; see table 5.1). The peripheral α_2-antagonist

L699,066 elicited, non significant, levels of substitution similar to those after the administration of saline, see table 5.3.

Figure 5.4). ***Ability of various noradrenergic ligands to antagonise the 2-BFI cue***



*p<0.05 significant difference between saline + 2-BFI and antagonist + 2-BFI

Figure 5.5). ***Ability of various noradrenergic ligands to antagonise the 2-BFI cue***

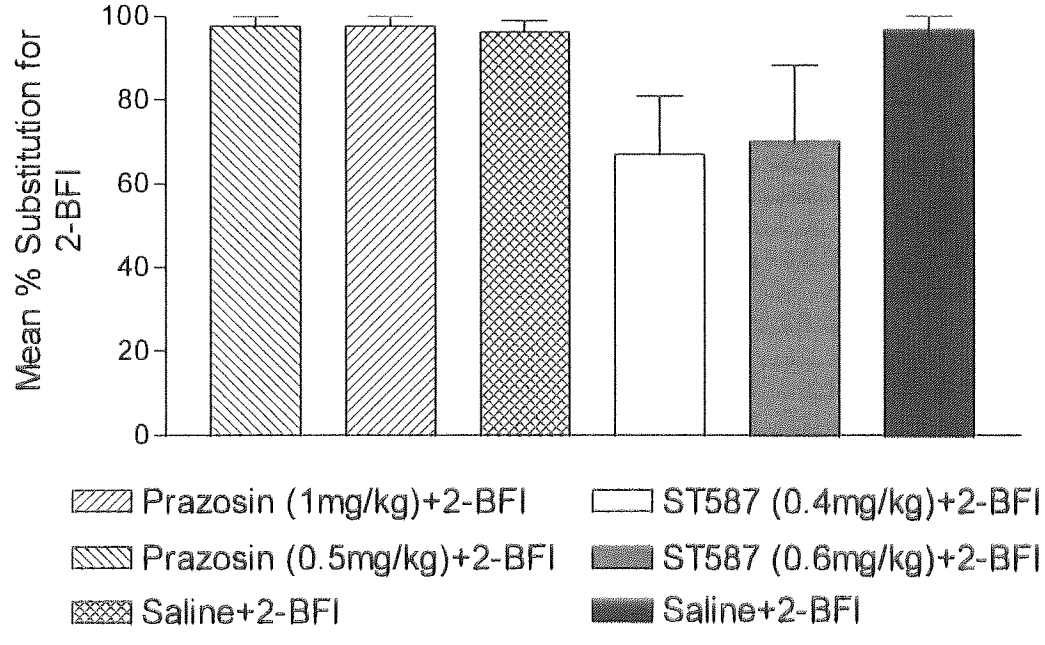


Table 5.1). ***Ability of various noradrenergic ligands to substitute for 2-BFI***

Test Drug	Dose	% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
Phenylephrine	0.50	15.9 ± 13.6	ns.	8/8	6.8 ± 2.1	ns.
Phenylephrine	1.00	43.1 ± 10.6	ns.	8/8	1.2 ± 0.8	p<0.05
Phenylephrine	1.50	42.1 ± 11.8	ns.	8/8	1.2 ± 0.6	p<0.05
Phenylephrine	2.00	36.4 ± 12.4	ns.	8/8	6.5 ± 3.9	ns.
Saline Control		6.8 ± 3.6		8/8	13.0 ± 4.2	
Methoxamine	0.185	22.3 ± 12.4	ns.	7/7	7.6 ± 2.0	ns.
Methoxamine	0.375	35.1 ± 12.3	ns.	8/8	5.7 ± 1.4	ns.
Methoxamine	0.75	63.5 ± 16.1	p<0.05	8/8	2.8 ± 0.4	ns.
Methoxamine	1.5	53.9 ± 14.8	ns.	7/7	1.6 ± 0.7	ns.
Saline Control		6.2 ± 2.4		8/8	4.7 ± 1.2	
ST587	0.2	7.7 ± 1.9	ns.	8/8	2.8 ± 0.8	ns.
ST587	0.4	11.0 ± 4.3	ns.	8/7	5.6 ± 1.7	ns.
ST587	0.6	13.5 ± 5.2	ns.	8/6	1.3 ± 0.3	p<0.05
Saline Control		7.1 ± 3.1		8/8	6.5 ± 1.0	
Dobutamine	3	6.3 ± 4.3	ns.	8/8	7.4 ± 1.2	ns.
Dobutamine	6	11.4 ± 8.1	ns.	8/8	5.8 ± 0.9	ns.
Saline Control		1.1 ± 1.1		8/8	7.3 ± 1.6	
Salbutamol	0.75	19.3 ± 8.9	ns.	8/8	2.6 ± 1.5	ns.
Salbutamol	1.5	24.3 ± 13.2	ns.	8/8	3.2 ± 1.0	ns.
Saline Control		12.0 ± 5.4		8/8	4.3 ± 1.0	
Clonidine	0.01	29.2 ± 16.1	ns.	8/6	2.66 ± 1.1	ns.
Clonidine	0.02	33.3 ± 33.3	ns.	8/3	5.04 ± 2.5	ns.
Clonidine	0.04	8.5 ± 4.9	ns.	8/2	2.17 ± 0.9	ns.
Saline Control		1.1 ± 1.1		8/8	7.3 ± 1.6	
Cirazoline	0.08	37.5 ± 11.9	p<0.05	8/8	5.5 ± 2.8	ns.
Saline Control		9.0 ± 3.7		8/8	10.3 ± 3.4	
D-amphetamine	0.2	15.3 ± 4.4	ns.	7/7	3.1 ± 0.9	ns.
D-amphetamine	0.4	27.5 ± 9.7	ns.	7/7	4.0 ± 1.7	ns.
D-amphetamine	0.6	62.5 ± 13.1	p<0.05	7/7	1.7 ±1.1	ns.
Saline Control		1.3 ± 1.3			3.9 ± 1.0	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug responses per minute.

Table 5.2). *Ability of various noradrenergic ligands to substitute for 2-BFI*

Test Drug	Dose	% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
WB4101	1.5	1.2 ± 1.2	ns.	8/7	3.2 ± 0.7	ns.
WB4101	3	11.2 ± 4.6	ns.	8/8	2.9 ± 0.8	ns.
Saline Control		6.1 ± 3.2		8/8	5.0 ± 1.7	
WB4101 + 2-BFI (7mg/kg)	1.5	60.7 ± 16.5	ns.	8/7	4.0 ± 1.8	ns.
WB4101 + 2-BFI (7mg/kg)	3.0	34.9 ± 14.0	p<0.05	8/7	5.6 ± 2.4	ns.
Saline + 2-BFI (7mg/kg)		89.9 ± 3.5		8/8	4.4 ± 1.6	
WB4101 + Methoxamine (0.75mg/kg)	1.5	57.2 ± 13.5	ns.	8/8	2.3 ± 0.8	ns.
WB4101 + Methoxamine (0.75mg/kg)	3.0	28.0 ± 13.1	ns.	8/8	3.6 ±0.7	ns.
Saline + Methoxamine (0.75mg/kg)		60.4 ± 6.7	ns.	8/8	3.2 ± 1.4	
Prazosin	0.5	11.8 ± 3.4	ns.	7/7	2.7 ± 0.8	ns.
Prazosin	1	17.1 ± 2.8	ns.	7/7	2.8 ± 0.7	ns.
Saline Control		7.0 ± 3.6			3.2 ± 0.6	
Prazosin + 2-BFI (7mg/kg)	0.5	96.2 ± 2.5	ns.	7/7	3.1 ± 1.6	ns.
Prazosin + 2-BFI (7mg/kg)	1	97.6 ± 2.3	ns.	7/7	4.7 ± 1.9	ns.
Saline ± 2-BFI (7mg/kg)		97.5 ± 2.4		7/7	6.4 ± 1.9	
ST587 + 2-BFI (7mg/kg)	0.4	67.0 ± 13.8	ns.	7/7	1.61 ± 0.58	p<0.05
ST587+ 2-BFI (7mg/kg)	0.6	70.1 ± 18.2	ns.	7/7	4.85 ± 0.90	ns.
Saline + 2-BFI (7mg/kg)		96.7± 3.3			7.79 ± 1.09	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug responses per minute.

Table 5.3). *Ability of various noradrenergic ligands to substitute for 2-BFI*

Test Drug	Dose	% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
L699, 066	8	13.4 ± 2.5	ns.	7/7	2.40 ± 0.55	ns.
Saline		12.5 ± 4.0		7/7	2.68 ± 0.51	ns.
Reboxetine	1	58.6 ± 12.2	p<0.05	8/8	2.6 ± 0.7	ns.
Reboxetine	5	11.6 ± 3.9	ns.	8/8	2.3 ± 0.9	ns.
Reboxetine	7.5	12.5 ± 4.0	ns.	8/4	3.3 ± 1.7	ns.
Saline		10.0 ± 8.0		8/8	5.3 ± 1.2	
Desipramine	2.5	54.2 ± 14.0	p<0.05	8/8	3.9 ± 1.0	ns.
Desipramine	5	39.3 ± 16.2	ns.	8/2	4.0 ± 0.2	ns.
Desipramine	10	0.0 ± 0.0	ns.	8/2	4.6 ± 2.8	ns.
Saline		4.8 ± 3.1		7/7	3.8 ± 0.9	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug response time.

Due to the ability of the α_1-adrenoceptor agonist methoxamine to show significant levels of 2-BFI appropriate responding we attempted to antagonise the 2-BFI cue by prior administration of specific α_1-adrenoceptor antagonists. When given alone the specific α_1-adrenoceptor antagonist WB 4101 failed to induce 2-BFI appropriate responding, see table 5.2. However, at the highest dose tested (3mg/kg) WB 4101, when administered 20 minutes prior to 2-BFI, significantly reduced levels of 2-BFI appropriate responding when compared with control days, i.e, test days in which saline was given prior to 2-BFI ($p<0.05$), see table 5.2 and figure 5.4. In a further experiment the ability of WB4101 to antagonise the significant levels of 2-BFI appropriate responding apparent after the administration of 0.75 mg/kg methoxamine was tested. However, although prior administration of WB4101 appeared to reduce levels of methoxamine induced responding, they were non-significant, see table 5.2 and figure 5.4. The administration of the α_1-adrenoceptor antagonist prazosin failed to reduce 2-BFI appropriate responding, see table 5.2. and figure 5.5.

The failure of the α_1-adrenoceptor agonist and imidazoline ST587 to show any appreciable levels of substitution for 2-BFI was surprising. Consequently ST587 was administered as an antagonist, i.e., twenty minutes before 2-BFI. However, ST587 failed to significantly reduce levels of 2-BFI appropriate responding, see table 5.2 and figure 5.5.

Administration of the selective noradrenaline reuptake inhibitor reboxetine at 1 mg/kg ($p<0.05$) and desipramine at 2.5 mg/kg ($p<0.05$) gave rise to significant levels of 2-BFI appropriate responding, see table 5.3.

3.2). Responses per minute (RPM)

Administration of the α_1-adrenoceptor agonists ST587, methoxamine and phenylephrine markedly reduced the average number of responses per minute (RPM). This reduction was significantly different from control test sessions, in which saline was administered; at 1 mg/kg phenylephrine ($p<0.05$), 1.5 mg/kg ($p<0.05$) phenylephrine and 0.6 mg/kg ST587 ($p<0.05$), see table 5.1. The administration of the β_1- and β_2– adrenoceptor agonists dobutamine and salbutamol failed to significantly reduce the RPM. The α_2-agonist clonidine failed to significantly reduce the RPM, but did have a marked sedative effect i.e., only two out of the eight rats completed the session, i.e., press either lever ten times over the thirty minute test period. see table 5.1. The administration of another α_2-adrenoceptor agonist cirazoline and the sympathomimetic d-amphetamine failed to significantly reduce the RPM. Non of the α_1-adrenocetor antagonists administered, i.e., WB4101 and prazosin, significantly reduced the RPM, see table 5.2. Consistent with its ability to reduce the RPM when given alone, ST587 given as an antagonist to 2-BFI significantly reduced the RPM at 0.4mg/kg ($p<0.05$). Administration of the selective noradrenaline reuptake inhibitor desipramine failed to alter the RPM. However, administration of the two higher doses, 5 and 10 mg/kg, had a marked effect on the ability of 2-BFI trained rats to press levers as six out of the eight rats failed to finish the session. Reboxetine had a similar effect on behaviour at the highest dose tested, i.e., four out of eight rats failed to finish the session.

4). Discussion

The aim of this study was to probe the psychopharmacology of 2-BFI by administering specific agonists and antagonists for each subtype of adrenoceptor. The results from this study suggest that the selective release of NA onto α_1-adrenoceptors contributes to the 2-BFI cue. Initially the peripheral α_2-antagonist L659,066 was administered to verify the psychopharmacological nature of the cue. L659,066 is an α_2-adrenoceptor antagonist that does not cross the blood brain barrier (Clineschmidt et al, 1988). Hence, if administration elicited saline lever pressing this would indicate these 2-BFI trained rats do not recognise drugs that associate with an increase of NA in the periphery, only those that are associated with an increase in the brain. Consistent with previous work that has indicated that L659,066 (8mg/kg) blocks α_2-adrenoceptors in the periphery but fails to substitute in ethoxy-idazoxan trained rats (Jordan et al, 1995; Jackson et al, 1991). Our 2-BFI trained rats failed to recognise the same dose. Consistent with there being a central noradrenergic component within the 2-BFI cue the specific NA reuptake inhibitors reboxetine (Benedetti et al, 1995) and desipramine (Checkley et al, 1981) significantly substituted for the cue. However, reboxetine and desipramine only showed significant levels of 2-BFI appropriate responding at the lowest doses administered, 1 and 2.5mg/kg. Time constraints made it impossible to administer lower doses and complete a dose response curve. The administration of the α_1-adrenoceptor agonist methoxamine (0.75mg/kg) gave rise to significant levels of 2-BFI appropriate responding. The α_1-adrenoceptor agonist phenylephrine (Wikberg et al, 1978) did elevate levels of 2-BFI appropriate responding but these did not reach significance. These data suggest the activation of α_1-adrenoceptors contributes to the 2-BFI cue.

The 2-BFI cue was significantly antagonised by prior administration of the selective α_1-antagonist WB4101 (Hayes et al, 1986). However, although WB4101 appeared to reduce levels of methoxamine 2-BFI lever pressing these decreases were non-significant. The selective α_1-adrenoceptor agonist ST587 failed to elicit levels of 2-BFI appropriate responding above

that of saline. We found this surprising as ST587 is an established α_1-adrenoceptor agonist and contains an imidazolidine moiety (De Jonge et al, 1981). We were unable to administer doses of ST587 above 0.6mg/kg as at this dose the RPM was significantly reduced, see table 5.1. Hence if we had administered higher doses this would have sedated animals to the extent they would fail to press levers. In the rat, the literature indicates 0.1mg/kg ST587 is psychopharmacologically active in modulating radial arm foraging (Pussinen and Sirvio, 1999). However, *in vivo* studies which assess α_1-adrenoceptor induced antinoception report doses of 1mg/kg are only moderately effective in reducing rat tolerance to increasing paw pressure. Doses of 3mg/kg are needed to induce substantial increases (Hayes et al, 1986). Hence, it is possible that the doses of ST587 were too low to have any significant adrenergic effect in 2-BFI trained rats. In the light of ST587's failure to elicit 2-BFI appropriate responding and the presence of an imidiazolindine moiety of its central ring structure it was felt appropriate to administer it as an antagonist. ST587 did not significantly reduce levels of 2-BFI appropriate responding at either dose administered.

In the light of WB40101's ability to antagonise the 2-BFI cue it is interesting that prazosin failed to. Prazosin is a selective α_1-adrenoceptor antagonist and as such should antagonise the 2-BFI cue if it is mediated by α_1-adrenoceptors (Cambridge et al, 1977). However, as mentioned above the data presented here suggests the 2-BFI cue is associated with the activation of α_1-adrenoceptors as the antagonism of methoxamine by WB4101 is an established method of assessing α_1-adrenergic effects *in vivo* (Hayes et al, 1986). Previous studies in rabbit brain hippocampal slices have indicated that prazosin fails to antagonise phenylephrine induced reductions in [^{3}H]noradrenaline efflux (Jackisch et al, 1984). Hence our result does not set a precedent. Previous drug discrimination studies have utilised between 0.1 and 10 mg/kg prazosin (Hughes et al, 1996; Arnt 1996). We administered 0.5 and 1 mg/kg. The literature indicates that 0.1-5mg/kg prazosin is ineffective at antagonising a 1mg/kg amphetamine cue in DD trained rats (Arnt 1996). 2-BFI trained rats recognise 0.6mg/kg amphetamine and

similarly to amphetamine trained rats do not recognise prazosin (Arnt 1996). Hence, the noradrenergic component associated with 2-BFI and amphetamine may be distinct to that associated with prazosin. Prazosin is a specific postsynaptic α_1-adrenoceptor antagonist (Cambridge et al, 1977). A variety of methods have indicted that α_1-adrenoceptors can separated into three classes, α_{1a}-, α_{1b} and α_{1d}-adrenoceptor subtypes (Piascik et al, 1996; Sirvio and MacDonald, 1999). Studies with [^{3}H]prazosin and [^{3}H]WB4101 have indicated these tritiated ligands can discriminate between the α_{1A}- and α_{1B}-subtypes (Morrow et al, 1985). Prazosin and noradrenaline have a similar affinity for both α_{1A}- and α_{1B}-adrenoceptor subtypes. However, WB 4101 is approximately 30 fold more potent for the α_{1A} subtype and each subtype exhibits a high degree of regional specificity within the brain (Morrow et al, 1985; Morrow and Creese, 1986; Blendy et al, 1991; Grimm et al, 1992). Further *in vivo* work has indicated that phenylephrine fails to cause a pressor response after treatment with SZL-49, a chemically reactive analogue of prazosin that has a reported ability to irreversibly reduce α_1-adrenergic populations by a maximum of 60% (Piascik et al, 1989). Such work indicates that phenylephrine is insensitive to this prazosin associated site. Hence, it is therefore possible that 2-BFI-induced NA release is selectively onto the α_{1A}-adrenoceptor subtype and the doses of WB4101 administered were sufficient, but the doses of prazosin were insufficient, to block this site. However WB4101 does have an affinity for the 5-HT$_{1A}$ receptor as an antagonist (Norman et al, 1985). In Chapter 7 we show that 2-BFI trained rats recognise fenfluramine, a potent 5-HT releaser. Hence, it could be suggested that WB4101 is in fact antagonising 5-HT acting at the 5-HT$_{1A}$ receptor. After an extensive literature search only one reference was found that indicated methoxamine and phenylephrine do not have an affinity for other receptors (Sirvio and MacDonald, 1999). However, this was a meta-analysis and did not cite any quantitative data for either compound. In a bid to clarify this situation antagonism of the 2-BFI cue was attempted by administration of the specific 5-HT$_{1A}$ antagonist WAY 100,635, see Chapter 7 (Fletcher et al, 1993). At 0.8 mg/kg, a dose far in excess of that needed to

antagonise the 5-HT_{1A} agonist 8-OH-DPAT (Kleven and Koek, 1998), all animals responded to the 2-BFI lever. Hence, this data suggests the ability of WB4101 to antagonise 2-BFI is through α_1-adrenoceptors not 5-HT_{1A} sites.

2-BFI-induced release of NA seems to be selective as the β_1 and β_2 agonists dobutamine and salbutamol failed to significantly substitute for 2-BFI (Sonnenblick et al, 1979; McElroy and O'Donnell, 1988). Previous drug discrimination studies have indicated doses between 0.1 and 1mg/kg of salbutamol are psychopharmacologically active in drug discrimination (Hughes et al, 1996; McElroy & O'Donnell, 1988). The highest dose we administered was 1.5mg/kg. Similarly, 3mg/kg dobutamine has also been shown to be psychopharmacologically active (Handley and Singh, 1986). Again we administered considerably more (6mg/kg) without any notable increase in 2-BFI appropriate responding. Hence, it is unlikely that any failure for these ligands to substitute is through low dosing.

The α_2-adrenoceptor agonist and I_1 site ligand clonidine (Haeusler, 1973; Bousquet et al, 1984) showed non significant low levels of substitution for the 2-BFI cue. The highest level of 2-BFI appropriate responding occurred at the lowest dose tested. This was probably due to clondine's marked ability to sedate 2-BFI trained rats. At highest dose, only six out of the eight rats failed to complete the session. It is likely that any imidazoline effects at these doses (clonidine does have a moderate affinity for the [^{3}H]2-BFI I_2 site - 207 nM; Alemany et al, 1997) would have been masked by the overwhelming α_2-adrenoceptor agonistic effects of the drug. Cirazoline is an α_2-adrenergic agonist and as such reduces extraneuronal levels of NA (Miralles et al, 1993). However, cirazoline exhibited low, but significant levels of 2-BFI appropriate responding. This is not surprising as cirazoline, dose have an high affinity for [^{3}H]2-BFI I_2 Sites (5.3 nM; Alemany et al, 1997). We were unable to administer higher doses as the one dose administered 0.08 mg/kg reduced the RPM, i.e., higher doses would have sedated the animals to the extent they would fail to finish test sessions. This was also the case for d-amphetamine. At the highest dose administered, 0.6 mg/kg, amphetamine

significantly substituted for 2-BFI. However, this was not full substitution (<80% of the animals responses were to the 2-BFI lever). It is possible that amphetamine, due to its ability release NA, would have substituted further. However, as was the case with cirazoline we could not administer higher dose because this would have reduced the RPM.

The results from this study are consistent with 2-BFI increasing levels of NA within the synaptic cleft. The administration of selective agonists from each major subclass of noradrenergic receptor indicated this increase may selectively activate α_1, but not β_1 and β_2 adrenoceptors.

Chapter 6). Drug Discrimination: Monoamine Oxidase Inhibitors 117

Chapter 6

Drug Discrimination: Monoamine Oxidase Inhibitors

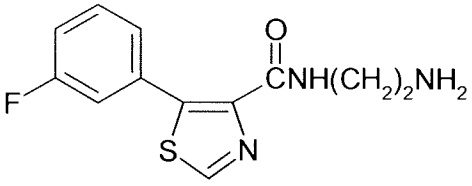
1). Introduction

I_2 sites have been repeatedly associated with the enzyme MAO [MAO; amine: oxygen oxidoreductase (deaminating, flavin containing); EC 1.4.3.4]. *In vitro* studies have indicated I_2 ligands inhibit MAO reversibly with a potency similar to that of the clinically effective antidepressant moclobemide (Brown et al, 1995; Carpene et al, 1995, Ozaita et al, 1997; Gargalidis-Moudanous et al, 1997: Sambunaris et al, 1997; Lalies et al, 1999; Anderson et al, 2000). Recently, a number of inhibitors selective for MAO-A or MAO-B have become available. Hence, the current study sought to examine the ability of various MAO-A and MAO-B selective inhibitors to substitute for 2-BFI.

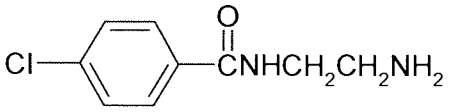
Monoamine oxidase catalyses the oxidative deamination of, serotonin, the catecholamine neurotransmitters and dietary amines (Von Korff, 1979). It has been convincingly established that MAO can be separated into two distinct isoforms - MAO-A and MAO-B (Johnson, 1968; Knoll & Magyar, 1972; Saura et al 1992; Bach et al, 1988; Luque et al, 1995). *In vitro*, MAO-A preferentially oxidises 5-HT (5-hydroxytryptamine, serotonin) and is inactivated irreversibly by clorgyline and reversibly by moclobemide and RO41-1049 (Johnson, 1968; Burkward et al, 1989; Cesura et al, 1989a; see figure 6.1). MAO-B shows a preference to benzylamine, β-phenylethylamine and is irreversibly inhibited by l-deprenyl, pargyline and reversibly by

lazabemide (Ro19-6327) and Ro 16-6491 (Knoll & Magyar, 1972; Cesura et al, 1987; Cesura et al, 1989b; see figure 6.1). Dopamine and noradrenaline have equal selectivity to the catalytic activity of both isoforms and drugs such as phenelzine and tranylcypromine are non-specific in their ability to inhibit MAO-A and -B (Rivett et al, 1982). However, *in vivo*, due to the regional localisation of MAO-A and –B, preference is defined by substrate availability (Westlund et al, 1988; Fagervall & Ross, 1986; Butcher et al, 1986; Luque et al 1995).

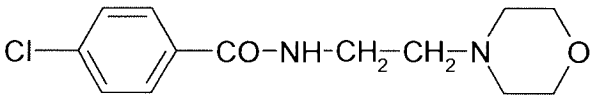
Ro 41-1049 (MAO-A)



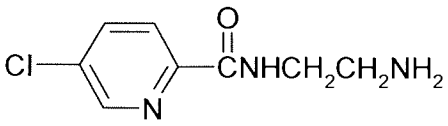
Ro 16-6491 (MAO-B)



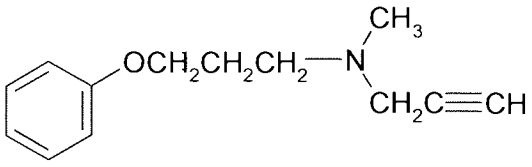
Moclobemide (MAO-A)



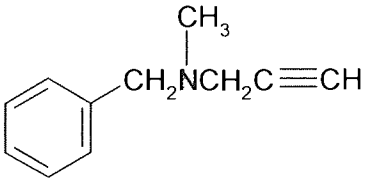
Ro 19-6327 (MAO-B)



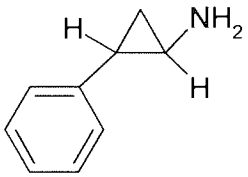
Clorgyline (MAO-A)



Pargyline (MAO-B)



Tranylcypromine (MAO-A+B)



R(-)-Deprenyl

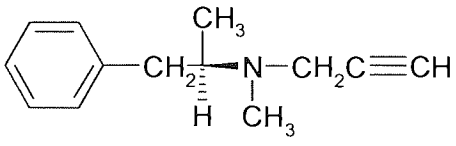


Figure 6.1). *Chemical structures of monoamine oxidase inhibitors*

A number of studies have indicated that I_2 ligands inhibit MAO in a reversible manner (Brown et al, 1995; Carpene et al, 1995, Ozaita et al, 1997; Gargalidis-Moudanous et al, 1997; Lalies et al, 1999). *In vitro*, the specific I_2 ligand 2-BFI and its analogues BU224 and BU226 show a slight preference for MAO-A over -B and inhibit MAO with a similar potency to the reversible MAO-A inhibitor moclobemide, see table 6.1 (Lalies et al, 1999). The imidazole moiety containing compounds, LSL 60101 and LSL 61122, show a preference for MAO-B over MAO-A and inhibit with moderate potency when compared with deprenyl or Ro 16-6491 (Ozaita et al, 1997). However, the relationship between MAO and I_2 sites is far from clear. Although I_2 ligands inhibit MAO with moderate potency, MAO inhibitors have a very low affinity for the I_2 site (Olmos et al, 1993; Alemany et al, 1995a). Hence, it has been proposed that the I_2 site represents a new regulatory site on the enzyme (Raddatz et al, 1999).

Table 6.1). *Ability of various compounds to inhibit MAO*

Drugs	[C^{14}] 5-HT MAO-A IC_{50} μM	[C^{14}] PEA MAO-B IC_{50} μM
2-BFI	11	23
BU226	2.9	16.9
BU224	4.8	44.8
BU216	36.1	88.9
BU239	33.5	102.8
LSL 60101	58	16
LSL 61122	100	32
Moclobemide	36	>1,000
Ro 41-1049	0.13	31
Ro 16-6491	85	0.12

Source: Ozaita et al, 1997; Lalies et al, 1999

Consistent with this theory previous drug discrimination work has indicated the administration of the reversible MAO-A inhibitor moclobemide gives rise to dose dependent, significant, levels of 2-BFI appropriate responding (Jordan et al, 1996). Similarly the administration of a single high (30mg/kg)

dose of pargyline gives rise to full substitution, i.e, over 80% of the animals lever presses were to the 2-BFI lever (Jordan et al, 1996). Pargyline, and most other MAO inhibitors irreversibly inactivate the enzyme, hence training animals for drug discrimination is impossible. However, Porsolt et al (1984) have trained rats to discriminate 0.6 mg/kg amphetamine from saline and administered various MAO inhibitors. The previous chapter reported data that indicated 2-BFI trained rats significantly recognise amphetamine, at the same dose utilised by Porsolt et al, (1984). In the light of this it is surprising that none of the MAO inhibitors administered, i.e., clorgyline, moclobemide or pargyline, gave rise to significant levels of amphetamine related lever pressing. However, deprenyl did show significant levels of lever pressing (Porsolt et al, 1984).

One of the major problems with drug discrimination is that drugs which irreversibly bind to receptors or enzymes, i.e, deprenyl, cannot be used because they essentially end the test life of the rat. However, recently a number of reversible and selective inhibitors of MAO-A and –B have become available and hence multiple doses can be administered to one group of animals. Moclobemide and RO 41-1049, see figure 6.1, are reversible inhibitors of MAO-A (Da Prada et a, 1989; Cesura et al, 1989a). Moclobemide inhibits MAO-A with a micromolar, and Ro 41-1049 with a nanomolar, potency. Both have a low potency for inhibiting MAO-B. Conversely, the reversible MAO-B inhibitors Ro 16-6491 and Ro 19-6327 (lazabemide) have a nanomolar potency for MAO-B and has a micromolar potency for inhibiting MAO-A (Ozaita et al, 1997; Lalies et al, 1999). Hence the purpose of the current study was to test the ability of MAO inhibitors to substitute for 2-BFI.

2). Method

2.1). Animals

All animals were trained following the drug discrimination schedule outlined in the method (Chapter 2).

The irreversible MAO-B inhibitor deprenyl was administered at a single dose, as the last test dose given to group two. Similarly, the irreversible MAO inhibitors tranylcypromine and clorgyline were administered as the final dose given to rat groups six and seven respectively. Moclobemide and Ro 19-6327 (lazabemide), were administered to group four. RO 41-1049 and Ro 16-6491 were administered to group six.

All drugs were dissolved in deionised water except for lazabemide and moclobemide. Lazabemide was dissolved in 0.9% physiological saline. Moclobemide was suspended in 9% tween 80 (Sigma, UK) dissolved further with 1 M HCl and brought back up to pH 4.5 with 5 M NaOH.

All doses of a single drug and associated saline day were given in a pseudo random order except for the irreversible MAO inhibitors which were administered at one dose which took place in one test session at the end of study of a particular rat group. Control data was drawn from the previous test day in which saline was administered. Deprenyl was administered at 4mg/kg, a dose which has previously significantly substituted in amphetamine trained rats (Porsolt et al, 1984). This was administered with a dosing time of 20 minutes. Clorgyline was administered at 2mg/kg a dose which is thought to be MAO-A specific (M. Lalies, personal communication). The time between dosing and testing was one hour. *In vivo* microdialysis studies have indicated the administration of clorgyline at this dose only increases levels of NA significantly after two hours (Lalies et al, 2000). Hence, animals were tested again one hour after this. Tranylcypromine was administered at 2.5mg/kg with a dosing time of one hour. Previous drug discrimination work has indicated higher doses reduce the RPM (Porsolt, et al, 1984).

3). Results

3.1). Levels of Substitution

Table 6.2 shows the mean percentage of various MAO inhibitors to substitute for 2-BFI. The reversible MAO-A inhibitors Ro41-1049 and moclobemide showed dose dependent levels of 2-BFI appropriate responding (see figure 6.2). These levels were significantly different from saline at every dose administered except for the lowest dose of moclobemide (5mg/kg; see table 6.2). The irreversible MAO inhibitor clorgyline, administered at a single dose and tested at one and two hours after this failed to elicit significant levels of 2-BFI responding, see table 6.2. Administration of the irreversible MAO-B inhibitor deprenyl gave rise to significant levels of 2-BFI appropriate levels of responding ($p<0.05$). However, the administration of the selective reversible MAO-B inhibitor RO16-6491 failed to elicit significant levels of 2-BFI appropriate responding. Lazabemide was administered at five doses but only one of these doses (3mg/kg; $p<0.05$) was significantly different from saline, see table 6.2 and figure 6.2. Tranylcypromine was given at one dose (2.5mg/kg), but had a marked sedative effect, i.e., only one of the eight animals successfully completed the session. Due to the low number of finishers statistical analysis was impossible, see table 6.2.

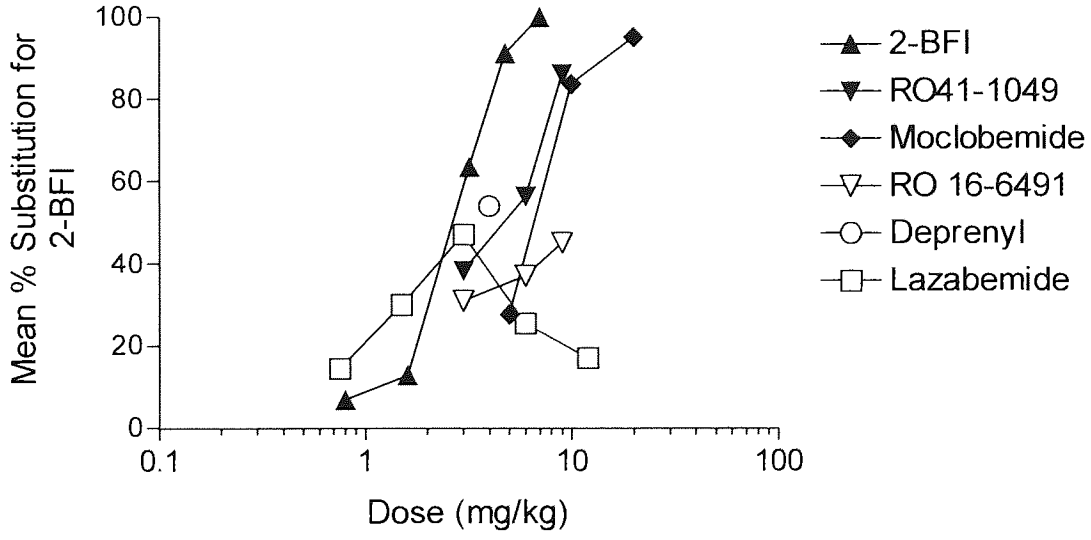


Figure 6.2). ***Ability of various MAO inhibitors to substitute for 2-BFI***

Table 6.2). ***Ability of various MAO inhibitors to substitute for 2-BFI***

Test Drug	Dose	% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
RO41-1049	3	38.1± 16.6	p<0.05	8/8	6.1 ± 1.7	ns.
RO41-1049	6	56.2 ± 9.4	p<0.01	8/8	3.8 ± 0.9	ns.
RO41-1049	9	85.9 ± 6.1	p<0.001	8/8	5.5 ± 1.9	ns.
Saline		2.2 ± 1.4		8/8	4.8 ± 1.0	
Moclobemide	5	27.5 ± 10.2	ns.	7/7	3.6 ± 1.5	ns.
Moclobemide	10	83.6 ± 10.8	p<0.001	7/7	4.7 ± 2.2	ns.
Moclobemide	20	95.0 ± 2.5	p<0.001	7/7	4.3 ± 1.6	ns.
Saline		8.5 ± 3.2		7/7	4.2 ± 1.5	
Clorgyline (1hr)	2	30.4 ± 9.2	ns.	8/8	2.9 ± 0.8	ns.
Clorgyline (2hr)	2	28.5 ± 11.3	ns.	8/8	0.8 ± 0.2	ns.
Saline (2hr)		7.1 ± 3.1			3.3 ± 0.9	
Deprenyl	4	53.8 ± 16.9	p<0.05	8/6	8.1 ± 5.1	ns.
Saline		10.2 ± 4.0		8/8	8.3 ± 2.4	
RO16-6491	3	30.8 ± 13.3	ns.	7/7	3.7 ± 1.2	ns.
RO16-6491	6	37.0 ± 13.3	ns.	7/7	2.0 ± 0.6	ns.
RO16-6491	9	44.9 ± 13.9	ns.	7/7	1.7 ± 0.5	ns.
Saline		5.8 ± 3.2		7/7	2.7 ± 0.5	
Lazabemide	0.75	14.3 ± 5.5	ns.	7/7	3.2 ± 0.8	ns.
Lazabemide	1.5	29.9 ± 4.3	ns.	7/7	3.2 ± 1.7	ns.
Lazabemide	3	47.0 ± 14.3	p<0.05	7/7	4.2 ±1.9	ns.
Lazabemide	6	25.3 ± 10.5	ns.	7/7	7.9 ± 3.7	ns.
Lazabemide	12	16.9 ± 10.9	ns.	7/7	5.4 ± 2.4	ns.
Saline		4.6 ± 3.3		7/7	6.3 ± 2.2	
Tranylcypromine	2.5	100	**	8/1	2.5	**
Saline		9.0 ± 3.7		8/8	4.75 ± 0.70	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug response time.
** number of finishers to low for statistical analysis.

3.2). Responses per minute (RPM)

None of the MAO inhibitors administered significantly reduced the average number of lever presses or responses per minute (RPM) elicited by 2-BFI trained rats, see table 6.2.

4). Discussion

The results from this study indicate that the reversible MAO-A inhibitors RO41-1049 and moclobemide dose dependently substitute for 2-BFI in a manner similar to 2-BFI itself. This effect seems to be specific as administration of the selective reversible MAO-B inhibitor lazabemide only gave rise to significant levels of 2-BFI appropriate responding at one dose, whilst another reversible MAO-B inhibitor (RO16-6491) failed to elicit significant levels of responding at any dose.

In vitro the I_2 specific ligands, 2-BFI, BU224, BU216 and BU226 inhibit MAO-A with a potency similar to that of moclobemide (Lalies et al, 1999). The data presented in Chapter 4 indicates that these ligands dose dependently substitute for 2-BFI, see figure 4.2. In this study we show moclobemide has a very similar dose response curve to these ligands, see figure 4.2 and figure 6.2. Moclobemide inhibits 50 % of MAO-A activity at concentrations of 36 μM (Lalies et al, 1999). Similarly, BU216 inhibits MAO-A to the same extent at 36μM (Lalies et al, 1999). The dose response curves associated with these two compounds are almost identical see figure 4.2 and figure 6.2. BU226, 2-BFI and RO41-1049 are more potent at inhibiting MAO-A than moclobemide and BU 216. Correspondingly, figure 6.3 shows the dose response curves for BU226, 2-BFI and RO41-1049 moving to the left. Indicating these compounds act at the same site but with a higher potency.

The administration of the specific irreversible MAO-B inhibitor deprenyl and one dose of the reversible MAO-B inhibitor lazabemide significantly substituted for 2-BFI. Recent work has indicated deprenyl, in addition to being an irreversible inhibitor of MAO-B, increases extraneuronal levels of NA in a manner not consistent with inhibition of the enzyme. Using the technique of *in vivo* microdialysis, the administration of 2mg/kg deprenyl but not 2mg/kg clorgyline, has been associated with a massive increase in NA overflow, this effect was maximal at 30 minutes with levels of NA peaking at 250% over basal (Lalies et al, 2000). It is unlikely that this increase was through the inhibition of MAO as the administration of clorgyline fails to significantly alter NA increase until two hours after administration and other

studies have indicated time periods of 2hrs are needed before MAO-B site density is markedly reduced (Richards et al, 1998; Lalies et al, 2000). As previously mentioned extraneuronal levels of NA were maximal 30 minutes after dosing. Hence, it is unlikely that this accumulation of NA is as a result of the inhibition of MAO. This would also account for the findings of Porsolt et al (1984). Who show deprenyl but not pargyline, clorgyline or tranylcypromine substitutes in amphetamine trained rats.

Surprisingly clorgyline at 2mg/kg failed to substitute for 2-BFI at one and two hours after dosing. Studies using the technique of *in vivo* microdialysis indicate that NA levels, after the administration of 2mg/kg clorgyline, only significantly increased from basal after two hours (Lalies et al, 2000). This failure is surprising in the light of moclobemide and Ro41-1049 ability to potently substitute for 2-BFI. It is unlikely that the dosing time for clorgyline was too short, but it may be the case that the dose was too low to mimic 2-BFI's psychopharmacological effect. Other data in this chapter indicate the 2-BFI cue associates significantly, although with a much lower potency, with the inhibition of MAO-B. Hence, the low dose administered here may have failed to alter MAO-B activity sufficiently to enable generalisation of clorgyline to 2-BFI.

A wide range of doses of lazabemide were administered and only one moderate dose, 3mg/kg, induced significant levels of 2-BFI appropriate responding. The dose response curve generated is consistent with a compound that has a low potency for the 2-BFI site. At the higher doses lazabemide generated a compound cue, i.e., alters other neurochemical systems which may mask the 2-BFI cue, and thus failed to substitute. However, lazabemide did significantly substitute suggesting that the inhibition of MAO-B does form part of the 2-BFI cue.

Enzyme inhibition studies indicate the imidazole compounds LSL 60101 and LSL 60125 show a slight preference for, and inhibit MAO-B at micromolar concentrations. Correspondingly data within Chapter 4 indicates LSL 60101 and LSL 60125 significantly substitute for 2-BFI, but at a lower potency (see figure 4.3). Similarly, the MAO-B inhibitors tested here have a much lower potency than MAO-A inhibitors in 2-BFI trained rats (see figure 6.2). Hence,

this data suggests that the imidazoline ligands have a closer psychopharmacological relationship to MAO-A inhibitors, whilst imidazole ligands (LSL60101 and LSL601125) share a closer relationship to MAO-B. (see figure 4.3).

The results of this study indicate that the specific MAO-A inhibitors, moclobemide and RO41-1049 show significant dose dependent levels of 2-BFI responding. These levels were very similar to the levels exhibited after administration of BU216, BU226 and 2-BFI itself. This data suggests that inhibition of MAO-A forms a substantial part of the 2-BFI cue. However, the potent reversible MAO-B inhibitor lazabemide, did show significant levels of 2-BFI appropriate responding, suggesting that a part of the 2-BFI cue may be associated with the inhibition of MAO-B.

Chapter 7

Drug Discrimination: β-carbolines

1). Introduction

It has recently been established that the endogenous beta carboline harmane has a high affinity for I_1 and I_2 sites *in vitro* (Moncrieff 1989; Hudson et al, 1999a; Alan Hudson, personal communication). The microinjection of harmane into the rat rostral ventrolateral medulla significantly and with a potency similar to clonidine reduces blood pressure (Musgrave and Badoer, 2000). However, as harmane has a high micromolar affinity for α_2-adrenoceptors it has been suggested by the authors that the hypotension reported here is mediated through I_1-sites (Hudson et al, 1999a; Musgrave and Badoer, 2000).

Table 7.1). *Affinity of β-carbolines for I_1 sites, I_2 sites and α_2-adrenoceptors*

	IC_{50} (nM)	K_i (nM)	K_i (μM)
Compound	I_1	I_2	α_2-adreno
Harmane	30.8	49.4	17.6
Norharmane	637.1	10.2	31.6
Harmine	629.2	10.2	21.3
Harmaline	13,800	22.1	21.2

Data reproduced with courtesy of Alan Hudson

Harmane (1-methyl-β-carboline) is a beta carboline indole alkaloid that is derived from a number of natural sources including the Middle Eastern plant *Peganum harmala*, the South American *Banisteriopsis caapi* and the Asian *Kopsia griffithii* (Naranjo 1967; Callaway et al, 1999; Kam et al, 1999). Anecdotal evidence has existed for a number of years that the consumption of beverages containing such compounds facilitates hallucinations and disordered thought (Naranjo 1967; Naranjo 1969; Strassman et al, 1994; Grella et al, 1998).

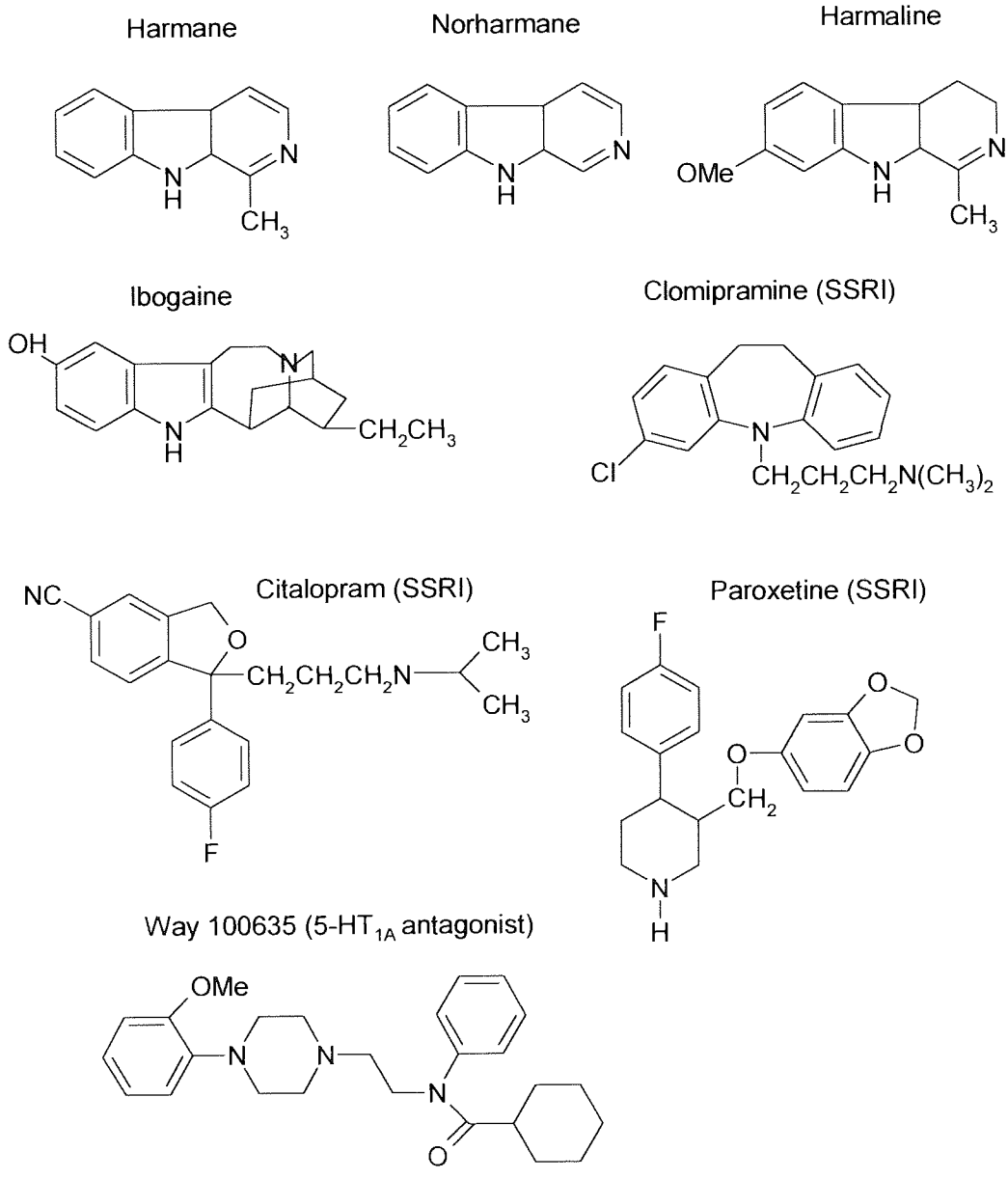


Figure 7.1). *Chemical structure of various β-carbolines and 5-HT ligands*

Behavioural studies have consistently indicated that the administration of harmaline reduces cocaine and morphine self-administration and increases tremor in the rat (Glick et al, 1994; Stanford and Fowler, 1998). *In vivo* microdialysis studies have indicated norharmane, the major metabolite of harmane significantly increases extraneuronal levels of dopamine within the nucleus accumbens (Baum et al, 1995). Pharmacologically speaking the literature does not contain a great deal of information about these compounds. A receptor mechanism has yet to be identified, but it has been firmly established that harmane, norharmane, harmaline and a number of other beta carbolines are potent and selective inhibitors of MAO-A and at high concentrations have an affinity for voltage dependent sodium channels (Buckholtz and Boggan, 1977; Meller et al, 1977; Nelson et al, 1979; Deecher et al, 1992). However drug discrimination studies have drawn a firm link between these compounds and a structurally related alkaloid ibogaine as harmane and harmaline administration gives rise to high levels of drug appropriate lever responding in Ibogaine trained rats (Helsley et al, 1997a; Helsley et al, 1998a).

Ibogaine, similarly to harmane is an alkaloid, which can be derived from the root bark of the African shrub *Tabernanthe iboga* (Evans-Schultes and Hoffman 1980) and has been used as a psychoactive stimulant during the initiation ceremonies of a number of secret societies (Fernandez 1982). Ibogaine has recently been patented, NIH 10567 - Endabuse™, as an anti addictive drug and is the currently the subject of a great deal of interest (Szumlinski et al, 2001).

However, the method by which ibogaine mediates its anti-addictive effect is unclear. Animals models suggest addiction associates with alterations in dopamine transmission within the extrapyrimidal and limbic forebrain structures (Berridge and Robinson, 1999). Some studies have indicated that acute ibogaine treatment blocks cocaine induced dopamine sensitisation within the mesolimbic pathway and behaviourally ibogaine can reduce self-administration of cocaine and morphine (Szumlinski et al, 2000). However, in other animal models of addiction ibogaine potentiates cocaine associated locomotor hyperactivity (Glick et al, 1994; Szumlinski et al, 1999).

The acute administration of ibogaine increases levels of the dopamine associated peptide dynorphin and substance P immuno-reactivity within the striatum and substantia nigra (Alburges and Hanson, 1999; Alburges et al, 2000). Studies utilising the technique of *in vivo* microdialysis have indicated that the administration of ibogaine associates with a decrease in dopamine, but an increase in 5-HT, overflow in the striatum and nucelus accumbens (Glick et al, 1994; Wei et al, 1998). Consistent with these studies ibogaine trained animals recognise the 5-HT_{2A} agonists MK-212 and mCPP and conversely fenfluramine trained rats recognise ibogaine (Schechter et al, 1997; Helsley et al, 1997b; Helsley et al, 1999). However, in spite of these pronounced *in vivo* effects, radiochemical binding studies indicate that ibogaine has a low micromolar affinity for nicotinic acetylcholine receptors, mu opioid receptors, serotonin (5-HT_2 and 5-HT_{1A}), dopamine (D_2), benzodiazepine receptors and unlike the harmala alkaloids does not inhibit MAO, but does have a nanomolar affinity for $sigma_2$ receptors (Nelson et al, 1979; Bowen et al, 1995 Mach et al, 1995; Codd, 1995; Mah et al, 1998; Glennon et al, 2000). Consistent with ibogaine's *in vitro* affinity for $sigma_2$ receptors rats trained with ibogaine recognise the $sigma_2$ ligand SKF 10,047 (Helsley et al, 1998b).

Hence, ibogaine and harmane share a number of similarities to I_2 specific ligands such as 2-BFI and BU224. Harmane and a number of other beta carbolines inhibit MAO-A with a low micromolar potency (Buckholtz and Boggan, 1977; Meller et al, 1977; Nelson et al, 1979; Deecher et al, 1992). More significantly I_2 ligands modulate opioid analgesia a property shared by both harmane and ibogaine (Glick et al, 1994; Stanford and Fowler, 1998). Therefore the current set of studies had three main aims. Firstly to compare the *in vivo* potency of harmane and harmaline with 2-BFI. Secondly, as drug discrimination studies have indicated ibogaine, harmane and harmaline are psychopharmacologically similar, the ability of the anti-addictive drug ibogaine to substitute for 2-BFI was also assessed. Finally, and largely due to ability of β-carbolines to increase the turn over of 5-HT *in vivo,* the potent 5-HT releaser fenfluramine (Rowland and Carlton, 1986) and the SSRI's

citalopram (Pawlowski et al. 1981), paroxetine (Tulloch et al. 1984) and clomipramine (Kinnier et al. 1984) were administered to probe their ability to generalise to 2-BFI.

2). Method

2.1). Animals

All animals were trained following the drug discrimination schedule outlined in the method (Chapter 2). Harmane, norharmane, harmaline, ibogaine and clomipramine were administered to group six. Paroxetine, citalopram, WAY100635, SKF10,047 and fenfluramine were administered to group seven. All drugs were dissolved in 0.9% physiological saline except for SKF10,047, paroxetine, harmane, norharmane, harmaline and ibogaine which were made up in deionised water. All doses of a single drug and associated vehicle day were given in a pseudo random order.

3). Results

3.1). Levels of Substitution

The beta carboline harmane elicited significant levels of 2-BFI responding at 3 ($p<0.05$), 6 ($p<0.001$) and 9 mg/kg ($p<0.001$), see table 7.2 and figure 7.2. Norharmane was slightly less potent but did significantly substitute for 2-BFI at 6 ($p<0.01$) and 9 ($p<0.001$) mg/kg, see table 7.2 and figure 7.2. The structurally related beta carboline harmaline proved to be more potent *in vivo*, as significant levels of 2-BFI appropriate responding occurred after administration of 0.75 ($p<0.05$), 1.5 ($p<0.01$), 3 ($p<0.01$) and 6 mg/kg ($p<0.001$). At the higher doses harmaline and harmane showed full substitution for the 2-BFI cue, i.e, over 80% of the animals first ten responses were to the 2-BFI lever, whilst all other doses showed partial substitution (between 20 and 80% animals first ten responses were to the 2-BFI lever). The alkaloid ibogaine also showed a high potency *in vivo*. All doses administered, 3 ($p<0.001$), 6 ($p<0.001$), 9 mg/kg ($p<0.001$) were significantly different from saline, see table 7.2 and figure 7.2. The $sigma_2$ agonist SKF10,047 failed to significantly substitute for 2-BFI at both of the doses administered, see figure 7.2 and table 7.2.

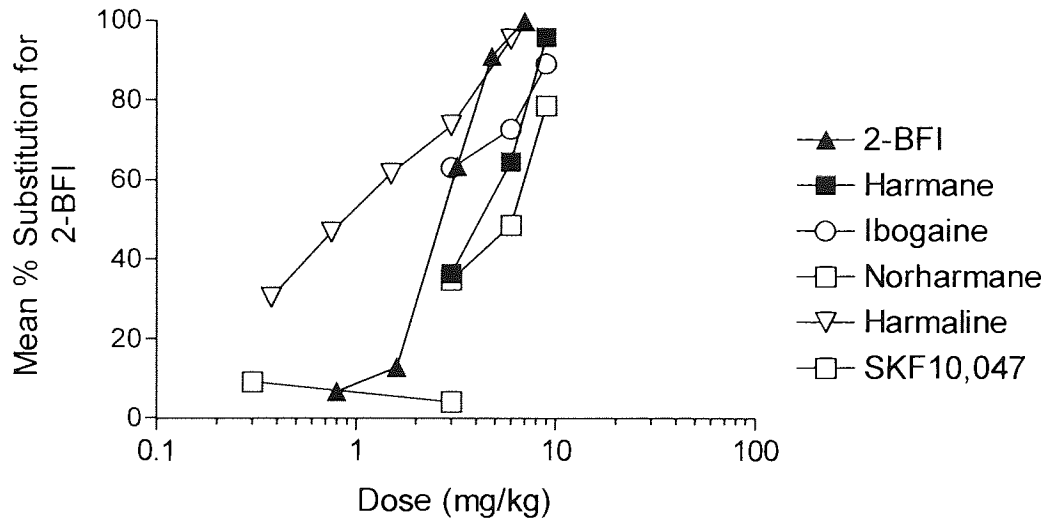


Figure 7.2). ***Ability of various compounds to substitute for 2-BFI***

The potent 5-HT releaser fenfluramine (Rowland and Carlton, 1986) significantly substituted for 2-BFI at the highest dose tested (2mg/kg; $p<0.001$), see table 7.3 and figure 7.3. Consistent with there being a serotonergic component to the 2-BFI cue administration of various SSRI's gave rise to moderate levels of significant 2-BFI lever pressing. Citalopram significantly substituted for 2-BFI at the two highest doses administered 10 ($p<0.01$) and 20 ($p<0.001$) mg/kg, see table 7.3 and figure 7.3. However, paroxetine proved to be less potent and only gave rise to significant levels of 2-BFI substitution at 10 mg/kg ($p<0.05$). The highest dose of paroxetine, 15mg/kg had a marked sedative effect on the rats and hence further testing was impossible. Consistent with the ability of citalopram and paroxetine to substitute for 2-BFI the SSRI clomipramine administered at 15 mg/kg ($p<0.05$) gave rise to significant levels of 2-BFI appropriate responding. Administration of the 5-HT_{1A} antagonist WAY 100,635 (0.8mg/kg) alone failed to significantly substitute for 2-BFI, see table 7.3. WAY 100,635 was also administered as an antagonist (twenty minutes prior to 2-BFI) and failed to reduce levels of 2-BFI appropriate responding.

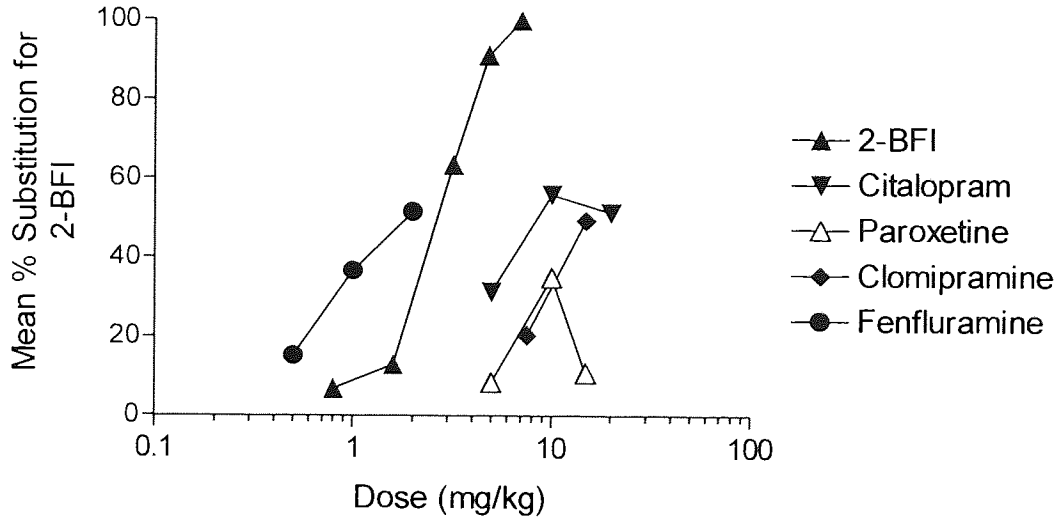


Figure 7.3). ***Ability of various serotonergic compounds to substitute for 2-BFI***

Table 7.2). ***Ability of various ligands to substitute for 2-BFI***

Test Drug	Dose	% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
Harmane	3	36.2 ± 14.0	p<0.05	8/8	4.4 ± 1.0	ns.
Harmane	6	64.3 ± 12.5	p<0.001	8/8	2.8 ± 0.7	ns.
Harmane	9	95.9 ± 4.0	p<0.001	8/7	2.3 ± 0.6	ns.
Saline		0.0		8/8	5.3 ± 0.9	
Norharmane	3	34.7 ± 13.8	ns.	8/8	2.6 ± 1.0	ns.
Norharmane	6	48.6 ± 10.9	p<0.01	8/8	3.2 ± 0.8	ns.
Norharmane	9	78.6 ± 9.2	p<0.001	8/8	1.0 ± 0.2	ns.
Saline		9.0 ± 3.7		8/8	2.5 ± 0.5	
Harmaline	0.375	30.4 ± 11.8	ns.	8/8	3.5 ± 0.9	ns.
Harmaline	0.75	46.8 ± 9.4	p<0.05	8/8	3.0 ± 0.9	ns.
Harmaline	1.5	54.9 ± 12.4	p<0.01	8/8	2.8 ± 0.7	ns.
Harmaline	3	76.8 ± 11.6	p<0.01	8/8	2.3 ± 0.7	ns.
Harmaline	6	95.9 ± 2.9	p<0.001	8/8	1.9 ± 0.5	ns.
Saline		10.3 ± 3.3		8/8	7.1 ± 3.3	
Ibogaine	3	62.9 ± 12.0	p<0.001	8/8	2.7 ± 0.6	ns.
Ibogaine	6	72.7 ± 11.1	p<0.001	8/8	6.0 ± 2.7	ns.
Ibogaine	9	89.0 ± 4.8	p<0.001	8/8	3.8 ± 1.1	ns.
Saline		6.6 ± 2.1		8/8	2.2 ± 0.4	
SKF 10,047	0.3	9.1 ± 3.7	ns.	8/8	2.5 ± 0.9	ns.
SKF 10,047	3	4.1 ± 4.1	ns.	8/5	2.9 ± 0.8	ns.
Saline		11.4 ± 3.4		8/8	6.1 ± 2.6	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug response time.

3.2). Responses per minute (RPM)

None of the beta carbolines tested had any significant effects on the number of responses (RPM) emitted by animals per minute see table 7.2. Non of the SSRI's tested significantly altered the RPM. However, these drugs, at the higher doses did have marked sedative effects, i.e., at 10 and 15mg/kg over half the group failed to finish the session, see table 7.3. However, administration of 2mg/kg, the highest dose of fenfluramine significantly reduced the RPM ($p<0.05$).

Table 7.3). ***Ability of various 5-HT ligands to substitute for 2-BFI***

Test Drug		% substitution for 2-BFI cue	[1]Sig.	n/ N	Responses per minute	[2]Sig.
	mg/kg	*M. ± SEM.*			*M. ± SEM.*	
Citalopram	5	31.2 ± 10.2	ns.	8/8	2.5 ± 0.7	ns.
Citalopram	10	55.9 ± 9.5	p<0.01	8/8	3.6 ± 1.3	ns.
Citalopram	20	51.2 ± 9.3	p<0.01	8/6	1.1 ± 0.4	ns.
Saline		3.3 ± 1.6		8/8	3.7 ± 1.0	
Paroxetine	5	8.3 ± 4.8	ns.	7/4	2.4 ± 0.4	ns.
Paroxetine	10	34.8 ± 6.3	p<0.05	7/2	4.0 ± 0.7	ns.
Paroxetine	15	10.7 ± 6.7	ns.	7/3	4.5 ± 0.8	ns.
Saline		10.2 ± 4.0		7/7	3.0 ± 0.6	
Clomipramine	7.5	20.2 ± 3.7	ns.	8/8	1.1 ± 0.3	ns.
Clomipramine	15	49.2 ± 15.1	p<0.05	8/8	2.6 ± 0.7	ns.
Saline		9.7 ± 2.5		8/8	2.3 ± 0.5	
Fenfluramine	0.5	15.1 ± 6.4	ns.	8/8	3.2 ± 0.5	ns.
Fenfluramine	1	36.6 ± 9.4	ns.	8/8	5.3 ± 2.1	ns.
Fenfluramine	2	51.5 ± 9.9	p<0.05	8/8	1.4 ± 0.2	p<0.05
Saline		12.8 ± 3.8			6.5 ± 0.8	
WAY 100635	0.8	9.0 ± 4.3	ns.	7/7	2.1 ± 0.5	ns.
Saline		10.7 ± 3.3	ns.	7/7	2.3 ± 0.5	
WAY 100635 + 2-BFI	0.8	97.9 ± 2.1	ns.	7/7	4.34 ± 1.16	ns.
Saline + 2-BFI		97.1 ± 2.9		7/7	4.38 ± 0.97	

n/ N: n, number of rats beginning session. N: number of rats completing session.
[1]Sig., significant difference between saline and drug % substitution.
[2]Sig., significant difference between saline and drug responses per minute.

4). Discussion

The aim of the current study was to evaluate the *in vivo* potency of a number of structurally related β-carbolines and ibogaine compared to 2-BFI. Due to the ability of these compounds to turn over levels of 5-HT within the brain (Glick et al, 1994; Wei et al, 1998) further DD studies probed for a possible serotonergic component within the 2-BFI cue. The results from this study indicate that the β-carbolines and Ibogaine potently and dose dependently significantly substitute for 2-BFI. The same is true, albeit at a lower potency for a number of SSRI's and the 5-HT releaser fenfluramine.

It has been firmly established that the β-carbolines harmane, norharmane and harmaline, inhibit MAO with potency similar to that of 2-BFI and its analogues (Buckholtz and Boggan, 1977; Lalies et al, 1999). A 2μM concentration of harmane inhibits 50% of MAO activity and slightly higher concentrations (20μM) are needed for harmane's metabolite noraharmane (Buckholtz and Boggan, 1977). The *in vitro* potency of norharmane to inhibit MAO is very similar to that of BU216 (36.1μM) and moclobemide (36μM) and correspondingly *in vivo* these three compounds give almost identical dose response curves. Hence, the levels of substitution that occur after the administration of, BU216, harmane and norharmane are likely to be through their ability to inhibit MAO. Harmaline has a low nanomolar potency for MAO and consistent with this hypothesis it shows a higher potency in 2-BFI trained rats than even 2-BFI, see figure 7.2 (Buckholtz and Boggan, 1977).

However, ibogaine has a high micromolar affinity for [^{3}H]harmaline sites and does not inhibit MAO (Nelson et al, 1979). Previous studies have indicated that ibogaine has a nanomolar affinity for the sigma$_2$ receptor and drug discrimination studies indicate the selective sigma$_2$ ligand SKF10,047 significantly substitutes in ibogaine trained rats (Nelson et al, 1979; Bowen et al, 1995 Mach et al, 1995; Codd, 1995; Helsley et al, 1998b; Mah et al, 1998; Glennon et al, 2000). In addition to this some imidazoline ligands have a micromolar affinity at the stomach [^{3}H]DTG (1,2-di(tolyl)guanidine) labelled sigma site and some sigma ligands have a high affinity for the [^{3}H]clonidine stomach I_1 site (Molderings et al, 1995b; Molderings et al,

1998). However, SKF10,047 failed to substitute for 2-BFI. It is possible that this failure was through administering too low a dose. In ibogaine trained rats (10mg/kg) Helsley et al, (1998b) show significant levels of substitution occur between 3 and 10mg/kg. The maximal dose used in this study was 3mg/kg. Two problems precluded administration of higher doses. Firstly, dissolution – SKF10,047 could only be dissolved at a maximum concentration of 3mg/ml. Secondly and more importantly at 3mg/kg two out of the eight rats failed to finish the session. Hence, the administration of higher doses would have compounded this problem and further rats would have failed to finish.

In vivo microdialysis studies have indicated that ibogaine administration leads to significant increase in extracellular levels of 5-HT (Glick et al, 1994; Wei et al, 1998). Having established that a number β-carbolines and ibogaine significantly substituted for 2-BFI the ability of various drugs that elevate extracellular concentrations of 5-HT were assessed for their potential to elicit levels of 2-BFI appropriate responding. Consistent with there being a serotonergic component to the 2-BFI cue significant levels of 2-BFI appropriate responding occurred after administration of the potent 5-HT releaser fenfluramine. The administration of the SSRI's paroxetine, clomipramine and citalopram led to significant levels of 2-BFI appropriate responding. Thereby confirming that in addition to NA the 2-BFI cue is also associated with increased availability of 5-HT. In a subsequent experiment the seroronergic component was probed further by administrating the selective 5-HT_{1A} antagonist WAY 100,635 prior to the administration the 2-BFI. At 0.8 mg/kg, a dose far in excess of that needed to antagonise the 5-HT_{1A} agonist 8-OH-DPAT (Kleven and Koek, 1998), WAY 100,635 failed to reduce levels of 2-BFI appropriate responding. Given alone the same dose of WAY 100,635 failed to increase levels of 2-BFI appropriate responding above that reported after the administration of vehicle. Hence, this data suggests that the serotonergic component of the cue does not act at 5-HT_{1A} receptors.

The method which some beta carbolines induce hallucinations has yet to be fully clarified. *In vitro* radiochemical binding studies indicate that ibogaine and the harmala alkaloids do not have an affinity for 5-HT receptor systems (Glennon et al, 2000). Recently, it has been suggested that the hallucinogenic properties of these 'tribal drinks' are not mediated by the beta carbolines, but by another compound – DMT (N,N-dimethyltryptamine) - which is also commonly found within the same concoction (Callaway et al, 1999). DMT is a 5-HT agonist and potent hallucinogen (Szara, 1956; Deliganis et al, 1991; Strassman et al, 1994). Hence, the harmala alkaloids inhibit MAO thereby increasing the availability of DMT released 5-HT to stimulate 5-HT receptors (Callaway et al, 1999). In support of this hypothesis, the co-administration of SSRI's fluoxetine, fluvoxamine (SSRI's), venelfaxine (SSNRI) with low doses of DOM in DOM trained rats; or LSD in LSD trained rats or ibogaine in ibogaine trained rats significantly potentiates levels of drug associated lever pressing (Winter et al, 1999).

The ability of ibogaine and harmaline to modulate opioid analgesia is particularly interesting, as it is a feature it shares with a number of I_2 ligands. As previously mentioned 2-BFI, LSL 60101, LSL 601122, agmatine significantly potentiate opioid analgesia and BU224 reduce some of the parameters associated with opiate withdrawal (Li et al 1999; Hudson et al, 1999b; Sanchez-Blazquez et al, 2000). The data presented here and in previous chapters suggests that all of these ligands, i.e, 2-BFI, LSL 60101, LSL 601122, agmatine, harmane, ibogaine, significantly substitute for 2-BFI. However, is difficult to draw any conclusions about their ability to modulate addictive behaviours, but these data indicate that all these compounds share similar pharmacological mechanisms

Hence the results from this study indicate that the β-carbolines are potent I_2 ligands *in vivo* as they are *in vitro*. The results here also suggest that ibogaine is also a potent I_2 ligand *in vivo*, it has yet to be clarified if ibogaine exhibits a similar affinity *in vitro*. Subsequent experiments, with a number of SSRI's and fenfluramine, indicated the 2-BFI cue contains a significant

serotonergic component, but this 5-HT component is probably not mediated through the 5-HT_{1A} receptor.

Chapter 8). Autoradiography: Localisation of [^{3}H]2-BFI I_2 Sites Within the Rat CNS 144

Chapter 8

Autoradiography: Localisation of [^{3}H]2-BFI I_2 Sites Within the Rat CNS

1). Introduction

A number of studies have examined the autoradiographical distribution of I_2 sites within a number of species, with a number of radioactive ligands. These include the frog, rabbit, and rat, using [^{3}H]idazoxan, [^{3}H]-RS-45041-190 and [^{3}H]2-BFI (Tyacke et al, 1999; Lione et al, 1997; Boyajian et al, 1987; Mallard et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). The initial study carried out by Boyajian et al (1987) mapped out I_2 and α_2 site distribution within the rat using [^{3}H]idazoxan and [^{3}H]rauwolscine. Their findings indicated that [^{3}H]rauwolscine autoradiographic distribution differed markedly from that of [^{3}H]idazoxan. However, this study assessed the binding parameters of [^{3}H]idazoxan without the presence of a saturating concentration of a non-imidazoline α_2-adrenoceptor antagonist (used to mask α_2-adrenoceptors, leaving only I_2 sites). Hence, their results indicated that [^{3}H]idazoxan bound to many more sites than [^{3}H]rauwolscine; particularly within the thalamus, hypothalamus, amygdala, and areas of the mesencephalon, i.e., the inferior colliculus, superior colliculus, central gray and median raphe (Boyajian et al, 1987).

The first study that looked solely at I_2 distribution, using [^{3}H]idazoxan and a micromolar mask of rauwolscine to preclude α_2-adreoceptor binding, was conducted by Mallard et al, (1992). This study, similarly to Boyajian et al

(1987), indicated that [^{3}H]idazoxan bound to sites other than α_2-adrenoceptors. High levels of [^{3}H]idazoxan I_2 binding were apparent within the lining of the ventricles and within the circumventricular organs, particularly the arcuate nucleus (102.2 fmol/mg wet tissue), area postrema (208.2 fmol/mg wet tissue), ependymal layer (101.9 fmol/mg wet tissue) and surface of the aqueduct (134.8 fmol/mg wet tissue). High areas were also found within the mesencephalon, notably the interpeduncular nucleus (123.4 fmol/mg wet tissue) and slightly lower areas of binding were found within the, central gray, superior and inferior colliculus (27.5, 47.9 and 16.6 fmol/mg wet tissue respectively). Moderate levels of [^{3}H]idazoxan I_2 binding were found within areas of the diencephalon, i.e., lateral and ventromedial hypothalamic regions (20 and 40.3 fmol/mg wet tissue respectively). Moderate levels of binding were also prevalent within the telencephalon, particularly the hippocampal fissure (61.6 fmol/mg wet tissue).

These studies were shortly followed by the autoradiographic distribution of [^{3}H]-RS-45041-190 - also within the rat brain (Mackinnon et al, 1995). [^{3}H]-RS-45041-190 was the first specific I_2 ligand synthesised and has a very low affinity for adrenoceptors, 5-HT receptors, dopamine and muscarinic receptors (Brown et al, 1995). This study showed a similar pattern of binding to that outlined by Mallard et al (1992) with [^{3}H]idazoxan. Namely, high levels of binding could be found within the circumventricular organs, i.e, the arcuate nucleus (247.3 fmol/mg tissue) and area postrema (215.6 fmol/mg tissue). However, [^{3}H]-RS-45041-190 also heavily labelled the subfornical organ (195.7 fmol/mg tissue) an area which was not reported by Mallard et al (1992). [^{3}H]-RS-45041-190 also heavily labelled areas of the mesencephalon, particularly the interpeduncular nucleus (217.4 fmol/mg tissue) and consistent with Mallard et al (1992) slightly lower areas of binding were found within the, central gray (93.4 fmol/mg tissue). As with [^{3}H]idazoxan I_2 binding, [^{3}H]-RS-45041-190 labelled the areas within the diencephalon, i.e., the dorsomedial hypothalamus, paraventricular thalamus, and medial habenular nucleus (237.6, 125.9 and 159.2 fmol/mg tissue respectively). [^{3}H]-RS-45041-190 also labelled a number of telencephalon

brain areas not reported by Mallard et al (1992). These include the dentate gyrus, subiculum, CA1, CA2 and CA3 (62.7, 97.8, 105.4, 97. 89.2 fmol/mg tissue respectively). High levels of binding were also prevalent within the rhombencephalon, particularly the locus coeruleus, dorsal raphe solitary tract, inferior olives and pyramidal tract (184.5, 135.1, 100.6, 76.4 and 66.3 fmol/mg tissue respectively).

As previously mentioned [^{3}H]idazoxan had been the radiolabelled ligand of choice for identifying I_2 sites. However, because of idazoxan's affinity for α_2-adrenoceptors and 5-HT_{1A} receptors, more specific radioligands were sought (Lachaud-Pettiti et al, 1991; Nutt et al, 1995; Llado et al 1996). 2-BFI is a specific I_2 ligand that has a high affinity for I_2 sites (1.71nM) and low affinity for α_2-adrenoceptors (1.86 μM; see figure 3.1; Lione et al, 1998; Alemany et al, 1997). Recently, Lione et al (1998) have published an extensive autoradiographical study of I_2 distribution, using both [^{3}H]2-BFI and [^{3}H]idazoxan, in adjacent rat brain sections. Levels of binding between the two radioactive ligands correlated significantly, indicating that [^{3}H]2-BFI and [^{3}H]idazoxan were labelling the same site – the I_2 site. The study labelled the areas outlined previously. As with [^{3}H]-RS-45041-190 and [^{3}H]idazoxan, [^{3}H]2-BFI labelled the lining of the ventricles and the circumventricular organs, i.e, the arcuate nucleus, area postrema, surface of the dorsal third ventricle, aqueduct and fourth ventricle (112.09, 96.73, 102.15, 25.4, 29.94 fmol/mg tissue respectively). [^{3}H]2-BFI, consistent with previous studies also labelled areas of the mesencephalon; particularly the interpeduncular nucleus and at low levels the peri-aqueductal gray, superior and inferior colliculus (88.75, 9.69, 6.36, 4.61 fmol/mg tissue respectively). Previous studies with [^{3}H]idazoxan and [^{3}H]-RS-45041-190 indicated I_2 sites showed a regional distribution within the diencephalon. Consistent with these findings [^{3}H]2-BFI showed low levels of binding in the dorsomedial and ventromedial hypothalamus (10.28 and 9.41 fmol/mg tissue respectively), and moderate levels within the lateral mammillary nucleus, paraventricular thalamus, posteromedian thalamic nucleus, medial habenular nuclei (54.33, 26.60, 17.21 and 22.52 fmol/mg tissue respectively). [^{3}H]2-BFI, consistent with [^{3}H]-

RS-45041-190, also labelled a number of telencephalon brain areas, ie., dentate gyrus, subiculum, CA1, CA2 and CA3 (9.11, 7.50, 8.86, 8.92, 8.84 fmol/mg tissue respectively). However, these levels are considerably lower than those reported for [^{3}H]-RS-45041-190.

Consistent with the [^{3}H]-RS-45041-190 study - moderate to high levels of [^{3}H]2-BFI binding were also prevalent within the rhombencephalon, particularly the dorsal raphe (32.66 fmol/mg tissue respectively). However, in contrast to the [^{3}H]-RS-45041-190 study the solitary tract, inferior olives and pyramidal tracts showed only low levels of [^{3}H]2-BFI binding (11.29, 4.62 and 2.2 fmol/mg tissue respectively).

Thus these studies utilising three I_2 ligands, [^{3}H]idazoxan, [^{3}H]-RS-45041-190 and [^{3}H]2-BFI, consistently identify discrete nuclei – these being the, arcuate nucleus, area postrema, subfornical organ, pineal gland, interpeduncular nuclei and dorsal raphe. However, a number of brain regions i.e, the subfornical organ, which have previously been shown to have high levels of I_2 specific [^{3}H]-RS-45041-190 binding have not been assessed with [^{3}H]2-BFI or [^{3}H]idazoxan (Mallard et al, 1992; MacKinnon et al, 1995; Lione et al, 1998). Thus, the first study (experiment 1) sought to follow the protocol of Lione et al (1998) and identify areas within the rat brain using [^{3}H]2-BFI that may have been previously missed.

Considering the dosing schedule of the drug discrimination animals we felt it important to characterise any alterations in [^{3}H]2-BFI binding that may occur after such treatment. Previous homogenate radioligand binding and immunocytochemical studies have indicated animals that have been chronically dosed with I_2 ligands show an increase in protein immunoreactivity, [^{3}H]idazoxan and [^{3}H]2-BFI I_2 site density (Olmos et al, 1994; Alemany et al, 1995; Escriba et al, 1996; Alemany et al, 1997). Molecular studies have indicated that chronic dosing (seven days) with high doses of idazoxan significantly increases the immunoreactivity of 29/30 and 66 kDa proteins isolated with the imidazoline immunoreactive antibody mentioned previously. However, similar treatment, using the I_2 selective ligand LSL 60101 significantly increased immunoreactivity levels of the 66 kDa protein only. These increases significantly correlated with an increase

in the [^{3}H]idazoxan I_2 binding maximum or B_{max}. Identical treatment using the specific α_2-adrenoceptor antagonists yohimbine and efaroxan failed to alter immunoreactivity or the [^{3}H]idazoxan adrenoceptor B_{max} thereby suggesting that these increases are associated with I_2 sites rather that α_2 adrenoceptors (Escriba et al, 1996). Chronic administration of, idazoxan, cirazoline, LSL 60101, but not LSL 60125, are also associated with an increase in rat brain glial fibrillary acidic protein immunoreactivity and the [^{3}H]idazoxan I_2 B_{max} (Olmos et al, 1994; Alemany et al, 1995). However, as previously mentioned in rodent models of global forebrain ischaemia GFAP levels are increased, but no changes where apparent in I_2 levels as assessed with [^{3}H]2-BFI (Conway et al, 1998).

To address this question two further experiments. The first of the two was designed to assess which areas of the brain, which have previously been shown to exhibit high levels of [^{3}H]2-BFI specific binding, could be reproducibly identified. We therefore took drug naive Wistar rats, the strain used in previous studies of this type, and targeted a number of areas that show high levels of [^{3}H]2-BFI binding. These being the, area postrema, pineal gland, arcuate nucleus, interpeduncular nucleus and the lateral mammillary nucleus. The third experiment within this chapter sought to assess any alterations that may occur between drug discrimination treated rats and saline controls, within the regions identified in experiment two. Hence, we dosed two groups of Hooded Lister rats. Hooded Lister rats were chosen for this experiment because they were the strain of rats used in drug discrimination. The first group followed the dosing schedule of an actual group of rats that had undergone drug discrimination training. Dosing ended at a point which we had previously deemed (96 sessions; see Chapter 3) to be sufficient for this group of rats to have developed the 2-BFI cue if they had actually been trained. The second group represented a control group and were only administered saline throughout the period.

Hence, the objectives of this study were three fold. Firstly we sought to map [^{3}H]2-BFI binding within the rat CNS to look for regions that have not been previously identified. Secondly, in drug naive rats, we targeted a number of

nuclei which show high levels of [^{3}H]2-BFI – these being the, lateral mammillary nucleus, aqueduct, area postrema, pineal gland, interpenducular and arcuate nuclei. Finally, we dosed a group of animals following the drug discrimination dosing schedule, and based on the results from part two, sought to image four of the afore mentioned CNS areas in a bid to assess any alterations that may occur as a result of this drug treatment.

2). Method

2.1). Animals

Four drug naive animals Wistar rats (Aston University, UK) were used for experiments one and two. Experiment three used ten Hooded Lister rats (Charles River, UK). Five of these rats acted as a control group (group 8B) and were dosed with only saline. The other group of five rats (group 8A) were dosed according to the drug discrimination dosing schedule (SDDSSDSSDD). They essentially shadowed the trained animals and received a total number of 96 pseudo-training doses (one pseudo-training dose per day). This breaks down to 50 drug days (at 7mg/kg 2-BFI) and 46 saline days.

Individual means for each individual nuclei were derived from a minimum of three sections per rat with three readings per section. Data for experiment one and two were derived from three to four rats. Data for experiment three was derived from four to five rats.

2.2). Autoradiographical Analysis

Rat brain sections were extracted and mounted for experiment one according to the method outlined previously. Experiments two and three used a slightly different method – once the nucleus had been isolated a 30μM section was taken for cresyl violet staining, then a section for total, then another for NSB. The total and NSB combination was repeated twice more and a final 30μM section was taken for cresyl violet staining – resulting in three sections, containing each nucleus, per animal.

All experiments were based on the protocol outlined by Lione et al (1998) which is outlined in the method. Brain structures were targeted - using Paxinos & Watson's (1998) stereotaxic atlas of the rat brain. Autoradiography experiments one and two used a concentration of 0.5 nM [^{3}H]2-BFI. In experiment three the concentration of [^{3}H]2-BFI was increased to 1 nM. Preliminary studies carried out on Hooded Lister rats indicated they show lower levels of [^{3}H]2-BFI binding than Wistar rats. Hence, it was felt appropriate to increase the concentration of [^{3}H]2-BFI to 1 nM.

Experiments were designed so that all autoradiography cases within one experiment contained equal proportions of each nucleus. However, data for the area prostrema reported in experiment two came from a single autoradiography case. In experiments which compared an experimental condition to a control condition, i.e., experiment 3, control and experimental brains were split across all boxes.

3). Results

3.1). General Binding Parameters of [^{3}H]2-BFI in the rat CNS

Figure 8.1A shows total binding of 0.5 nM [^{3}H]2-BFI within a 12 μM para-sagittal rat brain section approximately 50μM from the midline. Non specific binding was assessed by the presence of saturating concentrations (10μM) of BU224 in an adjacent 12μM brain section, see figure 8.1B. The images shown in figure 8.1A and B are reverse phase images of original autoradiography films. Data analysis using MCID defined that NSB (non-specific binding) was negligible (<10%; see figure 8.1B). This concentration (0.5nM) has been previously shown to be optimal for reducing NSB (Lione et al, 1998). Figure 8.1C shows a further 30μM rat brain section that lay adjacent to that used for figure 8.1A. Areas of tritiated 2-BFI binding were verified by comparison of autoradiograms to cresyl violet stained sections and the rat brain atlas (Paxinos and Watson, 1998). Figure 8.1A clearly shows high to moderate levels of 0.5 nM [^{3}H]2-BFI total binding within the arcuate nucleus (arc), subfornical organ (sfo) and to a lesser extent within the, pineal gland (pi), dorsal raphe (dr) and interpeduncular nuclei (ip). Total binding within the interpeduncular nucleus and area postrema is further in transverse rat brain sections, figure 8.2 and 8.3.

Analysis of these autoradiograms, using radioactive standards, gave rise to the fmol/mg levels reported in table 8.1. Data was derived from a minimum of three rats. For each nucleus identified densometric readings were taken from three sections within each individual rat brain. Three data readings were taken for each nucleus identified on each section. A number of areas showed high levels of [^{3}H]2-BFI binding (>70 fmol/mg tissue). These being the regions previously shown in figure 8.1A, i.e., the arcuate nucleus, subfornical organ and area prostrema. High to moderate levels of (70-40 fmol/mg tissue) of [^{3}H]2-BFI binding were found within the lateral mammillary nucleus, third ventricle and lateral ventricle. Moderate levels of [^{3}H]2-BFI binding (20-40 fmol/mg tissue binding) were found within many more brain regions; particularly, the pineal gland and dorsal raphe (see figure 8.1A),

anterior and posterior thalamus, dorso- and ventro-medial hypothalamus, lateral and tuberomammillary nuclei, rhinal fissure, interpeduncular nucleus, and optic chiasm. Low levels of [^{3}H]2-BFI binding (<20 fmol/mg tissue) were found within the hippocampus; i.e, the dentate gyrus, hippocampal fissure, stratum oriens and stratum pyrimidal; and basal ganglia, i.e., the caudate putamen. Low levels were also present within the cerebellum, forth ventricle and aqueduct. Within the brain sections assayed all remaining areas showed negligible levels of [^{3}H]2-BFI specific binding.

3.2). Targeting [^{3}H]2-BFI binding sites within the rat brain

Experiment two sought to target brain areas which had previously been shown to show moderate to high levels of [^{3}H]2-BFI binding. Experiment one indicated that a number of rat brain regions exhibit moderate to high levels of [^{3}H]2-BFI binding. Based on these results; the results of Lione et al (1998), the physical size of these nuclei and the neuroanatomical markers that surround them; the arcuate nucleus, interpeduncular nucleus, pineal gland, lateral mammillary nucleus, and area postrema were targeted. Assay conditions were the same as those for experiment 1. Hence, total binding was measured with 0.5 nM [^{3}H]2-BFI and NSB with 0.5 nM [^{3}H]2-BFI, in the presence of saturating concentrations (10μM) of BU224. Data analysis using MCID again confirmed that NSB was negligible (>10%). Data was derived from three to four rats. For each nuclei identified densometric readings were taken from three sections within each individual rat brain. Three data readings were taken for each nucleus identified on each section.

Table 8.2 shows the specific binding component of 0.5 nM [^{3}H]2-BFI. Consistent with experiment one the, arcuate nucleus (69.08 fmol/mg tissue), interpeduncular nucleus (35.81 fmol/mg tissue), pineal gland (32.38 fmol/mg tissue), lateral mammillary nucleus (45.17 fmol/mg tissue), and area postrema (51.58 fmol/mg tissue), showed high to moderate levels of [^{3}H]2-BFI binding see table 8.2.

Table 8.1). *Experiment 1: General Binding Characteristics of [^{3}H]2-BFI in the rat CNS*

Brain Region		Specific Binding 0.5 nM [^{3}H]2-BFI (fmol/mg tissue)	
		Mean	*SEM*
Telencephlon			
Hippocampus			
dentate gyrus	dg	9.08	1.23
stratum oriens CA1	CA1	12.08	0.71
stratum oriens CA2	CA2	11.68	0.83
stratum pyramidal	CA3	10.33	2.10
hippocampal fissure	Hif	17.36	2.96
Basal Ganglia			
caudate putamen	cpu	8.82	0.19
Diencephalon			
Thalamus			
paraventricular thalamus, anterior	pva	22.44	7.66
paraventricular thalamus, posterior	pvp	21.64	4.15
Hypothalmus			
dorsomedial	dm	26.77	8.47
ventromedial	vm	28.99	7.45
lateral mammillary nucleus	lm	41.60	8.38
tuberomammillary nucleus	tm	38.07	9.57
optic chiasm	ox/sox	38.88	3.75
Mesencephalon			
interpeduncular nucleus	Ip	32.15	4.70
Rhombencephalon			
dorsal raphe	dr	32.01	9.27
Ventricles			
rhinal fissure	rf	26.13	4.06
forth ventricle	4V	19.50	4.65
aqueduct	Aq	20.56	1.48
third ventricle	3V	66.74	9.23
lateral ventricle	LV	47.55	6.04
Circumventricular organs			
arcuate nuclei	Arc	96.46	15.22
pineal gland	Pi	32.15	6.54
subfornical organ	SFO	78.23	21.71
area postrema	Ap	78.09	6.85
Cerebellum	c	6.62	0.74

Data derived from three to four rats, with a minimum of three sections per rat, and three readings per section.

Table 8.2). *Experiment 2: Targeting [^{3}H]2-BFI binding sites within the rat brain*

Region		Specific Binding 0.5 nM [^{3}H]2-BFI (fmol/mg tissue)	
		Mean	***SEM.***
lateral mammillary nucleus	lm	45.17	3.68
Interpeduncular nucleus	Ip	35.81	3.63
arcuate nucleus	Arc	69.08	9.10
pineal gland	Pi	32.38	1.72
area postrema	Ap	51.58	3.68

Data derived from three to four rats, with a minimum of three sections per rat, and three readings per section.

3.3). Effects of Drug Discrimination Dosing on [^{3}H]2-BFI within the rat brain

Having reproducibly targeted a number of specific areas of [^{3}H]2-BFI binding in experiment two, experiment three focused on four of these regions; the arcuate nucleus, the interpeduncular nucleus, pineal gland and area postrema; in a bid to explore any alterations in [^{3}H]2-BFI binding may occur after chronic drug treatment. Assay conditions were the same as those for experiment one and two except total binding was measured with 1nM [^{3}H]2-BFI and NSB with 1nM [^{3}H]2-BFI, in the presence of saturating concentrations (10μM) of BU224. Data was derived from a minimum of four rats. For each nucleus identified densometric readings were taken from three sections within each individual rat brain. Three data readings were taken for each nucleus identified on each section.

Figure 8.4A depicts the total binding component of 1 nM [^{3}H]2-BFI in a transverse (–3.30mm from the bregma line) section taken from the brain of a saline treated rat. Despite increasing the concentration to 1nM [^{3}H]2-BFI NSB was still very low, see figure 8.4B. This Data analysis using MCID again confirmed that NSB was negligible (<10%). Comparison of an adjacent cresyl violet stained section verified the presence of the arcuate nucleus, see figure 8.4C.

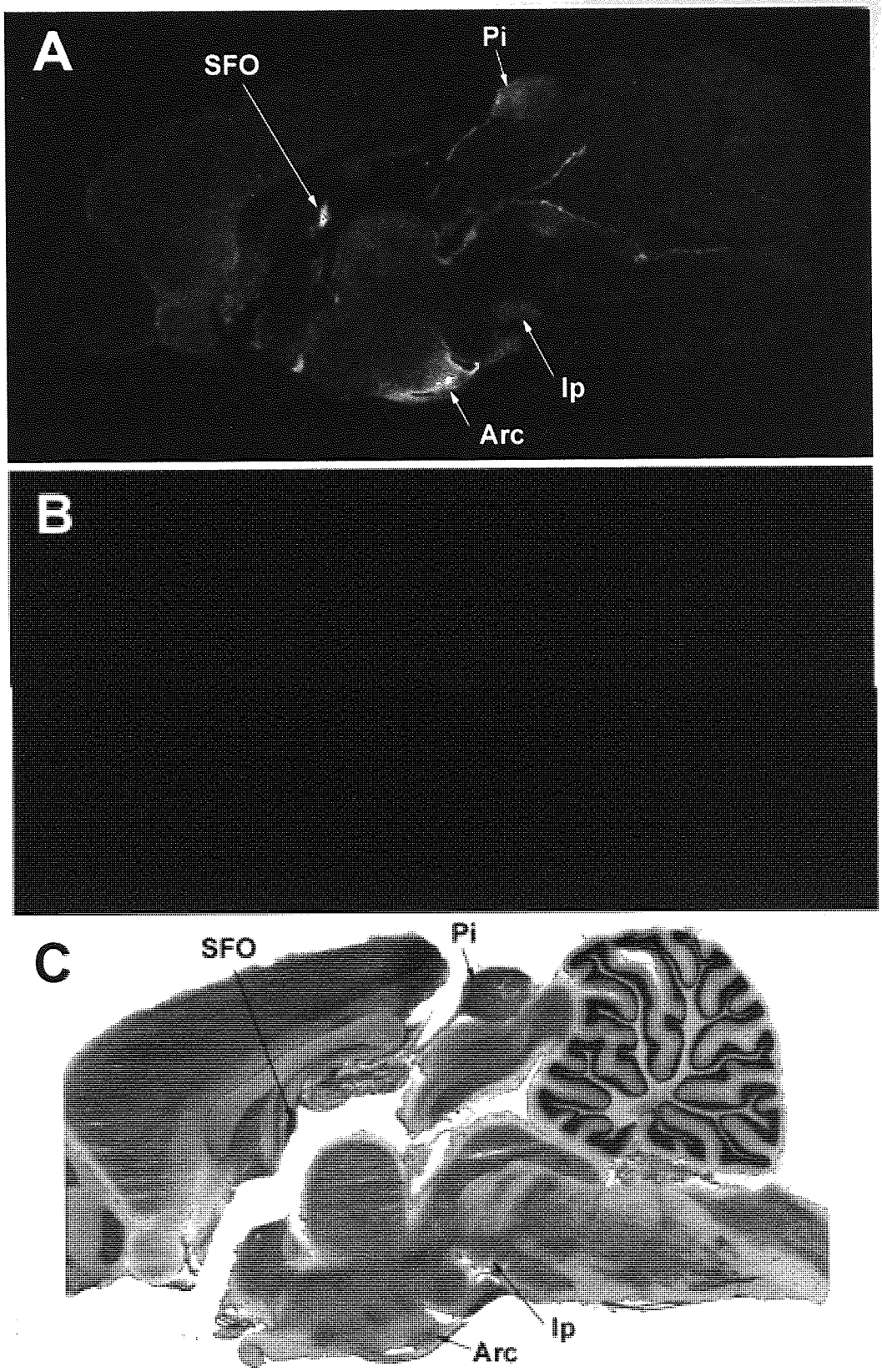
Table 8.3 shows specific binding levels (fmol/mg tissue) of 1nM [^{3}H]2-BFI in a number of discrete CNS nuclei, within the brains of saline dosed rats and those dosed under the drug discrimination schedule. Consistent with the previous experiments, the arcuate nucleus, interpeduncular nucleus, pineal gland and area postrema show high to moderate levels of binding. However, although these levels are high, they are not as high as those within experiments one and two. This not unexpected as we had previously established that Hooded Lister rats show lower levels of binding than Wistars, i.e., following protocol here with, 0.5 nM [^{3}H]2-BFI, Hooded Lister rats (n=2) show specific binding levels of 50.7 fmol/mg within the arcuate nucleus and 16.6 fmol/mg within the interpeduncular nucleus. Hence the figures we report here at 1nM are within the expected range, i.e, arcuate nucleus (70.5 fmol/mg tissue), interpeduncular nucleus (19.9 fmol/mg tissue), pineal gland (16.5 fmol/mg tissue), and area postrema (38.1 fmol/mg tissue). Comparison of these levels of specific binding to those of the DD dosed rats indicates this regime of 2-BFI administration increases [^{3}H]2-BFI within all areas of the brain. However, levels of specific [^{3}H]2-BFI binding were only significantly different from the corresponding regions within the brains of saline dosed animals (see table 8.3 and figure 8.4D, 8.4E and 8.4F) within arcuate nucleus and area postrema ($p<0.05$).

Table 8.3). *Experiment 3: Effects of Drug Discrimination (DD) dosing on [^{3}H]2-BFI binding sites within the rat brain*

Region		Control Specific Binding 1 nM [^{3}H]2-BFI (fmol/mg tissue)	DD Specific Binding 1 nM [^{3}H]2-BFI (fmol/mg tissue)	Sig[1]
		M. ± SEM.	*M. ± SEM.*	
interpeduncular nucleus	Ip	19.9 ± 2.18	23.28 ± 3.33	ns.
arcuate nucleus	Arc	70.5 ± 6.33	115.21 ± 16.22	p<0.05
pineal gland	Pi	16.5 ± 2.1	18.6 ± 1.6	ns.
area postrema	Ap	38.1 ± 5.10	63.90 ± 7.74	p<0.05

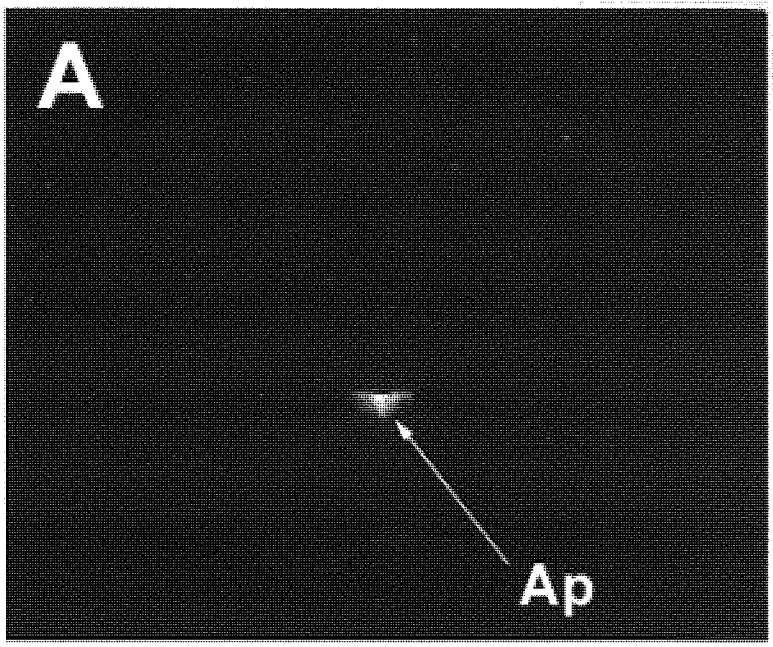
Data derived from four to five rats, with a minimum of three sections per rat, and three readings per section. * Mean ± Standard error of the mean. Sig[1], significant difference (one way ANOVA) between drug discrimination and saline treated rats.

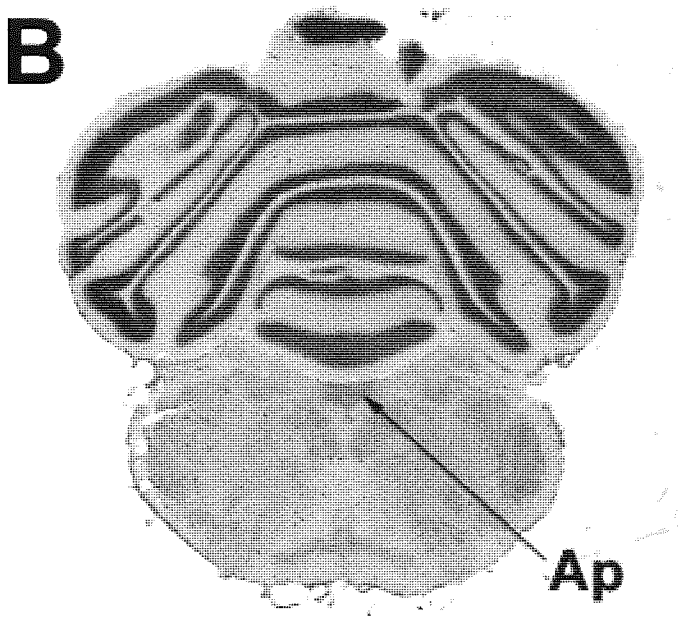
Figure 8.1). ***Total binding (section a), NSB binding (section b) of 0.5nM [^{3}H]2- BFI and cresyl violet stained (section C) rat para-sagittal brain sections***



Sections are approximately 150μM from the rat brain midline. Light areas represent high levels of tritiated ligand binding. See table 8.1 for abbreviations.

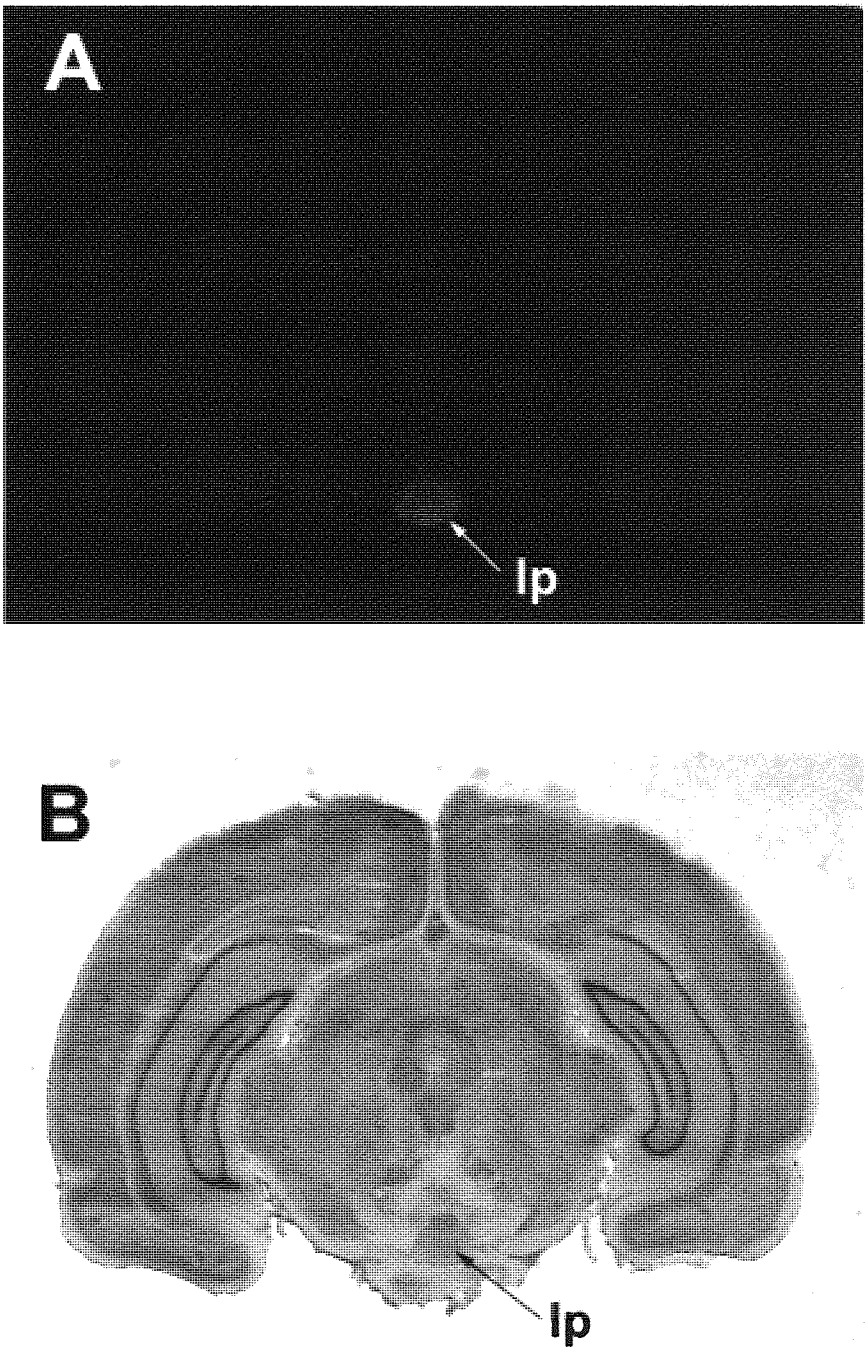
Figure 8.3). ***Total binding of 0.5nM [^{3}H]2-BFI (section a) and cresyl violet stained (section b) within rat brain transverse sections.***





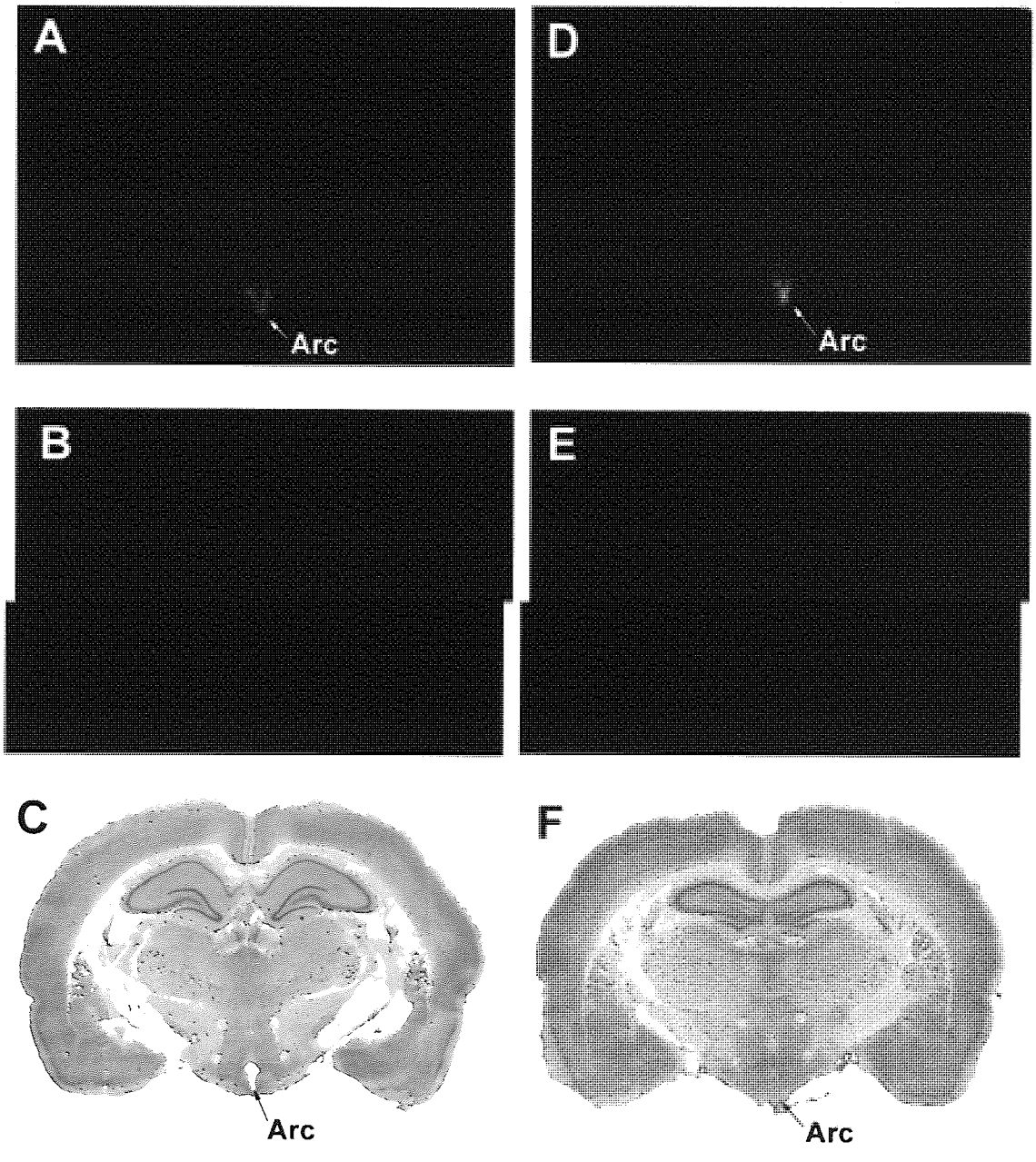
Sections are taken at approximately –13.68mm from the bregma line. Light areas represent high levels of tritiated ligand binding. See table 8.1 for abbreviations.

Figure 8.2). ***Total binding of 0.5nM [^{3}H]2-BFI (section a) and cresyl violet stained (section b) within rat brain transverse sections.***



Sections are taken at approximately –6.04mm from the bregma line. Light areas represent high levels of tritiated ligand binding. See table 8.1 for abbreviations.

Figure 8.4). ***Total binding (A - saline treated section, D - drug discrimination treated) and NSB binding (B - saline treated section, E - drug discrimination treated) of 1nM [^{3}H]2-BFI within rat brain transverse sections.***



Sections taken at –3.30mm from the bregma line. Light areas represent high levels of tritiated ligand binding. See table 8.1 for abbreviations.

4). Discussion

The results from this study confirm and add to the results from previous studies, i.e., [^{3}H]2-BFI binding is to a number of discrete brain nuclei; including the arcuate nucleus, area postrema, interpeduncular nucleus and pineal gland (Lione et al, 1998). However, this study reports high levels of binding to the subfornical organ, an area that has been identified with [^{3}H]-RS-45041-190 but not [^{3}H]2-BFI (Mackinnon et al, 1995; Lione et al, 1998). The subsequent studies reported here indicate the four [^{3}H]2-BFI I_2 associated brain areas mentioned above can be reproducibly targeted and dosing with 2-BFI significantly increases levels of [^{3}H]2-BFI binding over saline dosed controls in two of these regions.

Experiment one looked at the regional binding of [^{3}H]2-BFI within the rat brain. Consistent with previous studies [^{3}H]2-BFI heavily labelled a number of areas within the circumventricular organs. Lione et al, (1998) report heavy binding within the arcuate nucleus (112.09 fmol/mg tissue), area postrema (96.73), surface of the dorsal third ventricle (102.15 fmol/mg tissue), aqueduct (25.4 fmol/mg tissue) and fourth ventricle (29.94 fmol/mg tissue). We report similar figures; i.e., arcuate nucleus (96.46 fmol/mg tissue), area postrema (78.09), surface of the dorsal third ventricle (66.74 fmol/mg tissue), aqueduct (20.56 fmol/mg tissue) and fourth ventricle (19.50 fmol/mg tissue). Hippocampal levels of binding were also very similar. We report between 10 and 12 fmol/mg tissue for all layers of the hippocampus (CA1-CA3) whilst Lione et al (1998) report 8 to 9 fmol/mg tissue. Levels of [^{3}H]2-BFI binding within the dorsal raphe and paraventricular thalamus were virtually identical. We report 32.66 and 22.44 fmol/mg tissue for the dorsal raphe and paraventricular thalamus, whilst Lione et al, (1998) report 32.01 and 26.60 fmol/mg tissue.

However, inspite of these obvious similarities we report markedly lower levels of [^{3}H]2-BFI binding within the interpeduncular nucleus and pineal gland. Experiments one and two consistently show moderate levels of [^{3}H]2-BFI binding within the interpeduncular nucleus (32.15 and 35.81 fmol/mg tissue) and pineal gland (32.15 and 32.38) fmol/mg tissue. Lione et al,

(1998) report considerably higher levels for the interpeduncular (88.75 fmol/mg tissue) and pineal gland (63.99 fmol/mg tissue). We also report considerably higher levels of binding within the dorso- and ventro-lateral hypothalamus (26.77 and 28.99 fmol/mg tissue, respectively Vs 10.28 and 9.41 fmol/mg for Lione et al, 1998).

There are a number of reasons for this variance. Firstly the method of quantification would have lead to alterations. Lione et al (1998) use Quantimet 970 and we use MCID, which digitally subtracts the adjacent NSB section from total, then allows densomitry readings of specific binding to be taken directly from the digitally created specific section. Secondly it is possible that there is regional binding within each nucleus, i.e., the rat interpeduncular nuclei consists of four different subnuclei; the rostral, caudal, lateral and intermediate subnuclei (Paxinos and Watson, 1998). This may also be the case for the pineal gland. The pineal gland is clearly visible on figure 8.1A. However, levels of binding differ markedly across it. Rostrally the pineal gland shows high to moderate levels of [^{3}H]2-BFI binding, shown as lighter areas on figure 8.1A. However, caudally there are much lower levels of binding. However, having said this in general the results of these autoradiography studies agree well with those previously published (Mackinnon et al, 1995; Lione et al, 1998).

In addition to those areas mentioned previously this study found very high levels of [^{3}H]2-BFI binding within the subfornical organ (78.23 fmol/mg tissue). I_2 ligand binding within this brain region has not previously been reported for [^{3}H]2-BFI, only [^{3}H]-RS-45041-190 at 195.7 fmol/mg tissue (MacKinnon et al, 1995). Due to the small size of this nuclei, approximately 800μM, it is possible that previous studies using [^{3}H]2-BFI overlooked it or did not have sufficient sections for statistical analysis.

Experiment two was designed so as to develop a protocol in which areas of high to moderate [^{3}H]2-BFI binding could be reproducibly sectioned and thus experimented on. Depending on the size of the nucleus, the neuroanatomical markers that surrounded it and thus its potential for repeated identification the, arcuate nucleus, interpeduncular nucleus, pineal

gland, lateral mammillary nucleus, and area postrema were chosen for experiment two. Consistent with experiment one these areas showed high to moderate levels of [^{3}H]2-BFI binding. These levels of binding were comparable to those found in experiment one, i.e., arcuate nucleus (96.46 Vs 69.08 fmol/mg tissue, experiment 1 Vs experiment 2), interpeduncular nucleus (32.15 Vs 35.81 fmol/mg tissue), pineal gland (32.15 Vs 32.38 fmol/mg tissue), lateral mammillary nucleus (41.60 Vs 45.17 fmol/mg tissue), and area postrema (78.09 vs 51.58 fmol/mg tissue). However, although the levels of binding reported for experiments 1 and 2 are very similar within the interpeduncular nucleus, pineal gland and lateral mammillary nucleus (within 4 fmol/mg tissue or 5-8% of total) these differences are larger when looking at the arcuate nucleus and area postrema (27 fmol/mg tissue or 25-35% of total). Normally, equal numbers of each nucleus are spread across all autoradiography cases, so as to randomise any error over all experimental conditions. However, data used for the area postrema as mentioned in the method was derived in a separate experiment. Hence, if they had been assessed, this subsequent experiment might have given proportionally lower levels of binding within the pineal gland, interpeduncular nucleus. However, this does not account for the low levels of [^{3}H]2-BFI binding within the arcuate nucleus. The arcuate nucleus is one of the longest brain regions and as figure 8.1A shows exhibits differing levels of binding throughout its length. Hence, as is the case with the pineal gland, it is possible this variance occurred through sampling different sections of the arcuate nucleus.

Previous homogenate radioligand binding studies have indicated animals that have been chronically dosed with I_2 ligands such as idazoxan, cirazoline, and LSL 60101 show an increase in their brain [^{3}H]idazoxan or [^{3}H]2-BFI binding maximum (Olmos et al, 1994; Alemany et al, 1995; Escriba et al, 1996; Alemany et al, 1997). Further molecular studies which utilise a polyclonal antibody raised against a [^{3}H]idazoxan labelled protein, indicate levels of a 29/30, 66 kDa protein and GFAP are also elevated in response to chronic I_2 ligand dosing (Olmos et al, 1994; Alemany et al, 1995; Escriba et al, 1996). The results from this study corroborate these findings and identify

regions in which this upregulation is occurring. Those rats that had been dosed under the DD schedule showed significantly higher levels of [^{3}H]2-BFI binding within the arcuate nucleus and area prostrema when compared with their saline controls. However, we are unable to say if this is an increase in actual sites or an increase in affinity of the same number of sites. The studies mentioned above indicate such dosing regimes increase the number of binding sites not their affinity (Olmos et al, 1994; Alemany et al, 1995; Escriba et al, 1996). Hence, it is likely that we are reporting an increase in the number of [^{3}H]2-BFI I_2 binding sites. We are unable to state what these sites are. However, it is unlikely to be increases in GFAP as previous studies have shown increases in GFAP, but not [^{3}H]2-BFI, in the rodent model of global forebrain ischaemia (Conway et al, 1998).

The striking thing about autoradiographical studies of this type is the number of ventricular and circumventricular areas that show high quantities of I_2 ligand binding, i.e., the subfornical organ, arcuate nucleus, area postrema, pineal gland and ependyma/lateral ventricles. As previously mentioned, see table 1.6, these regions have been closely associated with MAO, particularly MAO-B (Saura et al, 1992; Luque et al, 1995). However, these regions are also associated with a number of other receptors. In situ hybridisation studies have indicated the presence of cannabinoid receptors within the interpeduncular and arcuate nucleus (Mailleux and Vanderhaedhen, 1992). The complete autoradiographical profile of [^{3}H]2-BFI closely matches that of glucagon like peptide (GLP) binding sites. Hence, dense levels of GLP binding sites were found within the area postrema, subfornical organ, interpeduncular nucleus, thalamus, hypothalamus, inferior olives and solitary tract (Goke et al, 1995). This is of particular interest as some imidazoline compounds have been reported to inhibit glucagon secretion from pancreatic α cells (Mourtada et al, 1997). Glucagon is primarily associated with the release of glucose from bodily stores. Whereas insulin secretion, another imidazoline induced process, is associated with high blood glucose levels and the uptake of carbohydrates from it (Chan et al, 1995; Dunne et al, 1995; Morgan et al, 1999; Zaitesev et al, 1999). Previous

in vivo work has also indicated that the administration of I_2 ligands increases food intake in the hours after dosing (Jackson et al, 1991; Menargues et al 1994; Menargues et al 1995; Brown et al, 1995; Polidori et al, 2000). These findings and the autoradiographical results mentioned here add to the accumulating evidence associating imidazoline sites with the regulation of food intake.

The results reported here and elsewhere indicate I_2 ligands bind to a number of circumventricular organs (Lione et al, 1998). However, in contrast to previous studies using [^{3}H]2-BFI we report the presence of high levels of binding within the subfornical organ. As mentioned in the introduction the subfornical organ has been closely associated with the regulation of blood pressure and water intake (Mangiapane and Simpson, 1980). More recent studies have indicated that the intracranial injection of the I_1 and α_2-adrenoceptor agonists rilmendine and clonidine into the paraventricular hypothalamus reduces the hypertensive effect apparent after angiotensin II application to the SFO (Arrais et al, 1997; Saad et al, 1998; Araujo-Almeida et al, 1999). Subsequent studies indicated that co-administration of the I_2 and α_2-adrenoceptor antagonists with clonidine or rilmendine antagonises their ability to reduce angiotensin II induced hypertension (Saad et al, 1998). However, as mentioned in the introduction it is unclear whether these effects are mediated by imidazoline receptors or α_2-adreoceptors. It is of note that it is I_1 ligands that have been associated with reducing blood pressure (Van Zwieten & Peters, 1999). *In vivo* the administration of the I_2 specific ligand RS-45041-190 has no effect on blood pressure or heart rate (Brown et al, 1995).

The results from this study confirm and extend that of others, i.e., I_2 binding within the rat brain is to discrete nuclei. A large number of these binding regions are ventricular or are circumventricular organs. In addition to those areas that have previously shown to have high levels of [^{3}H]2-BFI, i.e., the arcuate nucleus, pineal gland and area postrema, we report high levels of binding within the subfornical organ. The data presented here also indicates that chronic administration of 2-BFI significantly increases levels of [^{3}H]2-BFI

binding within discrete brain areas, i.e., the arcuate nucleus and area postrema. Again this is in accordance with previous tissue homogenate binding studies that indicate I_2 administration increases I_2 tritiated ligand binding within the rat brain as a whole.

Chapter 9). Autoradiography: Modulation of [^{3}H]2-BFI binding by MAO inhibitors 168

Chapter 9

Autoradiography: Modulation of [^{3}H]2-BFI binding by MAO inhibitors

1). Introduction

Molecular studies have recently localised an I_2 site to the enzyme monoamine oxidase B (Raddatz et al, 1999). However, a number of peptides with a range of molecular weight have been purified from a variety of species that do not posses the molecular characteristics of MAO-A or MAO-B (Bennai et al, 1996; Ivanov et al, 1998b; Garcia-Sevilla et al, 1996; Escriba et al, 1999). In a previous chapter using [^{3}H]2-BFI four nuclei were isolated that exhibited high to moderate levels of tritiated ligand binding. Therefore, the object of the current series of experiments was to gauge any alterations in [^{3}H]2-BFI binding that may occur after *in vivo* and *in vitro* pre-treatment of rat brain with a number of irreversible MAO inhibitors.

Deprenyl (selegiline) and clorgyline are acetylenic monoamine oxidase inhibitors, i.e., inhibition is a two step process which involves oxidation of the amine group by the flavin molecule and then subsequent irreversible covalent bonding of the inhibitor to the flavin molecule (Fowler et al, 1981). Generally speaking the distance between the carbon ring and the nitrogen group defines substrate and inhibitor isoform specificity. Hence, inhibitors or substrates that have three or more carbons between the carbon ring structure and nitrogen group, i.e., clorgyline or 5-HT, act at MAO-A (see figure 9.1). Conversely, inhibitors or substrates with fewer carbons, i.e.,

deprenyl or phenethylamine, act at MAO-B (see figure 10.1; Kalgutkar et al, 1995). Tranylcypromine is a cyclopropylamine and irreversible inhibition of MAO occurs via a similar mechanism, i.e., covalent bonding of the cyclopropyl ring to the flavin molecule (Kalgutkar et al, 1995). However, a different method of inhibition has been proposed for I_2 ligands. Molecular studies utilising antibodies raised against differing peptides from MAO-B suggest I_2 ligands reversibly bind to the portion between amino acids 149 and 222 (Raddatz et al, 1997). Hence, it is likely that irreversible inhibition via interactions with the flavin molecule do not occur.

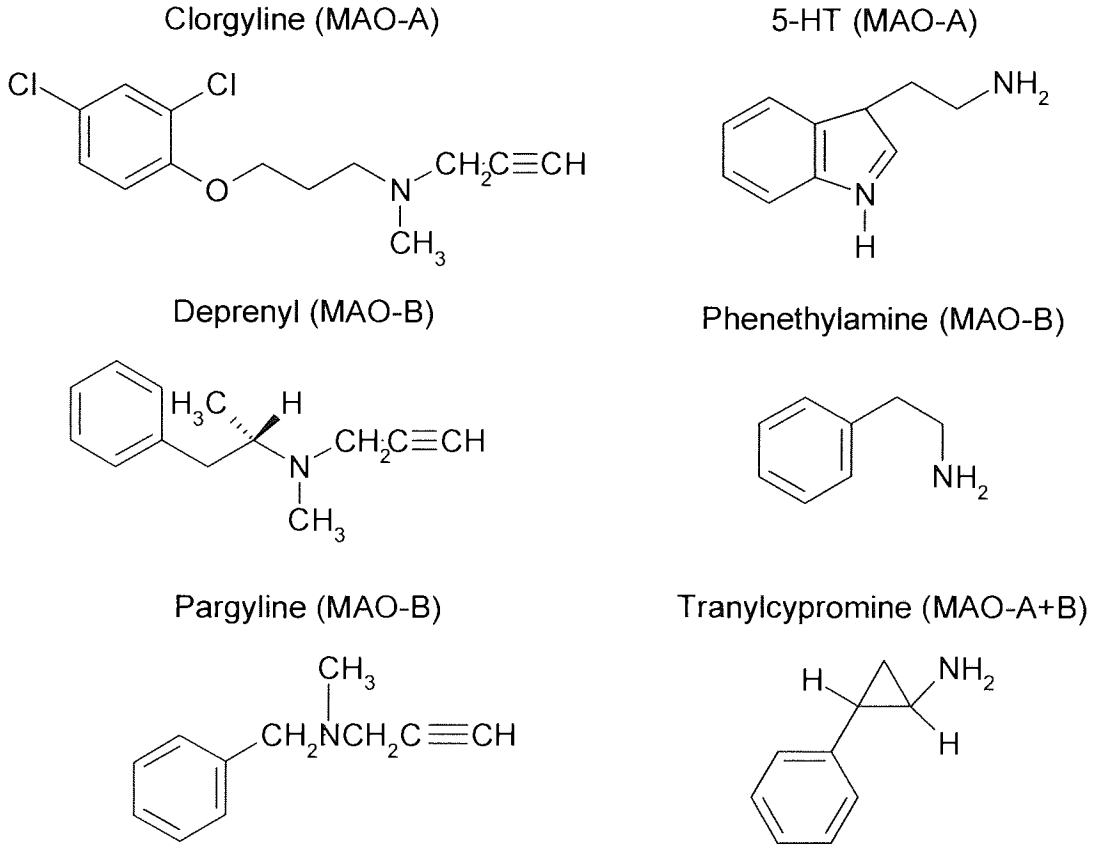


Figure 9.1). ***Chemical structures of MAO inhibitors and substrates***

It is therefore not surprising that MAO inhibitors have a low affinity for the I_2 site and previous studies have obtained conflicting evidence on the ability of MAO inhibitors to reduce tritiated I_2 ligand binding.

In vitro the application of clorgyline at two concentrations (10^{-9} and 10^{-6} M for 30 minutes) and pargyline at two concentrations (10^{-6} and 10^{-5} M for 30 minutes) to rat cerebral cortex membranes significantly reduces the [^{3}H]idazoxan I_2 density but not the affinity of [^{3}H]idazoxan to I_2 sites (Olmos et al, 1993). However, using the same protocol the application of phenelzine (10^{-4} M for 30 minutes), a hydrazine, fails to alter [^{3}H]idazoxan I_2 density (Alemany et al, 1995a). Phenelzine is a non-specific hydrazine MAO inhibitor and irreversible inhibition, as with the acetylenic and cyclopropylamine MAO inhibitors, occurs through covalent bonding of the ligand to the flavin molecule. However, unlike the acetylenic and cyclopropylamine MAO inhibitors, phenelzine binds to a different region of the flavin cofactor (Kalgutkar et al, 1995).

Chronic treatment (twice a day for 12 days) with the acetylenic monoamine oxidase inhibitors clorgyline (0.3-10mg/kg) and pargyline (10mg/kg) significantly reduces the binding maximum of [^{3}H]idazoxan to I_2 sites but not their affinity for tritiated idazoxan (Alemany et al, 1995a). Similar findings have been found after chronic treatment (twice a day for 7 days) with the cyclopropylamine tranylcypromine (10mg/kg) and the hydrazine phenelzine (3-20mg/kg; Alemany et al, 1995a). Hence, chronic treatment with these two non-specific inhibitors significantly reduces the binding maximum of [^{3}H]idazoxan to I_2 sites, but not the affinity (Alemany et al, 1995a). Studies that have administered pargyline (1-40mg/kg) acutely, i.e., twice during six to forty eight hours, have failed to yield significant differences in [^{3}H]idazoxan binding to I_2 sites (Alemany et al, 1995a).

Human brain homogenate binding studies using the nucelus reticularis lateralis (NRL) have indicated co-incubation of tranylcypromine and [^{3}H]2-BFI, but not clorgyline or pargyline and [^{3}H]2-BFI, potentiates tritiated 2-BFI binding (Wiest and Steinberg, 1997). Potentiation was maximal at 0.3μM which significantly increased the [^{3}H]2-BFI site density (2085 fmol/kg to 8393

fmol/mg) but not the affinity of the radioligand for its binding site. This effect was not observed within the rat cerebral cortex and could be cancelled by the prior incubation of human NRL membranes with either pargyline or clorgyline (Wiest and Steinberg, 1997). A subsequent study using cloned human MAO-B expressed in insect cells gave a similar result, i.e., the co-incubation of tranylcypromine and [^{3}H]2-BFI leads to a potentiation of [^{3}H]2-BFI binding (Steinberg et a, 1999). Again the potentiation was maximal at 0.3μM with the [^{3}H]2-BFI I_2 site density increasing 17 fold (Steinberg et a, 1999). This effect was not observed within insect cell cloned human MAO-A or rat MAO-B (Steinberg et a, 1999).

However, these studies were not site directed, i.e., they essentially assessed binding in cerebral cortex homogenates, an area which has low levels of [^{3}H]2-BFI binding (Olmos et al, 1993; Alemany et al, 1995a; Wiest and Steinberg, 1997). Hence, the experiments contained in this chapter had two main points. Firstly, the short term effects of irreversible MAO inhibitors on [^{3}H]2-BFI binding within the four nuclei that had identified in the previous chapter, i.e., pre-incubation of rat brain slices with various concentrations of MAO inhibitors and the effects of acute administration, i.e., in doses that we had previously shown to exhibit significant levels of 2-BFI appropriate responding, of the same inhibitors. Secondly a series of experiments looked at the long term effects, i.e., chronic administration, of the same MAO inhibitors on levels of [^{3}H]2-BFI specific binding in the same nuclei.

2). Method

2.1). Animals

All experiments used Wistar rats (Aston University, UK). *In vitro* pre-incubation experiments used four rats. *In vivo* acute administration experiments used a total of eight rats – four control, four drug dosed. Chronic dosing experiments used ten rats in total – five controls and five drug dosed.

In acute *in vivo* MAO inhibitor experiments; deprenyl (4mg/kg) was administered 20 minutes before brain extraction and cryo-protection; tranylcypromine (2.5mg/kg) was administered 45 minutes before brain extraction and cryo-protection. In chronic MAO inhibitor experiments; deprenyl (4mg/kg), tranylcypromine (2.5mg/kg) and pargyline (30mg/kg) were administered once a day for five days. Control groups were dosed with 0.9% saline vehicle (1ml/kg)

2.2). Autoradiographical Analysis

Rat brain sections were extracted and mounted according to the method outlined previously, except for the following modifications.

Pre-incubation and *in vivo* acute experiments used a concentration of 0.5 nM [^{3}H]2-BFI. The *in vivo* chronic treatment experiments used a concentration of 1 nM [^{3}H]2-BFI. Previous homogenate work has indicated chronic treatment markedly reduces [^{3}H]idazoxan binding. Hence, the concentration of [^{3}H]2-BFI was increased to 1 nM so as to retain some levels of specific binding, within drug treated rats, to aid nucleus identification.

For the pre-incubation experiments rat brains, from drug naive rats, were sectioned until the appropriate nuclei were isolated. An initial section was taken for cresyl violet staining, another for total, another for NSB, then a section for pre-incubation with 10^{-9} M MAO inhibitor, then another for 10^{-8} M MAO inhibitor and so on until a final section for pre-incubation with 10^{-4} M MAO inhibitor. Hence, specific binding (total – NSB) represents a control condition to compare to those adjacent sections that had been pre-incubated with the six concentrations of MAO inhibitors.

Acute and chronic experiments followed the sectioning protocol outlined for experiments in two and three in Chapter 9, i.e, once the nucleus had been isolated a 30μM section was taken for cresyl violet staining, then a section for total, then another for NSB. The total and NSB combination was repeated twice more and a final 30μM section was taken for cresyl violet staining – resulting in three sections for total and three for NSB, per animal.

In vitro pre-incubation experiments followed the protocol outlined in the method except after first 30 minute wash the MAO inhibitors (200μl; deprenyl, pargyline or tranylcypromine; dissolved in buffer) at six concentrations (10^{-9} M to 10^{-4} M) was applied to the brain sections. Buffer (200μl) was applied to the control specific and NSB binding sections. Deprenyl was given a 20 minute incubation time, tranylcypromine a 45 minute incubation time and pargyline a 45 minute incubation time; before one wash for 20 seconds in ice cold buffer, then rapid drying with a hairdryer (cool setting) and commencement with the assay outlined in general method.

Data for the pre-incubation experiment differed from that outlined in the general method. Rather than data for each nuclei being drawn from three sections, it was drawn from one section - due to the design of the experiment data was derived for each condition (total, NSB and 10^{-9} M MAO inhibitor to 10^{-4} M MAO inhibitor; area postrema, arcuate nucleus, interpeduncular nucleus or pineal gland) from one rather than three sections per animal. Data points represent the average of three readings from each section.

3). Results

Figure 9.2A shows total binding of 0.5 nM [^{3}H]2-BFI to a 12 μM transverse rat brain section, derived from a saline treated animal within the deprenyl acute administration experiment. As with previous experiments, non specific binding was assessed by the presence of saturating concentrations (10μM) of BU224 in an adjacent 12μM brain section and was low (<10%). All nuclei were verified by comparison of the autoradiogram to adjacent cresyl violet stained slides (see figure 9.2B). For all experiments data was derived from three or four rats. For each nuclei identified densometric readings were taken from three sections within each individual rat brain. Three data readings were taken for each nucleus identified on each section.

3.1). Effects of acute administration of MAO inhibitors.

Analysis of these autoradiograms, using radioactive standards, gave rise to the fmol/mg levels reported in tables 9.1 and 9.2. The administration of 4mg/kg deprenyl 20 minutes before brain extraction and cryo-protection gave rise to a significant ($p<0.05$) reduction in 0.5 nM [^{3}H]2-BFI specific binding within the pineal gland of deprenyl treated rats in comparison to saline injected controls, see table 9.1.

Table 9.1). ***Deprenyl in vivo acute – pre-treatment with a single dose of 4mg of Deprenyl***

Region	Control Specific Binding 0.5nM [^{3}H]2-BFI fmol/mg tissue	Deprenyl Specific Binding 0.5nM [^{3}H]2-BFI fmol/mg tissue	Sig[1]
	M. ± SEM.	*M. ± SEM.*	
Interpeduncular Nucleus (Ip)	38.10 ± 4.18	35.20 ± 3.46	ns.
Arcuate Nucleus (Arc)	77.82 ± 13.70	57.45 ± 7.87	ns.
Pineal Gland (Pi)	50.81 ± 2.53	23.95 ± 5.02	P<0.05
Area Prostrema (Ap)	91.06 ± 18.13	79.03 ± 14.09	ns.

*Mean ± Standard error of the mean. Sig[1], significant difference (one way ANOVA) between drug treated and saline treated rats.

In contrast to this the acute (2.5mg/kg) administration of tranylcypromine 45 minutes before brain extraction and cryo-protection, significantly increased levels of 0.5 nM [^{3}H]2-BFI specific binding in all nuclei assessed ($p<0.05$), see table 9.2. This increase can also been seen in pseudo-colour images shown in figure 9.3. Figure 9.3A depicts total binding of 0.5 nM [^{3}H]2-BFI in the arcuate nucelus of a saline treated animals, conversely, figure 9.3C shows specific binding within the arcuate nucleus of an animal that had experienced acute tranylcypromine administration. The presence of the arcuate was verified by staining adjacent sections with cresyl violet.

Table 9.2). *Tranylcypromine in vivo acute – pre-treatment with a single dose of 2.5mg/kg of Tranylcypromine*

Region	Control Specific Binding 0.5nM [^{3}H]2-BFI fmol/mg tissue	Tranylcypromine Specific Binding 0.5nM [^{3}H]2-BFI fmol/mg tissue	Sig[1]
	M. ± SEM.	*M. ± SEM.*	
Interpeduncular Nucleus (Ip)	28.47 ± 2.32	45.59 ± 0.64	P<0.001
Arcuate Nucleus (Arc)	56.58 ± 3.62	106.16 ± 6.56	P<0.001
Pineal Gland (Pi)	26.11 ± 1.61	44.95 ± 1.43	P<0.001
Area Prostrema (Ap)	38.62 ± 2.96	69.25 ± 4.28	P<0.005

Details as table 9.1

3.2). Effects of chronic administration of MAO inhibitors.

In a subsequent series of experiments we assessed the binding of 1nM [^{3}H]2-BFI within the aforementioned brain regions after rats had been chronically treated with deprenyl, tranylcypromine and pargyline. Figure 9.4a depicts total binding of 1nM [^{3}H]2-BFI to 12 μM transverse rat brain section, derived from a saline treated animal within the deprenyl chronic administration experiment. As with previous experiments, non specific binding was assessed by the presence of saturating concentrations (10μM) of BU224 in an adjacent 12μM brain section and despite increasing the concentration from 0.5 nM to 1nM [^{3}H]2-BFI NSB was low (<10%; see figure 9.4b).

Table 9.3). *Deprenyl in vivo chronic – pre-treatment, over five days, with five doses of 4mg of Deprenyl*

Region	Control Specific Binding 1nM [^{3}H]2-BFI fmol/mg tissue	Deprenyl Specific Binding 1nM [^{3}H]2-BFI fmol/mg tissue	Sig[1]
	M. ± SEM.	*M. ± SEM.*	
Interpeduncular Nucleus (Ip)	31.97 ± 4.10	13.60 ± 2.62	P<0.01
Arcuate Nucleus (Arc)	88.00 ± 3.44	23.60 ± 5.08	P<0.001
Pineal Gland (Pi)	32.51 ± 4.98	10.50 ± 1.18	P<0.01
Area Prostrema (Ap)	115.98 ± 26.16	24.24 ± 4.79	P<0.01

Details as table 9.1

Comparison of autoradiograms to radioactive standards, indicated that the chronic administration of deprenyl gave rise to significant reductions in [^{3}H]2-BFI within all nuclei assessed, see table 9.3. Within the arcuate nucleus this decrease can also been seen when comparing the autoradiograms from a saline treated rat (figure 9.5A) and chronically treated rat (figure 9.5C). As mentioned in the method the presence of the arcuate nucelus was verified with adjacent cresyl violet stained slides, see figure 9.5B and 9.5D.

Table 9.4). *Tranylcypromine in vivo chronic – pre-treatment, over five days, with five doses of 2.5mg/kg of Tranylcypromine*

Region	Control Specific Binding 1nM [^{3}H]2-BFI fmol/mg tissue	Tranylcypromine Specific Binding 1nM [^{3}H]2-BFI fmol/mg tissue	Sig[1]
	M. ± SEM.	*M. ± SEM.*	
Interpeduncular Nucleus (Ip)	52.00 ± 2.11	28.95 ± 4.98	P<0.01
Arcuate Nucleus (Arc)	169.89 ± 6.97	122.26 ± 13.54	P<0.05
Pineal Gland (Pi)	41.62 ± 4.51	23.76 ± 2.40	P<0.05
Area Prostrema (Ap)	104.98 ± 5.73	43.01 ± 9.69	P<0.005

Details as table 9.1

The chronic administration of tranylcypromine gave similar results, see table 9.4. Levels of [^{3}H]2-BFI binding were significantly reduced within the interpeduncular nucleus (P<0.01), arcuate nucleus (P<0.05), pineal gland (P<0.05) and area postrema (P<0.005). The chronic administration of

pargyline significantly reduced levels of [^{3}H]2-BFI within arcuate nucleus (P<0.05) and area postrema (P<0.005), see table 9.5.

Table 9.5). *Pargyline in vivo chronic – pre-treatment, over five days, with five doses of 30mg/kg of Pargyline*

Region	Control Specific Binding 1nM [^{3}H]2-BFI fmol/mg tissue	Pargyline Specific Binding 1nM [^{3}H]2-BFI fmol/mg tissue	Sig[1]
	M. ± SEM.	*M. ± SEM.*	
Interpeduncular Nucleus (Ip)	36.50 ± 2.18	28.75 ± 2.63	ns.
Arcuate Nucleus (Arc)	124.50 ± 16.89	80.20 ± 7.69	P<0.05
Pineal Gland (Pi)	21.33 ± 4.81	19.40 ± 2.18	ns.
Area Prostrema (Ap)	112.75 ±10.44	59.80 ± 7.34	P<0.005

Details as table 9.1

3.3). Effects of in vitro pre-incubation.

Table 9.6 to 9.8 show the effects of various concentrations of deprenyl and pargyline on levels of [^{3}H]2-BFI specific binding. Pre-incubation for 45 minutes with 10^{-9} to 10^{-6} deprenyl or pargyline failed to significantly alter levels of specific [^{3}H]2-BFI binding. The same was true for rat brain sections that had been pre-incubated with tranylcypromine. However, pre-incubation with tranylcypromine did have an effect on [^{3}H]2-BFI in the arcuate nucleus, see figure 9.6.

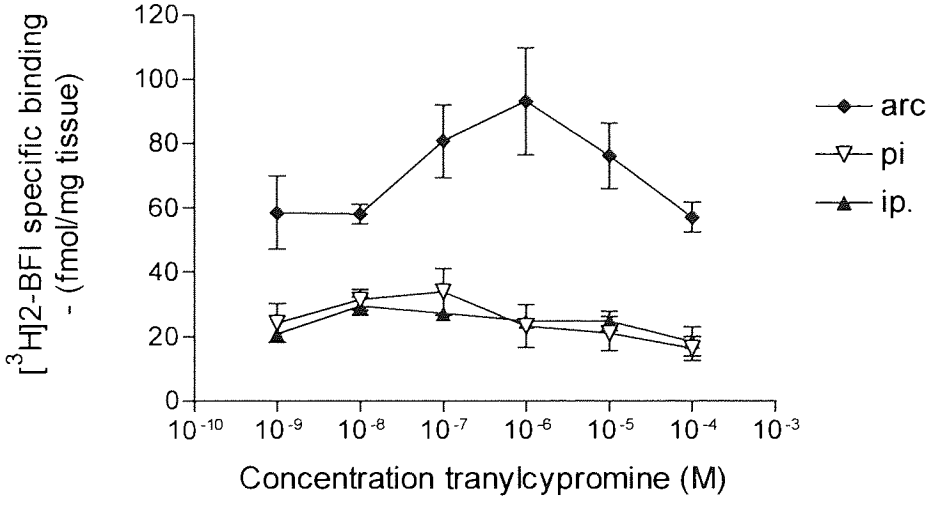


Figure 9.6). *Alterations of [^{3}H]2-BFI specific binding by tranylcypromine*

Table 9.6). ***In vitro effect of various deprenyl concentrations on [^{3}H]2-BFI binding in the rat CNS***

Region	0.5 nM [^{3}H]2-BFI specific binding (fmol/mg tissue)						
		Concentration deprenyl					
	Control	*1nM*	*10nM*	*100nm*	*1μM*	*10μM*	*100μM*
Interpenduncular Nucleus (IP)	30.57 ± 3.45	36.74 ± 3.64	33.54 ± 2.83	35.74 ± 4.77	31.65 ± 5.06	34.65 ± 4.59	31.25 ± 2.03
Arcuate Nucleus (Arc)	109.43 ± 14.32	86.40 ± 12.30	100.21 ± 8.78	89.34 ± 5.11	100.95 ± 0.52	117.29 ± 6.77	97.24 ± 6.21
Pineal Gland (Pi)	27.99 ± 2.12	26.92 ± 0.73	24.19 ± 1.40	25.16 ± 2.32	25.88 ± 3.29	30.33 ± 3.04	25.63 ± 5.47
Area Prostrema (Ap)	97.76 ± 25.89	94.70 ± 14.07	100.94 ± 21.98	102.41 ± 18.45	113.65 ± 12.78	110.58 ± 17.28	113.08 ± 2.17

No significant differences were found between any of the variables. Data for each nucleus, under each condition (total, NSB, and each concentration of deprenyl) was derived from one section per rat. Data derived from four rats. *Mean ± Standard Error of the Mean.

Table 9.7). ***In vitro effect of various tranylcypromine concentrations on [^{3}H]2-BFI binding in the rat CNS***

Region	0.5 nM [^{3}H]2-BFI specific binding (fmol/mg tissue)						
		Concentration tranylcypromine					
	Control	*1nM*	*10nM*	*100nm*	*1μM*	*10μM*	*100μM*
Interpenduncular Nucleus (IP)	23.10 ± 4.79	20.87 ± 1.15	28.77± 2.09	27.24 ± 0.93	24.89 ± 1.53	24.80 ± 2.99	16.33 ± 3.75
Arcuate Nucleus (Arc)	72.99 ± 4.61	58.51 ± 11.4	58.12 ± 2.99	80.71 ± 11.28	93.05 ± 16.63	76.15 ± 10.14	57.04 ± 4.67
Pineal Gland (Pi)	26.16 ± 6.61	24.18 ± 6.00	32.93 ± 4.04	33.86 ± 7.19	23.25 ± 6.68	21.04 ± 5.35	16.33 ± 3.75

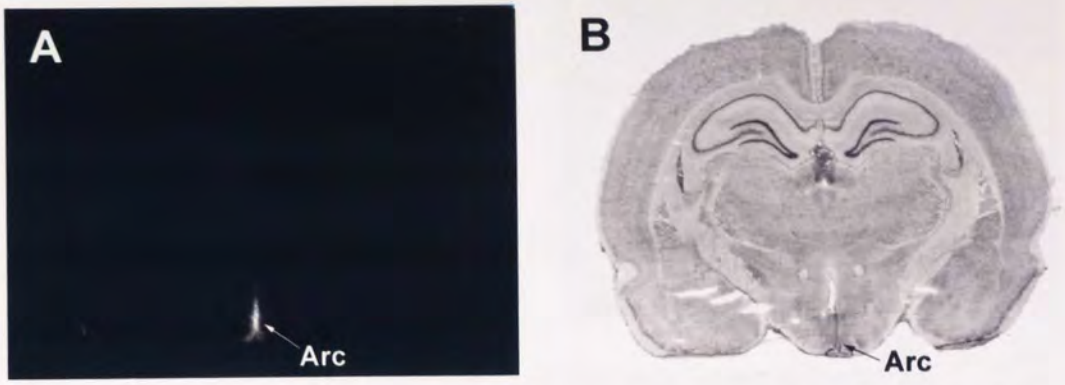
No significant differences were found between any of the variables. Data for each nucleus, under each condition (total, NSB, and each concentration of tranylcypromine) was derived from one section per rat. Data derived from four rats. *Mean ± Standard Error of the Mean.

Table 9.8). ***In vitro effect of various pargyline concentrations on [^{3}H]2-BFI binding in the rat CNS***

Region	0.5 nM [^{3}H]2-BFI specific binding (fmol/mg tissue)						
		Concentration pargyline					
	Control	*1nM*	*10nM*	*100nM*	*1μM*	*10μM*	*100μM*
Interpenduncular Nucleus (IP)	25.89 ± 2.03	32.56 ± 4.56	29.09 ± 3.93	29.26 ± 2.47	30.18 ± 4.55	31.07 ± 2.95	25.70 ± 3.68
Arcuate Nucleus (Arc)	39.80 ± 9.40	59.66 ± 2.98	65.24 ± 11.08	68.17 ± 9.11	45.39 ± 5.70	48.08 ± 3.79	49.16 ± 5.02
Pineal Gland (Pi)	22.07 ± 2.28	32.81 ± 2.17	28.56 ± 3.50	28.09 ± 1.67	27.66 ± 1.92	27.64 ± 1.24	21.93 ± 1.75
Area Prostrema (Ap)	41.88 ± 5.03	40.70 ± 2.98	45.74 ± 3.89	48.24 ± 5.04	59.22 ± 6.11	53.75 ± 4.81	62.03 ± 3.62

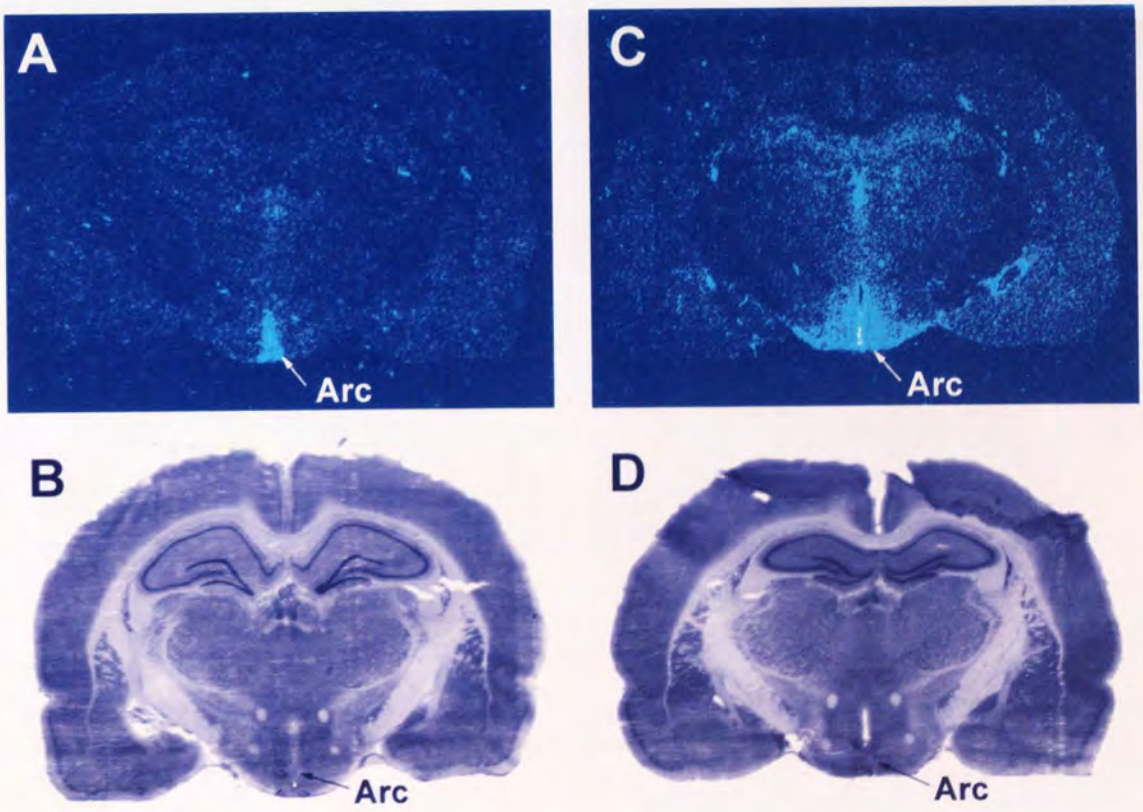
No significant differences were found between any of the variables. Data for each nucleus, under each condition (total, NSB, and each concentration of pargyline) was derived from one section per rat. Data derived from four rats. *Mean ± Standard Error of the Mean.

Figure 9.2). ***Total binding (section A) of 0.5nM [^{3}H]2-BFI to rat transverse brain section and adjacent cresyl violet (section B) stained rat transverse brain section.***



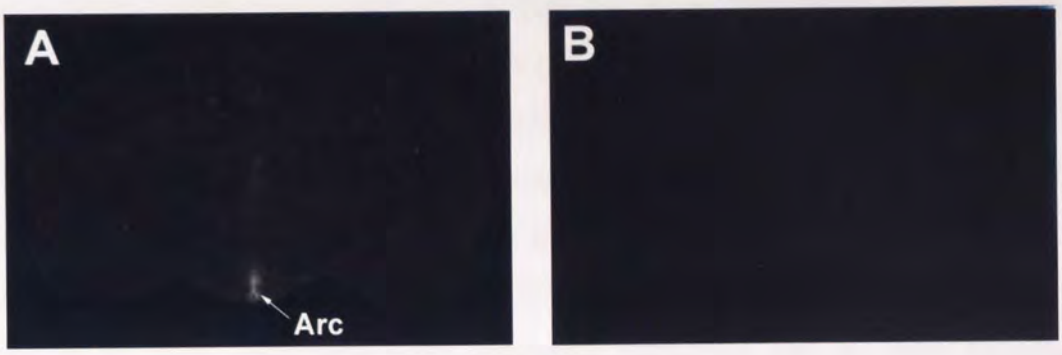
Data derived from saline treated rat in acute deprenyl experiment. Section taken –3.30mm from the bregma line. See table 9.1 for abbreviations.

Figure 9.3). ***Pseudo-colour images of 0.5nM [^{3}H]2-BFI total binding and adjacent cresyl violet stained section, in saline treated rats (section A, section B respectively) and rats treated acutely with tranylcypromine (section C, section D respectively).***



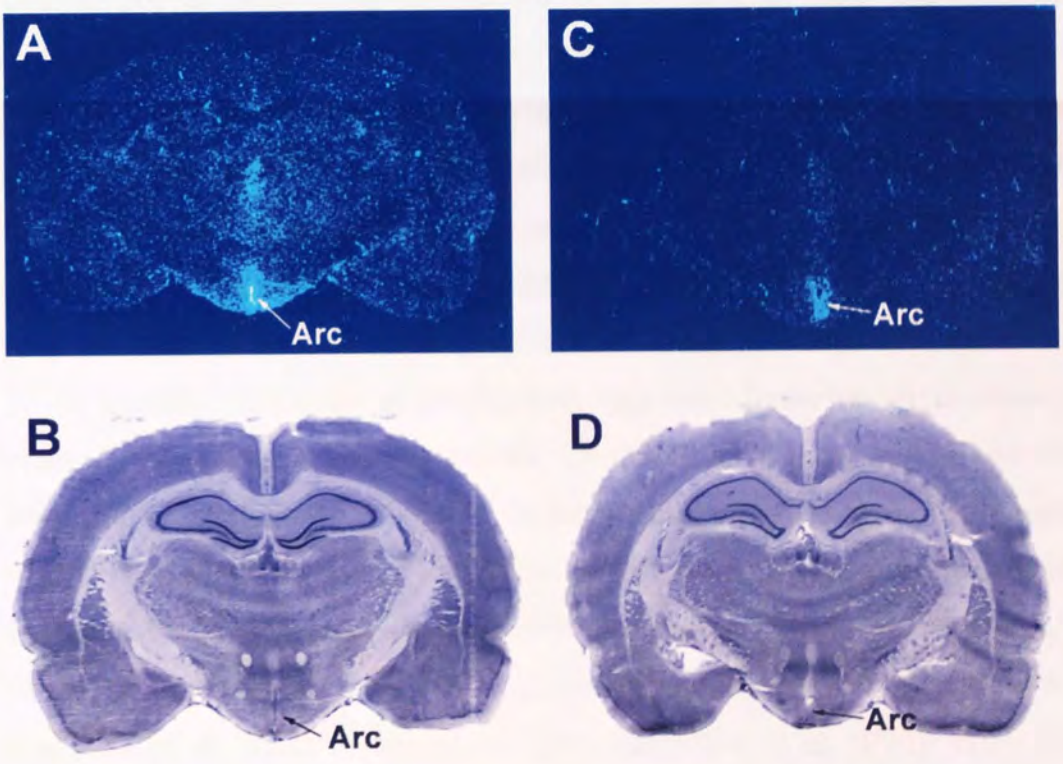
See table 9.1 for abbreviations. Sections taken -3.30mm from the bregma line.

Figure 9.4). ***Total binding (section A) and NSB binding (section B) of 1nM [^{3}H]2- BFI in rat transverse brain sections***



Section taken –3.30mm from the bregma line. See table 9.1 for abbreviations.

Figure 9.5). ***Pseudo-colour images of 1nM [^{3}H]2-BFI total binding and adjacent cresyl violet stained sections, in saline treated rats (section A, section B respectively) and rats chronically treated with deprenyl (section C, section D respectively).***



Section taken –3.30mm from the bregma line. See table 9.1 for abbreviations.

4). Discussion

The aim of this study was to explore the effects of monoamine oxidase inhibitors on four rat brain nuclei that we have previously shown to have high levels of [^{3}H]2-BFI binding. The pre-incubation of rat brain sections with tranylcypromine, deprenyl and pargyline, at variety of concentrations failed to alter [^{3}H]2-BFI binding, within any of the nuclei assessed. The *in vivo* acute administration of 2.5 mg/kg tranylcypromine significantly increased levels of [^{3}H]2-BFI binding within all regions assessed. In contrast to this, acute *in vivo* treatment with deprenyl (4mg/kg) led to non significant reductions in all regions except the pineal gland which showed significant reductions in [^{3}H]2-BFI binding. The chronic treatment of rats with deprenyl and tranylcypromine significantly reduced [^{3}H]2-BFI binding in all regions assessed. Similarly chronic treatment with pargyline reduced binding in all regions, but only significantly within the arcuate nucleus and area postrema. The failure of short-term i.e., acute *in vivo* and *in vitro* pre-incubation, experiments with acetylenic MAO inhibitors (deprenyl and pargyline) to alter [^{3}H]2-BFI specific binding is probably through two factors. Firstly, autoradiographical studies using radiolabelled MAO inhibitors indicate MAO specific binding is reduced to non-specific binding levels 2 hours after the *in vivo* administration of irreversible MAO inhibitors and levels within the brain only return to 50% of their original amount after 13 days (Richards et al, 1998). Hence, incubation or dosing time may have been too short. However, at the dosing times used, 45 minutes, we would have expected to see some alterations. The failure of these short-term experiments may be for a second reason, i.e., their method of inhibition and thus binding site on the enzyme. The acetylenic MAO inhibitor pargyline and deprenyl have a low affinity for the I_2 site and inhibit MAO by covalently binding to the flavin cofactor (Carpene et al, 1995; Olmos et al, 1993; Kalgutkar et al, 1995). Whereas I_2 ligands bind to a site that is reported to be distinct to this region (Raddatz et al, 1997). Hence, in the short term, reductions in [^{3}H]2-BFI binding would not occur as these MAO inhibitors bind to different sites on the enzyme and do not block pargyline or deprenyl's access to it. However, short term

experiments with the cyclopropylamine tranylcypromine did show a potentiation in levels of [^{3}H]2-BFI binding within a number of nuclei. In rats treated acutely with tranylcypromine (one dose of 2.5mg/kg tranylcypromine 45 minutes before brain extraction and cryo-protection) levels of [^{3}H]2-BFI were significantly elevated in all areas assessed. In their initial study, Wiest and Steinberg (1997) report that tranylcypromine failed to potentiate [^{3}H]2-BFI binding within the rat cerebral cortex. In the light of the levels of rat cerebral [^{3}H]2-BFI binding reported in the previous chapter their result is not surprising – we imaged discrete I_2 associated nuclei that have high to moderate levels of [^{3}H]2-BFI binding.

In the short term experiments reported here and elsewhere, the ability of some MAO inhibitors to alter I_2 ligand binding, whilst others do not probably lies in their method of MAO inhibition. Previous short term experiments have indicated that pre-incubation of rat cerebral membranes with the acetylenic MAO inhibitors clorgyline or pargyline, but not with the hydrazine phenelzine, reduces [^{3}H]idazoxan I_2 site density. Hydrazines, in comparison to acetylenic and cyclopropylamine MAO inhibitors, covalently bond to a different site on the flavin co-factor and this may modulate a change in MAO conformation. This theory would account for the ability of tranylcypromine to potentiate [^{3}H]2-BFI binding. Although, the [^{3}H]2-BFI I_2 site density increases with tranylcypromine co-incubation, it is unlikely that this indicates an increase in MAO. Rather it indicates an increase in the number of binding sites within the already existing population of MAO. The literature does indicate that in addition to covalently bonding to the flavin co-factor tranylcypromine associates with the protein (Kalgutkar et al, 1995). This theory is supported by the failure of pargyline and deprenyl in the short term to alter [^{3}H]2-BFI binding. However, the pineal gland did show a significant reduction in levels of [^{3}H]2-BFI binding when compared with saline vehicle dosed controls. This is not consistent with the underlying theory. As mentioned in Chapter 8 levels of [^{3}H]2-BFI binding differ markedly across the pineal. Hence, it is possible that this is an experimental artefact. However, an alternate explanation would suggest that the pineal gland

contains a subgroup of MAO sites in which deprenyl and [^{3}H]2-BFI bind to the same site.

The results of the chronic experiments support the above hypothesis, i.e., I_2 ligands bind to a site distinct to that of other irreversible MAO inhibitors, as after chronic treatment levels of [^{3}H]2-BFI specific binding were reduced within the selected nuclei. It is unclear if this represents a loss in the actual enzyme or only its catalytic ability. The data in this case allows the suggestion that reductions observed after chronic, but not with acute, treatment may be due to the turnover of the enzyme and thus loss of binding site. Interestingly animals that had been chronically dosed with deprenyl, but not pargyline, significant reductions in [^{3}H]2-BFI specific binding were found in all areas suggesting certain rat brain nuclei are more sensitive to reduction by particular MAO inhibitors.

Previous studies have indicated after *in vivo* chronic treatment with either pargyline or deprenyl will irreversibly inhibit both MAO-A and –B activity (Butcher et al, 1990). Hence, it is speculative to suggest that these subpopulations are associated with MAO-A or MAO-B and in any case deprenyl is the more specific MAO-B inhibitor but shows the lower specificity of the two acetylenic MAO inhibitors tested (Butcher et al, 1990), i.e., deprenyl significantly reduces levels of [^{3}H]2-BFI in all nuclei, whereas pargyline only reduces it in the arcuate nucleus and area postrema. If these subpopulations were associated with the -A and -B isoforms the opposite should theoretically occur. It may be the case that the I_2 MAO site may be located on a subpopulation of both MAO-A and –B. These populations may differ with respect to post-translational modifications or may even be unidentified enzymes that share the pharmacological characteristics of MAO. In support of this hypothesis a novel form of MAO has been isolated within the arcuate nucleus. The enzyme isolated had an apparent molecular weight of less than 20 kDa, metabolised [^{14}C]dopamine to DOPAC (MAO-B associated), but was inhibited by clorgyline (MAO-A specific), with complete inhibition occurring at 1 mM. High concentrations of deprenyl (MAO-B specific; 1-10 mM) showed only partial inhibition of the dopamine metabolising enzyme (Sim & Lim, 1992).

The results from this study indicate that the chronic treatment of rats with a number of MAO inhibitors dramatically reduces levels of [^{3}H]2-BFI specific binding. Chronic treatment with two out of three of the irreversible inhibitors reported here reduced levels of [^{3}H]2-BFI specific binding in all nuclei assessed. However, the failure of pargyline to have this effect suggests subpopulations of I_2-sites exist which differ in their sensitivity to these inhibitors. However, this decrease was not found after acute administration or *in vitro* pre-incubation with the same MAO inhibitors. This data is consistent with the theory that I_2 ligands bind to a site on monoamine oxidase that is distinct from the acetylenic or cyclopropylamine site and suggests alterations in [^{3}H]2-BFI specific binding can only be gauged after sufficient time has accumulated for the enzyme MAO to be metabolised.

Chapter 10

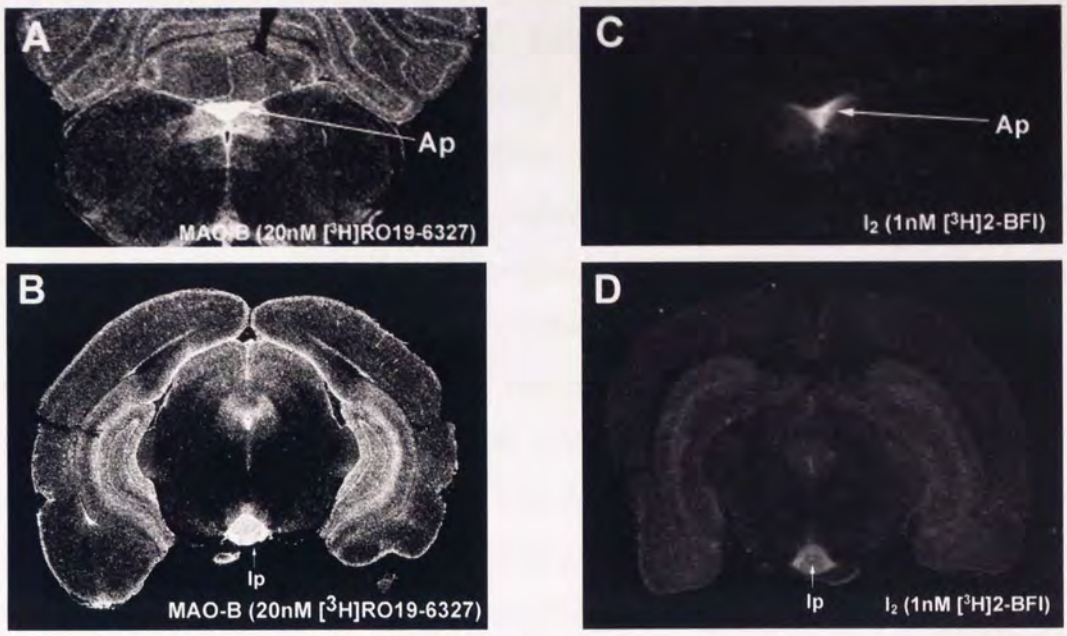
Autoradiography: Levels of [^{3}H]2-BFI binding in transgenic and control MAO-B C57/B mice

1). Introduction

Data presented in previous chapters and elsewhere has indicated that a close relationship exists between I_2 sites and MAO-B (Chapter 7; Chapter 9; Lalies et al, 1999; Raddatz et al, 1995; Alemany et al, 1995a). Recently, transgenic animals who express the MAO-B isoform an estimated 4 to 6 fold more than control litter mates, have been reported in the literature (Richards et al, 1998). Hence, the current chapter mapped the regional distribution of [^{3}H]2-BFI I_2 sites within these transgenic mice and compared them to that found in control animals.

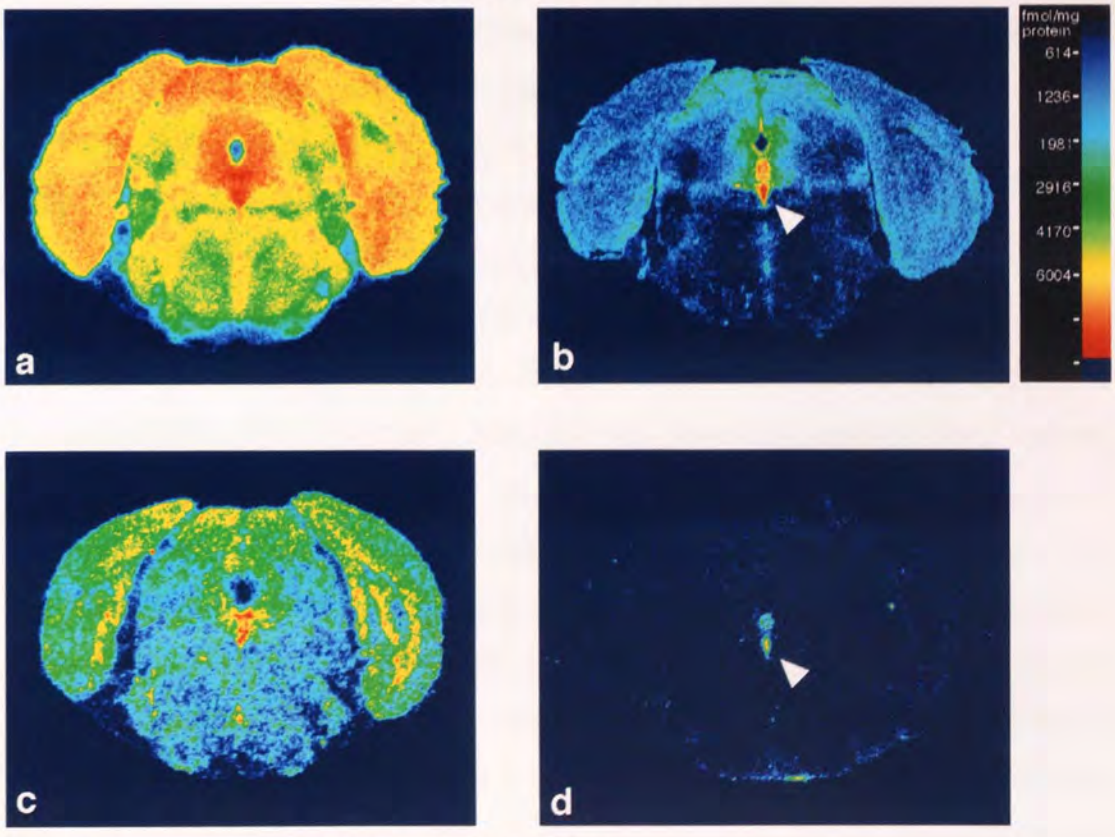
A great deal of previous evidence has linked the enzyme monoamine oxidase with I_2 sites. Imidazoline ligands inhibit MAO with a good potency, antibodies specific for MAO immuno-precipitate I_2 radiolabelled proteins and the *in vivo* chronic administration of monoamine oxidase ligands reduces I_2 radioligand binding (Lalies et al, 1999; Raddatz et al, 1995; Alemany et al, 1995a). Further studies which express human MAO-B in yeast and bacterial cells show these enzymes exhibit spontaneous tritiated I_2 ligand binding (Tesson et al, 1995; Steinberg, et al, 1999). However, this relationship is far from clear as monoamine oxidase inhibitors have a low affinity for the I_2 site (Olmos et al, 1993; Alemany et al, 1995a).

Figure 10.1). *[^{3}H]Ro19-6327 (section A and B) and [^{3}H]2-BFI (Section C and D) binding within the area postrema and interpeduncular nucleus.*



A and B reproduced with courtesy of Saura et al, (1992) C and D derived from experiment 3 Chapter 8.

Figure 10.2). *Distribution of MAO-B protein (a,b) and mRNA (c,d) in adjacent transverse sections of transgenic (a,c) vs wild type (b,d) mouse brains. Arrow depicts dorsal raphe*



Reproduced with courtesy of Richards et al, (1998)

Rat brain studies have indicated that [^{3}H]2-BFI binding bears a closer resemblance to the autoradiographical localisation of MAO-B, not MAO-A (Chapter 7 and 8; Lione et al, 1998; Saura et al, 1992). Figure 10.1 shows the total binding component of 20nM [^{3}H]Ro19-6327 (section A and B; reproduced with courtesy from Saura et al, 1992) and 1nM [^{3}H]2-BFI (section C and D, derived from experiment 3 Chapter 8) to the area postrema (section A and B) and interpeduncular nuclei (section C and D). [^{3}H]Ro19-6327 is a specific reversible inhibitor of MAO-B and autoradiographical studies indicate binding within the previously mentioned nuclei are associated with MAO-B not MAO-A (Saura et al, 1992). In addition to this, levels of MAO-B are high in a number of other regions identified with [^{3}H]2-BFI. Namely, the arcuate nucleus, subfornical organ, area postrema, pineal gland and dorsal raphe (see Chapter 8; Saura et al, 1992). These regional differences also match the distribution of MAO-B mRNA, see table 1.6 (Luque et al, 1995). Chapter 9 contained data which indicated the chronic administration of irreversible MAO inhibitors reduced levels of [^{3}H]2-BFI I_2 site binding. Consistent with there being a relationship between I_2 sites and MAO-B, levels of [^{3}H]2-BFI binding were significantly reduced within the, interpeduncular nucleus, arcuate nucleus, area postrema and pineal gland. In addition to this patterns of binding by I_2 and MAO-B radioligands to MAO-B peptide fragments has indicated that I_2 ligands inhibit MAO at a site distinct to that of other inhibitors (Raddatz et al, 1997; Raddatz et al, 1999). Hence, the differing binding sites for MAO inhibitors and I_2 ligands on MAO could readily explain the low affinity of these inhibitors have for the I_2 site, but this does not resolve other issues, i.e., the success of MAO-A, over MAO-B inhibitors, in drug discrimination or the preference of I_2 ligands for inhibiting MAO-A over MAO-B *in vitro*. The data presented in Chapters 7 and 8 indicate a number of reversible selective inhibitors of MAO-A significantly substitute for 2-BFI. However, similar studies using specific reversible inhibitors of MAO-B fail to substitute for 2-BFI. In addition I_2 ligands such as 2-BFI, BU224 and BU216 inhibit MAO-A *in vivo* selectively and with a good potency (Lalies et al, 1999). These data are consistent with there being a relationship with MAO-A rather than MAO-B,

The literature contains only one autoradiographical study that reports the distribution of MAO-A and MAO-B in mice (Saura et al, 1994). However, this study looks at age related changes in [^{3}H]Ro41-1049 (MAO-A) and [^{3}H]Ro19-6327 (MAO-B) and only assesses specific binding in a limited number of nuclei. The highest levels (>2000 fmol/mg tissue) of [^{3}H]Ro19-6327 specific binding were detected within the substantia nigra, nucleus accumbens and superior colliculus (Saura et al, 1994). Moderate to high levels of specific binding (1500-2000 fmol/mg tissue) were found within the frontal cortex, inferior colliculus and caudate putamen. Low to moderate levels of [^{3}H]Ro19-6327 specific binding (1000-1500 fmol/mg tissue) were found within a larger number of nuclei, i.e, parietal cortex, occipital cortex and granular layer of the cerebellum. The lowest levels (<1000 fmol/mg tissue) of specific binding were found within the, corpus callosum, molecular layer and white matter of the cerebellum (Saura et al, 1994).

Recently a line of transgenic mice has been generated that over-express human MAO-B protein under the control of the neuron-specific enolase promoter (Richards et al, 1998). Verification of these transgenic animals was established with in situ hybridisation and receptor autoradiography, see figure 10.2. These heterozygotic mice over express MAO-B in the brain an estimated four to six fold more than non-transgenic littermates. Levels of MAO-B within the liver were the same as that of control litter mates, but as illustrated in figure 10.2 levels of MAO-B protein and mRNA are markedly increased. Analysis of brain monoamines indicated transgenic mice showed a 30-40% increase in basal levels of the dopamine metabolites DOPAC and HVA, but no change in levels of dopamine.

Hence, the current study sought to initially localise the levels of [^{3}H]2-BFI within C57/B mouse brains and then compare these to levels found within their transgenic littermates.

2). Method

2.1). Animals

Six transgenic MAO-B drug naive male SPF (specific pathogen free; 25-30g; Dr Grayson Richards, Hoffman La Roche, Basel Switzerland) C/57B mice and six non-transgenic litter mates were anaesthetised with fluothane. These animals were killed by decapitation and their brains rapidly removed and placed in a mouse brain blocker. All brains were blocked at approximately 0.8mm from the Bregma line, placed on cork disks, then frozen by gently sprinkling crushed dry ice around them.

2.2). Autoradiographical Analysis

Brain sections were extracted and mounted according to the method outlined previously. All experiments were based on the protocol outlined in the method except for a modification to wash times (see below: 2.2.1. Wash times). Brain structures were identified by comparison of autoradiograms and adjacent cresyl violet stained slides to the mouse brain atlas (Franklin and Paxinos, 1997). The K_D of [^{3}H]2-BFI was estimated to be between 2 and 4 nM (Alan Hudson, personal communication). Preliminary experiments indicated 0.5 nM [^{3}H]2-BFI give rise to levels of specific [^{3}H]2-BFI binding no greater than NSB. Hence, specific binding was assessed with 2nM [^{3}H]2-BFI. Brain sections and experimental conditions were randomised across boxes in the method described in Chapter 8.

2.2.1). Wash times

Preliminary scintillation experiments were conducted to optimise post wash times. Briefly, the autoradiographical assay was conducted as normal, except radioactive sections were scraped in scintillation vials instead of apposed to tritium sensitive film. Scintillation vials, radioactive sections, 100μM tissue solubilizer (NCS II; Amersham, UK) and 4ml scintillation fluid (Optiphase Hisafe 3; Wallac, UK) were placed in a Packard 1900 scintillation counter (Packard, USA). This data indicated post wash times of

two washes for 10 seconds were optimal for giving the largest difference between total and NSB components of ligand binding.

3). Results

Figure 10.3A (control) and 10.3D (transgenic) show total binding of 2nM [^{3}H]2-BFI within 12 μM rat transverse brain sections. Non specific binding was assessed by the presence of saturating concentrations (10μM) of BU224 in an adjacent 12 μM brain section, see figure 10.3B and 10.3E. These images reverse phase images of original autoradiography films. Data analysis using MCID defined that NSB (non-specific binding) was negligible (<20%; see figure 10.3B and 10.3E). Figure 10.3C and 10.3E show 30μM mouse brain sections, stained with cresyl violet, that lie adjacent to each total binding section. Areas of tritiated 2-BFI binding were verified by comparison of autoradiograms to cresyl violet stained sections and the mouse brain atlas (Franklin and Paxinos, 1997). Data was derived from five or six mice. For each nucleus identified densometric readings were taken from three sections within each individual mouse brain. Three data readings were taken for each nucleus identified on each section.

3.1). Levels of [^{3}H]2-BFI specific binding within control C57/B mice

Analysis of these autoradiograms, using radioactive standards, gave rise to the fmol/mg levels of specific binding reported in table 10.1. All cortical areas, except the olfactory tubercle (24.54 fmol/mg tissue) showed low levels (<20 fmol/mg tissue) of [^{3}H]2-BFI specific binding, see table 10.1. Similarly all layers of the cerebellum showed low levels of binding except the Purkinje cells which exhibited low to moderate (20-40 fmol/mg tissue) levels of [^{3}H]2-BFI specific binding. Virtually all areas of the telencephalon showed low levels of [^{3}H]2-BFI specific binding except for one notable exception the nucleus accumbens showed moderate to high (40-60 fmol/mg tissue) levels (59.20 fmol/mg tissue; see figure 10.4A). Control C57/B mice also showed high levels of [^{3}H]2-BFI specific binding (>60 fmol/mg tissue) within the posterior (72.19 fmol/mg tissue), medial (70.52 fmol/mg tissue) and anterior paraventricular thalamus (63.16 fmol/mg tissue). Levels of [^{3}H]2-BFI total binding within the posterior paraventricular thalamus can also be seen on the pseudo-colour images generated from the original autoradiograms (see

figure 10.5A). All other thalamic areas, except the reuniens thalamic group which exhibited moderate to high levels of specific binding (40-60 fmol/mg tissue), showed low levels of [^{3}H]2-BFI specific binding (<20 fmol/mg tissue). Most hypothalamic areas also showed low levels of [^{3}H]2-BFI specific binding, i.e., the ventromedial hypothalamic nuclei and the medial mammillary nucleus. However, the suprachiasmatic nucleus (20.13 fmol/mg tissue), dorsomedial hypothalamic nucleus (21.71 fmol/mg tissue), posterior hypothalamus (21.71 fmol/mg tissue) showed low to moderate levels (20-40 fmol/mg tissue) of specific binding and the ventral tuberomammillary nucleus (44.59 fmol/mg tissue) exhibited moderate to high levels.

The interpeduncular nuclei (Ip) also showed moderate to high levels of specific [^{3}H]2-BFI binding, see table 10.1. However, levels of binding were localised to subnuclei within the Ip, see figure 10.3A. The rostral and caudal interpeduncular subnuclei only exhibited low to moderate levels of specific binding, 27.45 and 24.13 fmol/mg tissue respectively, whilst the lateral/intermediate subnuclei exhibited moderate to high levels (51.74 fmol/mg tissue). Other areas of the mesencephalon also showed moderate to high levels of [^{3}H]2-BFI specific binding, notably the granular layer of the superior colliculus (28.51 fmol/mg tissue) and the peri-aqueductal gray (23.11 fmol/mg tissue).

The highest levels of [^{3}H]2-BFI specific binding were found within the dorsal raphe (dr; 76.04 fmol/mg tissue), see figure 10.6A. Figure 10.7A indicates that levels of [^{3}H]2-BFI total binding were also elevated in the locus coeruleus. Correspondingly table 10.1 indicates the LC exhibits moderate to high levels of [^{3}H]2-BFI specific binding (49.81 fmol/mg tissue). A number of other areas within the rhombencephalon exhibited low to moderate levels of [^{3}H]2-BFI specific binding, i.e., solitary tract (27.47 fmol/mg tissue), lateral parabracial nucleus (21.37 fmol/mg tissue) and the inferior olives (23.31 fmol/mg tissue). All other areas of the rhombencephalon showed low levels of specific binding (<20 fmol/mg tissue).

High levels of specific binding were also found within the lining of the lateral forth ventricle (L4V; 65.73 fmol/mg tissue) and aqueduct (Aq; 65.3 fmol/mg tissue) and the levels of binding within the lining of the L4V can be seen on

the pseudo-colour images found in figure 10.8A. Moderate to high levels (40-60 fmol/mg tissue) were found within the lateral ventricle (54.14 fmol/mg tissue, see figure 10.5A), 4th ventricle (47.66 fmol/mg tissue), dorsal 3rd ventricle (57.34 fmol/mg tissue), but not the 3rd ventricle (21.80 fmol/mg tissue) which showed moderate to low levels of [^{3}H]2-BFI specific binding, i.e., 20-40 fmol/mg tissue. The area postrema (43.57 fmol/mg tissue) showed moderate to high levels of [^{3}H]2-BFI specific binding. However, all other circumventricular organs, i.e., the arcuate nucelus, median eminence and subfornical organ all showed low levels of binding (<20 fmol/mg tissue).

3.2). Levels of [^{3}H]2-BFI specific binding within transgenic C57/B mice

In comparison to control mice, transgenic C57/B brain sections generally showed large increases in [^{3}H]2-BFI specific binding, see table 10.1. Most transgenic cortical areas showed high levels (300 to 500% increase) of [^{3}H]2-BFI specific binding when compared with controls. However, the entorhinal cortex, basolateral amygdaloid group, anterior commissure showed moderate increases (100-300% increase) whilst the granule layer of the olfactory bulb showed very high % increases in specific binding (>500% increase).

Percentage increases in [^{3}H]2-BFI specific binding in areas of the cerebellum varied. Data from transgenic white matter indicates % increases in specific binding is low when compared with controls (75%). However, actual levels of [^{3}H]2-BFI specific binding was very low in both regions (<10 fmol/mg tissue). This is contrast to the levels reported within the molecular layer which show very high % (>500%) increases in specific binding between controls and transgenic (7.62 Vs 51.48 fmol/mg tissue, respectively). The Purkinje cells within the control mouse cerebellum, as previously mentioned show low to moderate levels of [^{3}H]2-BFI specific binding (21.19 fmol/mg tissue). This level of binding increases to 81.69 fmol/mg tissue in transgenic mouse brain sections. However, this is only an increase of 286%. The remaining layer of the cerebellum, the dense granules, within transgenic mouse brains showed much higher % increases

in [^{3}H]2-BFI specific binding when compared with controls (450%; 9.71 Vs 53.40 fmol/mg tissue, respectively).

A similar pattern of specific binding was found within the telencephalon, i.e., high % increases (300-500%) in [^{3}H]2-BFI specific binding were found between the transgenic and control mouse hippocampus and septum. The fornix and corpus callosum showed only moderate % increases (100-300%) when compared with controls. However, actual levels of specific binding were low (<5 fmol/mg tissue) in both controls and transgenic mice, see table 10.1. Similarly all areas of the basal ganglia showed only moderate increases (100 to 300% increase) in specific binding when compared with control mice. However, in comparison to other areas of the basal ganglia the % increase between control and transgenic within the nucleus accumbens was not great (128%), see figure 10.4A and 10.4B.

In contrast to telencephalon, some areas of the diencephalon within transgenic mouse brains, showed very low % (<50%) increases in [^{3}H]2-BFI specific binding over controls, i.e., the posterior paraventricular thalamus, whilst other areas showed large increases (300 to 500 % increase; i.e., the mammillary nucleus and lateral hypothalamus). As previously mentioned in control animal brains all regions of the paraventricular thalamus show high levels of [^{3}H]2-BFI specific binding (63-72 fmol/mg tissue). In comparison, transgenic mouse brain sections show only 102-119 fmol/mg tissue, i.e, increases were very low. Levels of binding within the paraventricular thalamus showed low % increases in specific binding (50-100% increases), see figure 10.5A and 10.5B. Levels of specific binding within the control mouse ventral tuberomammillary nucleus (vtm), as previously mentioned, were moderate to high (44.59 fmol/mg tissue). However, levels of specific binding within the transgenic mouse vtm (100.88 fmol/mg tissue) were only increased by 115%. This is in contrast to levels of specific binding within other regions of the hypothalamus in transgenic animals, these were increased by over 300%.

Low increases (50-100% increase) in specific binding were seen between control and transgenic mouse areas of the mesencephalon, notably the lateral/intermediate subnuclei of the interpeduncular nuclei (ipi), see figure

10.3A and 10.3D. Analysis of control animal brain sections indicated the ipi bound moderate to high levels of [^{3}H]2-BFI (51.74 fmol/mg tissue). In transgenics this increased to 100.88 fmol/mg tissue (95% increase). This is in contrast to the other subnuclei of the interpeduncular, the rostral and caudal sections, which show lower levels in control animals (<28 fmol/mg tissue) but larger % increases when control specific binding is compared with transgenic specific binding (160-250% increase over control; see table 10.1). All other transgenic brain regions within the mesencephalon regions showed moderate increases (100-300% increase) in specific binding in comparison to controls.

In comparison to controls, most areas of the transgenic mouse rhombencephalon showed moderate increases in [^{3}H]2-BFI specific binding, i.e, 100-300% increases between control and transgenic levels of specific binding. However, two notable exceptions exist, the dorsal raphe and the locus coeruleus which both show very low % increases (<50%) in specific binding between controls and transgenics. In control mouse brains the dorsal raphe shows very high levels of [^{3}H]2-BFI specific binding (76.04 fmol/mg). However, transgenic mouse brains only showed 82.46 fmol/mg tissue binding. This is only an increase of 8%, see figure 10.6A and 10.6B.

A similar trend was appharent in the locus coeruleus, i.e., very low increases (<50%) occur in the specific binding reported for the locus of coeruleus between control and transgenic mice, see figure 10.7A and 10.7B.

Three areas of the circumventricular organs were assessed in control and transgenic mouse brain. Within the area postrema levels of [^{3}H]2-BFI specific binding within control animals were moderate to high (40-60 fmol/mg tissue). However, levels of specific binding only increased to 55.78 fmol/mg tissue in transgenic brain. This was only a 28% increase in specific binding. This is in contrast to the % increases recorded within the arcuate nucleus (320% increase), median eminence (384% increase) and subfornical organ (171% increase) between control and transgenic animals.

Table 10.1). *[^{3}H]2-BFI specific binding in control C/57 mice and transgenic MAO over-expressers.*

		Control		Transgenics		% I
		Specific Binding 2nM [^{3}H]2-BFI fmol/mg tissue				
		Mean	*SEM*	*Mean*	*SEM*	
Cortical Areas						
visual cortex	V	12.18	0.74	60.19	4.67	394
motor cortex	M	11.09	1.07	54.88	4.66	395
sensory cortex	S	10.19	0.83	54.76	3.87	437
auditory cortex	A	10.00	1.53	54.90	5.36	449
cingulate cortex	Cg	16.57	1.68	71.77	3.74	333
granule layer olfactory bulb	gro	5.63	0.95	50.23	4.59	793
ventral orbital cortex	lo/vo	10.92	1.69	54.01	3.78	395
anterior olfactory nucleus	ao	9.03	1.48	56.27	4.74	523
olfactory tubercle	tu	24.54	2.01	97.25	7.08	296
mitral cell layer	mit	16.28	2.38	113.17	10.55	595
anterior commissure	ac	4.62	0.72	12.89	1.38	179
piriform cortex	pir	9.40	0.96	39.97	2.99	325
entorhinal cortex	Ent	14.63	0.90	48.40	4.61	231
retrosplenial granular cortex	rsg	15.09	1.61	87.72	4.43	481
basomedial amygdaloid group	bma	12.82	0.95	55.36	4.01	332
basolateral amygdaloid group	bla	15.08	1.54	54.63	3.61	262
Cerebellum						
white matter	1	4.77	0.48	8.36	0.85	75
dense granules	2	9.71	0.80	53.40	4.59	450
purkinje cells	3	21.19	1.13	81.69	6.76	286
molecular layer	4	7.62	0.58	51.48	5.17	576
Telencephlon						
Hippocampus						
CA1 field of the hippocampus	CA1	9.30	1.31	49.52	4.25	432
CA2 field of the hippocampus	CA2	7.90	0.96	46.55	4.50	489
CA3 field of the hippocampus	CA3	9.05	1.09	41.92	3.45	363
dentate gyrus	dg	11.90	1.38	54.33	5.14	357
hippocampal fissure	Hif	16.44	1.00	87.37	5.12	431
subiculum	s	12.86	1.92	50.23	2.12	291
bed nuc. stria terminalis	bst	14.16	2.25	63.14	3.59	346
Septum						
lateral septal nucleus						
dorsal	lsd	13.50	1.79	58.79	4.86	335
intermediate	lsi	13.93	1.72	55.87	4.68	301
ventral	lsv	20.15	2.32	83.79	3.77	316
corpus callosum	cc	3.59	0.53	11.11	1.24	209
fornix	f	5.33	0.81	16.84	1.54	216

Data derived from five to six mice, with a minimum of three sections per mouse, and three readings per section. % I, percent increase in specific binding between control and transgenic.

Table 10.1). *[^{3}H]2-BFI specific binding in control C/57 mice and transgenic MAO over-expressers.*

		Control		Transgenics		% I
		Specific Binding 2nM [^{3}H]2-BFI fmol/mg tissue				
		Mean	*SEM*	*Mean*	*SEM*	
Telencephlon (cont)						
Basal Ganglia						
lateral globus pallidus	lgp	12.03	1.02	39.03	3.88	224
caudate putamen	cpu	18.83	1.78	66.81	4.03	255
accumbens nucleus	acbc	59.20	4.26	134.94	11.01	128
Diencephalon						
Thalamus						
paraventricular thalamus						
posterior	pvp	72.19	8.81	102.05	6.01	41
medial	pv	70.52	8.13	109.23	7.18	55
anterior	pva	63.16	8.25	119.44	5.35	89
lateral geniculate nucleus	lg	13.58	1.77	60.15	4.36	343
medial geniculate nucleus	mg	15.05	1.76	52.78	3.55	251
lateral posterior thalamic nucleus	lp	15.79	1.92	59.69	5.36	278
ventral posterior thalamic nucleus	vp	7.14	1.15	40.58	2.25	468
mediodorsal thalamic nucleus	md	18.64	2.20	61.10	4.98	227
posterior thalamic nuclear group	po	10.72	1.31	46.66	3.10	335
reuniens thalamic group	re	42.41	4.51	88.90	5.64	110
Hypothalamus						
lateral hypothalamus area	lh	17.10	0.77	78.03	6.33	356
posterior hypothalmic area	ph	22.51	2.59	84.85	4.71	277
ventromedial hypothalamic nuclei	vm	16.25	1.75	73.10	3.36	350
dorsomedial hypothalamic nuclei	dm	21.71	2.45	73.08	3.36	237
medial mammillary nuc. medial	mm	18.62	2.73	78.84	7.12	323
medial mammillary nuc. lateral	ml	18.05	2.16	73.64	3.45	308
ventral tuberomammillary nucleus	vtm	44.59	6.90	95.96	7.96	115
Suprachiasmatic nucleus	scn	20.13	2.26	77.88	5.98	287
Mesencephalon						
Tectum						
superior colliculus	su	14.95	1.44	55.50	4.69	271
superior colliculus superficial gray	sug	28.51	2.27	101.87	6.61	257
inferior colliculus	ic	5.16	0.34	47.80	15.54	208
Tegmentum						
interpeduncular nucleus						
rostral subnuclei	ipr	27.45	2.34	73.26	5.73	167
caudal subnuclei	ipc	24.13	1.48	85.06	6.37	253
lateral/intermediate subnuclei	ipi/lpi	51.74	4.11	100.88	8.12	95
substantia nigra	snr	19.62	1.22	76.03	5.57	287
peri-aqueductal gray	pag	23.11	0.69	65.37	5.14	183

Data derived from five to six mice, with a minimum of three sections per mouse, and three readings per section. % I, percent increase in specific binding between control and transgenic.

Table 10.1). *[^{3}H]2-BFI specific binding in control C/57 mice and transgenic MAO over-expressers.*

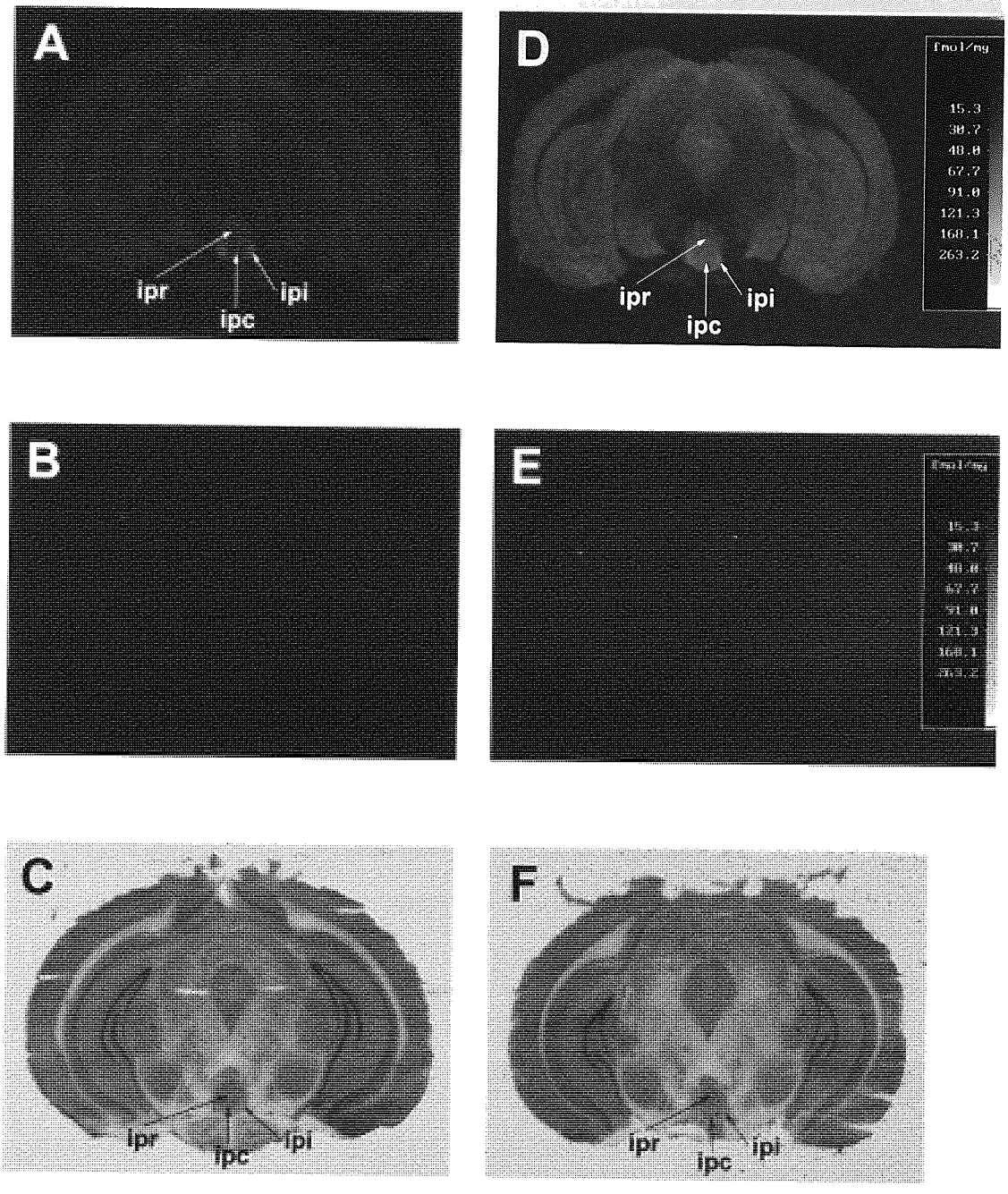
		Control		Transgenics		% I
		Specific Binding 2nM [^{3}H]2-BFI fmol/mg tissue				
		Mean	*SEM*	*Mean*	*SEM*	
Rhombencephalon						
solitary tract	sol	27.47	2.12	59.51	3.88	117
hypoglossal nucleus	12	17.99	0.78	48.75	3.75	171
prepositus nucleus	pr	18.74	1.68	50.97	2.14	172
inferior olives	io	23.31	1.07	50.31	3.93	116
pontine nuclei	pn	12.69	0.95	40.73	1.98	221
locus coeruleus	LC	49.81	3.07	69.19	3.00	39
dorsal raphe	dr	76.04	2.30	82.46	2.48	8
median rahpe	mnr	16.90	2.39	41.01	2.57	143
lateral parabracial nucleus	lpb	21.37	1.35	55.08	4.37	158
central grey pons	CGP	15.96	1.29	53.58	2.89	236
medial vestibular nucleus	mve	13.76	1.44	54.92	3.63	299
spinal trigeminal tract	sp	12.16	0.62	34.18	1.96	181
medullary reticular nucleus	md	9.66	0.82	31.59	1.98	227
Circumventricular organs						
area postrema	Ap	43.57	2.97	55.78	5.19	28
arcuate nucleus	Arc	17.50	2.95	73.49	5.12	320
median eminence	me	14.73	1.79	71.25	5.98	384
subfornical organ	SFO	16.86	2.44	45.69	3.37	171
Ventricles						
aqueduct	Aq	65.33	6.58	74.09	6.62	13
3rd ventricle	3V	21.80	3.17	61.02	4.69	180
dorsal 3rd ventricle	D3V	57.31	4.50	61.41	2.43	7
lateral ventricle	LV	54.14	6.81	54.89	6.71	1
lateral 4th ventricle	L4V	65.73	4.51	92.35	7.88	41
4th ventricle	4V	47.66	5.00	50.28	2.97	5

Data derived from five to six mice, with a minimum of three sections per mouse, and three readings per section. % I, percent increase in specific binding between control and transgenic.

Levels of [^{3}H]2-BFI specific binding within the lining of the ventricles were moderate to high (>45 fmol/mg tissue) within control animals. However, these levels were not substantially increased in transgenic animals. Levels of specific binding within the control mouse lateral ventricle was 54.14 fmol/mg tissue, see figure 10.5A. These levels only increased by 1% to 54.89 fmol/mg tissue in transgenic mouse lateral ventricle, see figure 10.5B. A similar pattern was observed, i.e, control brains have moderate to high

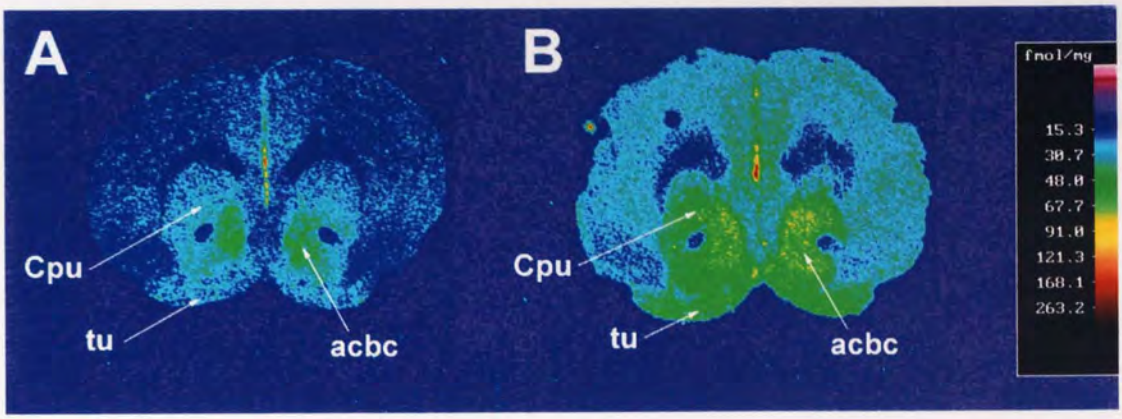
levels of specific binding, but in comparison to levels of specific binding in transgenic brains % increases are low (<50% increase), within the lining of the aqueduct (13% increase), dorsal 3rd ventricle (7% increase), lateral 4th ventricle (41% increase) and 4th ventricle (5% increase). However, in contrast, levels of binding in the control mouse lining of the 3rd ventricle were lower (21.80 fmol/mg tissue) than in other ventricles, but showed much larger increases in specific binding when compared with transgenics.

Figure 10.3). ***Reverse phase images of 2nM [^{3}H]2-BFI total (Section A and D), NSB binding (section B and E) and cresyl violet (Section C and F) within control (Section A, B and C) and transgenic (Section D, E and F) mouse brain***



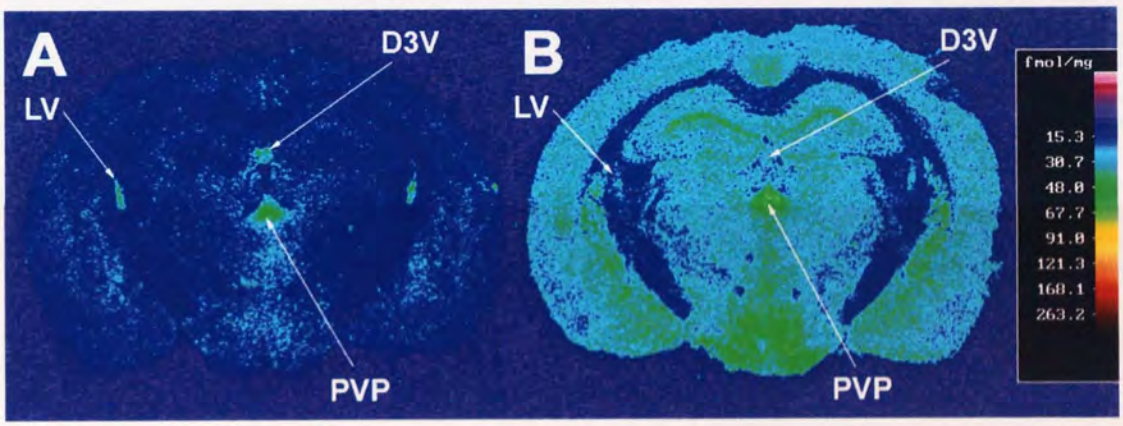
Section taken –3.80mm from the bregma line. See table 10.1 for abbreviations.

Figure 10.4). ***Pseudo-colour images of 2nM [^{3}H]2-BFI total binding within wildtype (Section A) and transgenic (section B) mouse brains***



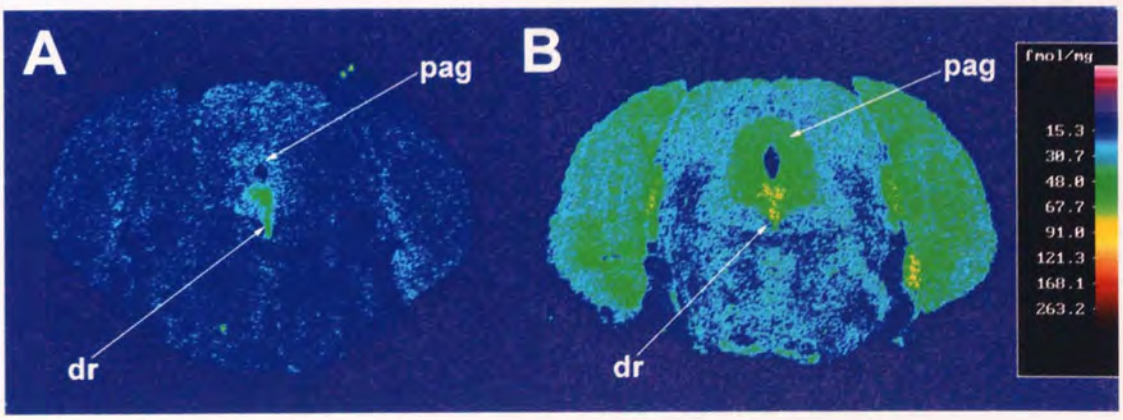
Section taken 1.34mm from the bregma line. See table 10.1 for abbreviations.

Figure 10.5). ***Pseudo-colour images of 2nM [^{3}H]2-BFI total binding within wildtype (Section A) and transgenic (section B) mouse brains***



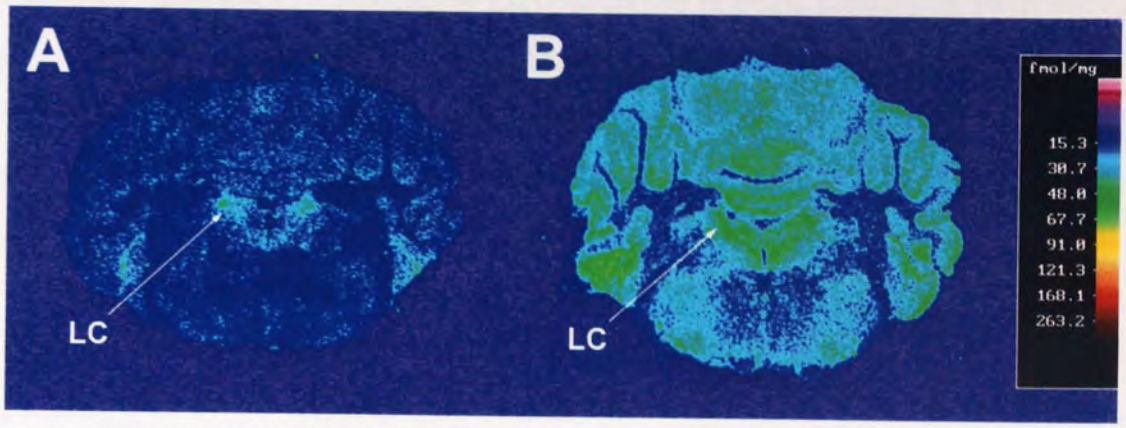
Section taken –2.18mm from the bregma line. See table 10.1 for abbreviations.

Figure 10.6). ***Pseudo-colour images of 2nM [^{3}H]2-BFI total binding within wildtype (Section A) and transgenic (section B) mouse brains***



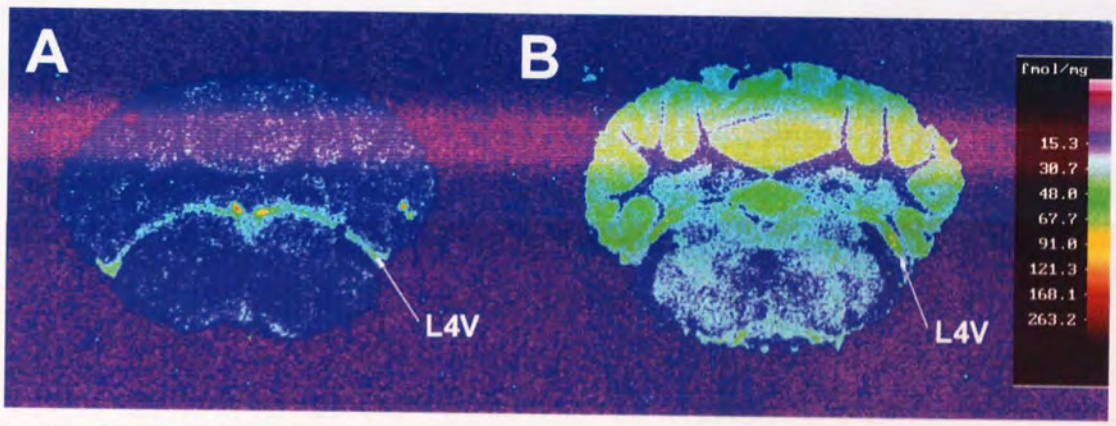
Section taken –4.48mm from the bregma line. See table 10.1 for abbreviations.

Figure 10.7). ***Pseudo-colour images of 2nM [^{3}H]2-BFI total binding within wildtype (Section A) and transgenic (section B) mouse brains***



Section taken –5.80mm from the bregma line. See table 10.1 for abbreviations.

Figure 10.8). ***Pseudo-colour images of 2nM [^{3}H]2-BFI total binding within wildtype (Section A) and transgenic (section B) mouse brains***



Section taken –6.12mm from the bregma line. See table 10.1 for abbreviations.

4). Discussion

The data reported in this chapter compared the specific binding of [^{3}H]2-BFI in C57/B mice to transgenic litter mates that reportedly over express MAO-B 4-6 fold. The reason for this was two fold. Firstly, the evidence within Chapters 5, 6, 7 and 8 indicate the characteristics of the I_2 MAO site does not conform to the established nomenclature for MAO-A or –B. As a consequence comparison between control and transgenic brains with [^{3}H]2-BFI may indicate a subpopulation of MAO-B, ie., the I_2 MAO site. Secondly, the potential exits of finding discrete nuclei, in both controls and transgenics, which show similar levels of [^{3}H]2-BFI binding – thereby suggesting a location for the elusive non-MAO I_2 site.

The results from this study indicate that the specific I_2 ligand [^{3}H]2-BFI binds to discrete nuclei within the mouse brain. High levels (>60 fmol/mg tissue) of specific [^{3}H]2-BFI binding were found within the dorsal raphe, lateral 4th ventricle, aqueduct, posterior, medial, and anterior paraventricular thalamus. Moderate to high levels (40-60 fmol/mg tissue) of specific binding was found within a number of other mouse brain regions, notably the nucleus accumbens, ventral tuberomammillary nucleus, interpeduncular lateral/intermediate subnuclei, locus coeruleus, lateral ventricle, 4th ventricle, and dorsal 3rd ventricle. The mouse brain also contained a similar number of regions that exhibited low to moderate (20-40 fmol/mg tissue) levels of specific binding, i.e., the rostral and caudal interpeduncular subnuclei, olfactory tubercle, purkinje cells of the cerebellum, solitary tract, inferior olives, superior colliculus, suprachiasmatic nucleus, dorsomedial hypothalamic nucleus, posterior hypothalamus, lateral parabracial nucleus, peri-aqueductal gray, and the 3rd ventricle. All other regions assessed had low levels (<20 fmol/mg tissue) of [^{3}H]2-BFI specific binding notably the arcuate nucelus, all other areas of the thalamus and hypothalamus, all remaining cortical areas, all other layers of the cerebellum, median eminence, subfornical organ and hippocampus.

The distribution of [^{3}H]2-BFI specific binding within the mouse favourably matches that found within the rat with [^{3}H]2-BFI and [^{3}H]idazoxan (Lione et

al, 1998; this thesis). In the rat high levels [^{3}H]2-BFI specific binding can be found within circumventricular organs and lining of the ventricles i.e., the area postrema (96.73 fmol/mg tissue), surface of the dorsal third ventricle (102.15 fmol/mg tissue), aqueduct (25.4 fmol/mg tissue) and fourth ventricle (29.94 fmol/mg tissue; Lione et al, 1998). In the mouse these regions are also labelled and with broadly similar levels of [^{3}H]2-BFI, i.e., the area postrema (43.57 fmol/mg tissue), surface of the dorsal third ventricle (57.31 fmol/mg tissue), aqueduct (65.33 fmol/mg tissue) and fourth ventricle (47.66 fmol/mg tissue). However, in contrast the rat arcuate nucleus shows high levels of specific [^{3}H]2-BFI binding (112.09 fmol/mg tissue; Lione et al, 1998), whereas the mouse contains low levels (17.50 fmol/mg tissue).

In the control mouse high levels of [^{3}H]2-BFI specific binding are found within all regions (posterior, 72.19; medial, 70.52; and anterior, 63.16 fmol/mg tissue) of the paraventricular thalamus. In the rat the anterior and posterior sections (22.44 Vs 21.64 fmol/mg tissue) of the paraventricular thalamus also show, albeit lower levels of, [^{3}H]2-BFI specific binding (see Chapter 8). For the rat data within Chapter 8 indicates that high levels of [^{3}H]2-BFI specific binding can be found within the tuberomammillary nucleus (38.07 fmol/mg tissue). Similar levels of binding are also found within the mouse ventral tuberomammillary nucleus (44.59 fmol/mg tissue).

In the rat high levels of specific binding have been found within the interpeduncular nucelus (88.75 fmol/mg tissue; Lione et al, 1998). High to moderate levels of [^{3}H]2-BFI (51.74 fmol/mg tissue) are also found within the mouse interpeduncular nucelus. However, unlike the rat this binding is regionalised to the interpeduncular lateral/intermediate subnuclei, whilst the other interpeduncular subnuclei show much lower levels (rostral, 27.45 fmol/mg tissue; caudal, 24.13 fmol/mg tissue). Both the rat and the mouse superior and inferior colliculus show low levels (<20 fmol/mg tissue) of [^{3}H]2-BFI binding. However, the rat superficial grey layer of the superior colliculus does show slightly higher levels of [^{3}H]2-BFI binding than the surrounding superior colliculus. The same is also true, albeit at much higher levels, within the mouse superior colliculus.

In both mice and rat brains [^{3}H]2-BFI binds to the dorsal raphe (Chapter 8; Lione et al, 1998; this Chapter). However, in the rat the dorsal raphe only shows moderate levels (<35 fmol/mg tissue; Chapter 8; Lione et al, 1998) of specific binding, whilst the mouse dorsal raphe shows very high levels (>75 fmol/mg tissue).

In contrast to the rat, the mouse brain shows high to moderate levels of [^{3}H]2-BFI specific binding within the locus coeruleus (Chapter 8; Lione et al, 1998; This Chapter). [^{3}H]2-BFI binding in this area has not been previously reported in the literature and was not detected in Chapter 8. Consistent with data from the rat the mouse solitary tract and inferior olives also show moderate levels of [^{3}H]2-BFI specific binding.

At this point in time autoradiographical data derived from these mice with tritiated specific MAO-A and MAO-B inhibitors is unavailable (J.G. Richards, personal communication) and the only mouse data available for comparison is that discussed in the introduction. The data is immediately striking with respect to the number or density of [^{3}H]Ro19-6327 MAO-B sites in comparison to [^{3}H]2-BFI I_2 sites. Within the mouse brain the area that shows the lowest number of [^{3}H]Ro19-6327 MAO-B sites is the white matter of the cerebellum (653 fmol/mg tissue). This level of binding is far in excess of that reported for the white matter in this study (4.77 fmol/mg tissue). The K_D of the [^{3}H]2-BFI I_2 site has not been assessed, but has been suggested to be between 2 and 4 nM (Alan Hudson, personal correspondence). The results of Saura et al, (1994) are based on a much higher concentration of 15 nM which is the K_D reported for the rat (Cesura et al, 1989b). Hence, as we used a concentration which is probably lower than the K_D (2 nM) of [^{3}H]2-BFI it is likely that this study is imaging less than 50% of available I_2 sites. However, this alone cannot account for the considerable differences between levels of [^{3}H]Ro19-6327 and [^{3}H]2-BFI specific binding. These results suggest that [^{3}H]2-BFI may be binding to a subset of MAO enzymes.

The literature indicates that high levels of [^{3}H]Ro19-6327 MAO-B specific binding have been detected within the mouse substantia nigra, nucleus accumbens and superior colliculus (Saura et al, 1994). In this study and in relation to other mouse sites we report low levels (<20 fmol/mg tissue) of

[^{3}H]2-BFI specific binding within the substantia nigra and superior colliculus. However, this study does report moderate to high levels of specific binding in the nucleus accumbens (40-60 fmol/mg tissue). A number of other sites show moderate to high levels of [^{3}H]Ro19-6327 MAO-B specific binding, i.e., parietal cortex, occipital cortex, granular layer of the cerebellum, frontal cortex, inferior colliculus and caudate putamen. In contrast to these results and in relation to the other sites labelled by [^{3}H]2-BFI levels of [^{3}H]2-BFI specific binding were low within all of these nuclei (Saura et al, 1994). Hence based on these results the autoradiographical distribution of MAO-B does not bear many similarities to that of I_2 distribution within the mouse (Saura et al, 1994; this Chapter). However, as mentioned in the introduction Saura et al (1994) assess less than 15 CNS regions and the autoradiograms shown within the paper clearly show very high levels of total binding within the paraventricular thalamus and lining of the lateral ventricles. However, levels of specific binding within these regions are not reported in the text (Saura et al, 1994).

A more detailed analysis of the distribution of MAO-B (mRNA and protein) has been reported within the rat and this is similar to that reported for [^{3}H]2-BFI within the mouse (Saura et al, 1992; Luque et al, 1995; This Chapter). Hence, high levels of MAO-B and [^{3}H]2-BFI specific binding are reported in the rat, subfornical organ (SFO), ependyma and lateral ventricles, interpeduncular nuclei (Ip) and area postrema (ap; see figure 10.1; Saura et al, 1992; Lione et al, 1998; Chapter 8). Similarly in the mouse [^{3}H]2-BFI binds to all these regions and shows high to moderate levels of [^{3}H]2-BFI specific binding. However, although levels of specific [^{3}H]2-BFI and MAO-B binding within the rat arcuate nucleus are high, in the mouse arcuate nucleus they are low (Saura et al, 1992; Lione et al, 1998; Chapter 8). The comparison of control to transgenics brains shows a general massive increase in levels of specific [^{3}H]2-BFI binding. In control mouse brain virtually all areas of the cerebral cortex show low levels (<20 fmol/mg tissue) of specific binding. The same brain areas within transgenic mice show large % increases in specific binding, i.e, 300-700%. The same pattern is seen throughout all other brains regions, i.e, control areas which show low levels

of specific binding show large % increases when compared with the same regions within transgenic animals. As these animals over-express levels of MAO-B an estimated 4 to 6 fold and an I_2 binding site has been localised to MAO-B (Raddatz et al, 1997; Richards et al, 1998) [^{3}H]2-BFI it is probably binding to MAO-B. However, patterns of [^{3}H]2-BFI specific binding are more complex than this. In control animals the lining or surface of the ventricles and a number of circumventricular areas show moderate to high levels of specific binding (43-65 fmol/mg tissue). The corresponding regions within transgenic brains show very similar actual levels of specific [^{3}H]2-BFI binding (54-90 fmol/mg tissue), i.e., percentage increases between control and transgenic brain regions were very small (<45% increase). This pattern is particularly evident within the surface of the third ventricle within this region. Control (54.14 fmol/mg tissue) and transgenic (54.89 fmol/mg tissue) animals exhibit virtually identical levels of [^{3}H]2-BFI specific binding. Expression of MAO-B within these transgenic mice was under the control of a neuron-specific enolase promoter (Richards et al, 1998). Hence, it is feasable that the use of neuron specific promoter can account for the failure of [^{3}H]2-BFI specific binding to increase in transgenic MAO-B non-neuronal areas, i.e., the lining of the ventricles. However, this factor cannot account for the failure of [^{3}H]2-BFI specific binding to increase in neuronal areas, i.e., dorsal, between control (76.04 fmol/mg tissue) and transgenic (82.46 fmol/mg tissue) animals. This data suggests that [^{3}H]2-BFI is labelling two populations within the mouse brain. The first population is of low density, found throughout the whole brain and is greatly increased in transgenic animals that over-express MAO-B. The second is of high density, regionalised to discrete brain nuclei and is not increased in the transgenic mouse brain. Radiochemical homogenate studies have indicated I_2 ligands such as 2-BFI label two different components – a high affinity site and a low affinity site (Lione et al, 1996). Hence, it could be suggested that the low affinity site detected in homogenate studies could be the low-density diffuse site detected here, i.e., MAO-B. In this model the high affinity site would be associated with [^{3}H]2-BFI binding in high quantities to discrete nuclei, does not increase in transgenics that over-express MAO-B, and may be MAO-A or

alternatly a non I_2 MAO site. However, without verification of percentage increases, within [^{3}H]2-BFI labelled areas, between control and transgenics with tritiated MAO-B inhibitors it is impossible to credibly suggest that this is a non MAO-B I_2 site.

Hence the possibility exists that these are non-MAO imidazoline sites. In mouse brain the distribution of [^{3}H]2-BFI is similar to that of angiotensin II receptors (Hauser et al, 1998; This chapter). Iodinated angiotensin II labels the area postrema, arcuate nucelus, inferior olives, locus coeruleus and solitary tract. Of particular interest is the ability of [^{125}I][sar^1]-angiotensin II to bind to discrete subnuclei within the interpeduncular nucleus, an ability it shares with [^{3}H]2-BFI (Hauser et al, 1998). Hauser et al (1998) report high levels of specific binding within the dorsomedial, but low to moderate levels within the central and lateral, interpeduncular nuclei. As mentioned in the introduction the injection of angiotensin II into the rat subfornical organ, another area which shows high levels of [^{3}H]2-BFI binding, increases blood pressure (Mangiapane and Simpson, 1980; Chapter 8). This hypertensive response can be modulated by the application of I_2 ligands to the hypothalamus (Arrais et al, 1997; Saad et al, 1998; Araujo-Almeida et al, 1999). These are interesting in the light of some I_1 site ligands ability to modulate blood pressure (Ernsberger, 1998 & 1999, Van Zwieten & Peters, 1999).

In contrast to the rat, high levels of [^{3}H]2-BFI specific binding were found in the mouse brain locus coeruleus (Chapter 8; Lione et al, 1998; This chapter). Previous studies have indicated that I_2 ligands modulate opioid withdrawal and potentiate opioid analgesia in both rats and mice. (Hudson et al, 1999b; Li et al 1999; Sanchez-Blazquez et al, 2000). *In vivo* work in the rat has indicated micro injection of clonidine into the locus coeruleus (LC) reduces the increase in firing associated with opiate withdrawal, i.e., the withdrawal response (Aghajanian, 1978). The LC is a major noradrenergic nucleus, and as a consequence these effects were thought to be mediated through the noradrenergic system (Aghajanian, 1978; Gold et al, 1978). However, it is interesting that the administration of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), a purported noradrenergic neurotoxin, in

massive doses (50 and 100mg/kg) fails to alter opioid tolerance as measured with the tail flick assay (Dossin et al, 1995). In the mouse I_2 ligands potentiate morphine analgesia and prevent the development of tolerance (Li et al, 1999; Jin et al, 1999). Hence, it has been suggested that clonidine's ability to modulate LC firing and possibly alter these analgesic or addictive effects occurs through imidazoline receptors (Ruiz-Ortega et al, 1995). However, data from rat brain slices has indicated there are no functional imidazoline sites within the LC (Szabo et al, 1996) and it is activation of an efferent pathway that stretches to the LC from the nucleus paragigantocellularis that may account for any imidazoline mediated effects that occur within the LC (Ruiz-Ortega and Ugedo, 1997).

A number of other lines of evidence associate drug dependence with the regions identified here. The LC also contains a large number of mu-morphine and galanin receptors (Abbadie et al, 2000; Zachariou et al, 2000). Galanin is a neuropeptide and activation of galanin receptors reduces the spontaneous firing of LC neurones, an effect it shares with clonidine (Aghajanian, 1978; Pieribone et al, 1995). Chronic treatment with morphine upregulates galanin binding within the mouse LC and further drug associated changes in c-*fos* immunoreactivity occur within the LC after the administration of cocaine (Nikulina et al, 1998; Zachariou et al, 2000). In addition to the LC, cocaine administration increases c-fos expression in the dorsal raphe, an area shown in this study to contain a large number of [^{3}H]2-BFI binding sites (Nikulina et al, 1998). C-fos increases in the LC are also associated with the exposure of mice to a stressful environment, i.e, the dominant animal (Nikulina et al, 1998). Other stress models i.e., restraint stress, indicate noradrenaline reuptake sites are increased in stressed mice (Hwang et al, 1999). As previously mentioned animal models of depression, namely the rat olfactory bulbectomy, have indicated chronic treatment with the clinically effective antidepressant imipramine significantly decreases [^{3}H]clonidine I_1 binding but increases [^{3}H]idazoxan I_2 binding in rat brain (Zhu et al, 1997a; Zhu et al, 1997b).

The data presented here suggest that [^{3}H]2-BFI binds to specific regions within the mouse brain. Comparison of specific binding between control

animals and transgenic mice who over express MAO-B indicates a proportion of I_2 sites are associated with MAO-B. However, in control animals [^{3}H]2-BFI also identified areas which showed high levels of specific binding, but these regions did not show increases in binding when compared with their transgenic litter mates. The regions isolated by [^{3}H]2-BFI within the mouse are also associated with functions that have previously been associated with I_2 ligands, i.e., regulating blood pressure, opioid analgesia and opiate withdrawal.

Chapter 11

General Discussion

The concept of the imidazoline site arrived in the eighties with the publication of three papers (Bousquet et al, 1984; Ernsberger et al, 1987; Boyajian et al, 1987). Data within these papers indicated that α_2-adrenoceptors that contained an imidazoline moiety bound to sites other than α_2-adrenoceptors. These findings and others led the first international symposium on imidazoline preferring receptors to describe two imidazoline sites or receptors, the I_1 site which preferentially binds [^{3}H]PAC (in the presence of 10μM noradrenaline to saturate α-adrenoceptors) and the I_2 site which preferentially binds [^{3}H]idazoxan under similar conditions (Michel & Ernsberger, 1992). However, a suitable second messenger system has yet to be found for either subtype and whether I_1 and I_2 sites are actually functional receptors is still a matter of controversy (Eglen et al, 1998; Ernsberger, 1999).

A great deal of evidence links I_2 sites with the enzyme MAO i.e., I_2 ligands inhibit MAO with a good potency, MAO specific antibodies immunoprecipitate imidazoline labelled proteins with a similar molecular weight to the two isoforms MAO (–A and –B) and *in vivo* chronic treatment with irreversible MAO inhibitors reduces I_2 density (Olmos et al, 1993; Alemany et al, 1995; Raddatz et al, 1995; Brown et al, 1995; Carpene et al, 1995, Ozaita et al, 1997; Gargalidis-Moudanous et al, 1997: Lalies et al,

1999). However, a variety of I_2 associated proteins that do not share the molecular characteristics of MAO have been isolated from the brain (Bennai et al, 1996; Ivanov et al, 1998b; Garcia-Sevilla et al, 1996; Escriba et al, 1999) and so the search for a functional receptor continues. Behavioural studies have given mixed results. I_2 ligands have been associated with increasing food intake, modulating opioid analgesia, altering levels of GFAP and *in vivo* microdialysis studies have firmly established that the administration of I_2 ligands leads to a marked increase in extracellular NA (thorough a mechanism independent of direct antagonism of the α_2-adrenoceptor) and dopamine within the rodent brain (Jordan, 1993; Lalies and Nutt, 1993; Nutt et al, 1995; Hudson et al, 1999b; Sanchez-Blazquez et al, 2000; Jackson et al, 1991; Menargues et al 1994; Menargues et al 1995; Brown et al, 1995; Prasad and Prasad, 1996; Polidori et al, 2000). However, although *in vivo* microdialysis studies indicate I_2 ligands instigate marked changes in the turnover of NA and dopamine other studies show the administration of I_2 specific ligands gives rise to little overt behavioural pharmacology (Jordan 1993; Brown et al, 1995). As a result of this, other behavioural techniques such as drug discrimination have had to be utilised in a bid to probe the psychopharmacology of I_2 ligand administration (Jordan et al, 1996). The initial findings of Jordan et al (1996) indicated 2-BFI (7mg/kg) could generate a CNS cue in drug discrimination and the administration of a number of α_2-antagonists and reversible MAO inhibitors give rise to significant levels of 2-BFI appropriate responding. Hence as outlined in the introduction the aims of the studies included in the thesis were to initially find the minimal dose of 2-BFI needed to elicit a discriminable cue and compare the potency of a range I_2 ligands to the 2-BFI cue.

The first group of rats were trained on 3.5, 4 and 5 mg/kg 2-BFI to find the minimum dose of 2-BFI needed to elicit a discriminable cue. However, after a large number of training sessions it became apparent that DD rats would not be able to successfully discriminate 2-BFI, at the afore mentioned doses, from saline. Hence, all other animals were trained on the dose (7mg/kg)

utilised by Jordan et al (1996). Previous work has indicated that eight rats could be trained to discriminate 2-BFI (7 mg/kg) from saline with over 80% accuracy after an average of 44 sessions (Jordan et al, 1996). This compares favourably with the number of sessions reported for the subsequent groups trained on 2-BFI (Chapter 3), i.e., the fastest learners were group 5 who took an average of 38.2 sessions, whilst the slowest learners were within group 4 who underwent an average of 60.3 sessions before being able to discriminate 7 mg/kg 2-BFI from saline with 80% accuracy.

After successfully training rats to discriminate 7 mg/kg 2-BFI from saline a number of I_2 specific ligands were administered. This included the quinoline and isoquinoline analogues of 2-BFI, BU224, BU226 and BU216. All of these compounds exhibited full and significant levels of substitution for the 2-BFI cue (see chapter 4). Previous studies have indicated that 2-BFI is an antagonist whilst BU224 and idazoxan are agonists at the I_2 site (Sanchez-Blazquez et al, 2000). As mentioned in chapter 4 the results within this study and the *in vivo* microdialysis studies of others (Hudson et al, 1999b) do not support this assertion. The data presented in chapter 4 indicate the administration of BU224 and 2-BFI have similar effects *in vivo*, i.e., they are of one class which is either agonists or antagonists. It is possible that the results derived from 2-BFI trained rats and I_2 ligands in morphine analgesia form two distinct pharmacological pathways. It is true to say that a variety of imidazoline associated proteins have been purified from the brain (Bennai et al, 1996; Ivanov et al, 1998b; Garcia-Sevilla et al, 1996; Escriba et al, 1999) and the rank order of potency of I_2 ligands in modulating morphine analgesia does not match that reported in 2-BFI trained rats (Sanchez-Blazquez et al, 2000; chapter 4).

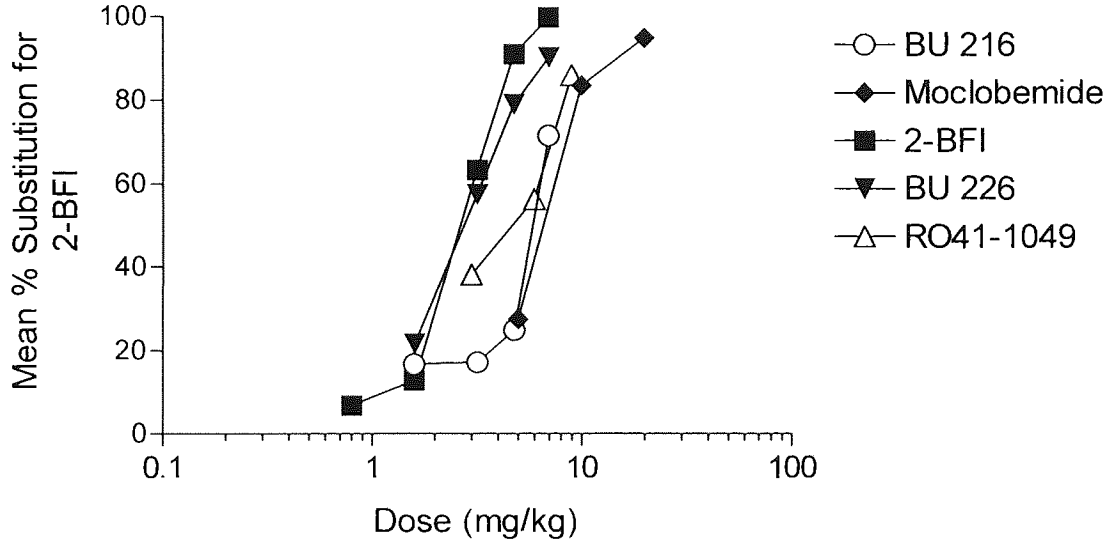
Another aim of this thesis was to investigate the noradrenergic component of the 2-BFI cue. Utilising the technique of *in vivo* microdialysis it has been firmly established that the administration of I_2 ligands such as BU224 and 2-BFI increases extracellular levels of NA and to a smaller extent dopamine (Jordan, 1993; Lalies and Nutt, 1993; Hudson et al, 1999b). Hence in chapter 5 a number of adrenoceptor agonists specific for each subtype were

administered to 2-BFI trained rats. The results indicate that the α_1-agonist methoxamine at 0.75 mg/kg shows significant levels of 2-BFI appropriate responding and the levels of 2-BFI appropriate responding that occur after the administration of 2-BFI can be antagonised by prior administration of the α_1-adrenoceptor antagonist WB4101. Hence, this data suggests the activation of α_1, but not β_1 or β_2 adrenoceptors contributes to the 2-BFI cue. Interestingly the α_1-adrenoceptor antagonist prazosin failed to antagonise the 2-BFI cue. As mentioned in chapter 5 WB4101 has a 30 fold higher affinity for the α_{1a}-adrenoceptor subtype, whilst prazosin has an equal but lower affinity for both the α_{1a}- and α_{1b}-adrenoceptor subtypes (Morrow et al, 1985; Morrow and Creese, 1986; Blendy et al, 1991; Grimm et al, 1992). Bearing in mind the doses used, see chapter 5, it is feasible to suggest that in part the 2-BFI cue is associated with the selective activation of α_{1a}-adrenoceptors.

A great deal of evidence links I_2 sites and MAO and as a result of this a further aim of this thesis was to probe the relationship between MAO and I_2 sites. As mentioned in the Chapter 6, *In vitro* 2-BFI, BU224, BU226, BU239 and BU216 inhibit MAO-A in a reversible manner with a similar potency to moclobemide (Carpene et al, 1995, Ozaita et al, 1997; Lalies et al, 1999) and *in vivo* the chronic treatment with a number or irreversible inhibitors results in the down regulation of rodent [^{3}H]-idazoxan I_2 binding sites (Olmos et al, 1993; Alemany et al, 1995). In chapter 6 a number of MAO inhibitors were administered to 2-BFI trained rats. The results indicated that reversible MAO-A inhibitors moclobemide and Ro41-1049 potently and dose dependently substitute for 2-BFI (Chapter 6). *In vitro* moclobemide and BU216 inhibit MAO-A with a similar potency (Lalies et al, 1999). Correspondingly moclobemide shows an almost identical potency to BU216 in 2-BFI trained rats, see figure 11.1. BU226, 2-BFI and RO41-1049 are more potent at inhibiting MAO-A than moclobemide and BU 216 and correspondingly figure 6.3 indicates lower doses of these compounds give rise to similar levels of 2-BFI appropriate responding.

Two reversible MAO-B inhibitors were administered over a wide range of doses. Bearing in mind the molecular and autoradiographical work that has associated I_2 sites with MAO-B (Raddatz et al, 1997; Chapter 8; Chapter 9) it was surprising that only one of these inhibitors (lazabemide; 3 mg/kg) at one dose showed significant levels of 2-BFI appropriate responding. Although reversible inhibitors of MAO-A have a high potency in 2-BFI trained rats this data suggests that a small but significant part of the 2-BFI cue is associated with the reversible inhibition of MAO-B.

Figure 11.1). ***Potency of I_2 ligands and MAO-A inhibitors in 2-BFI (7 mg/kg) trained rats***

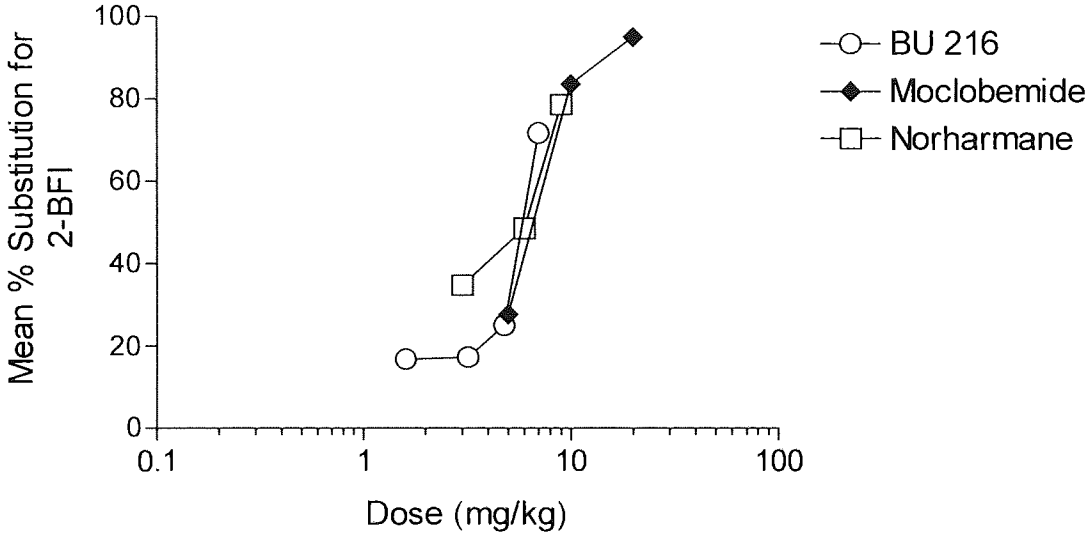


Data for BU216, BU226 and 2-BFI derived from chapter 4. Data for moclobemide and Ro41-1049 derived from chapter 6.

Consistent with there being an association between I_2 sites and MAO-A a number of beta carbolines have recently been shown to have a high affinity for I_2 sites *in vitro* and results within this thesis indicate that these β-carbolines significantly substitute for 2-BFI (Chapter 7; Hudson et al, 1999a; Alan Hudson, personal communication). It has been firmly established that β-carbolines such as harmane, norharmane and harmaline, inhibit MAO with potency similar to that of 2-BFI and its analogues (Buckholtz and Boggan, 1977; Lalies et al, 1999). A 2μM concentration of harmane inhibits 50% of MAO activity and slightly higher concentrations (20μM) are needed for

harmane's metabolite norharmane (Buckholtz and Boggan, 1977). The *in vitro* potency of norharmane to inhibit MAO is very similar to that of BU216 and moclobemide and correspondingly *in vivo* these three compounds give almost identical dose response curves, see figure 11.2. Hence, the levels of substitution that occur after the administration of, BU216, harmane and norharmane are likely to be through their ability to inhibit MAO.

Figure 11.2). ***Potency of moclobemide, BU216 and norharmane in 2-BFI (7 mg/kg) trained rats***



Data for BU216 derived from chapter 4. Data for moclobemide derived from chapter 6. Data for norharmane derived from chapter 7.

As mentioned throughout this thesis a number of I_2 ligands potentiate morphine analgesia (Kolesnikov et al, 1996; Li et al 1999; Sanchez-Blazquez et al, 2000). This is an ability the β-carboline harmaline also possesses (Glick et al, 1994). It is possible that opioid analgesia is potentiated by imidazoline ligands through the inhibition of MAO. It has been proposed that the hallucinogenic properties associated with South American tribal drinks are attributed not to the β carbolines but to the potent hallucinogen DMT (N,N-dimethyltryptamine) which is also commonly found within the same concoction (Callaway et al, 1999; Szara, 1956; Deliganis et al, 1991; Strassman et al, 1994). Hence, the β carbolines inhibit MAO thereby increasing the availability of DMT - released 5-HT to stimulate 5-HT

receptors (Callaway et al, 1999). Previous drug discrimination studies indicate that the co-administration of SSRI's with low doses of DOM ([-]-2.5-dimethoxy-4-methyl-amphetamine) in DOM trained rats potentiates levels of drug associated lever pressing. This data indicates the co-administration of an SSRI increases the availability of the low levels of 5-HT released by DOM (Winter et al, 1999). Hence, it is possible that the inhibition of MAO by I_2 specific ligands reduces the breakdown of a substance (substance X) within the pharmacological mechanism that modulates analgesia. The increased availability of this substance X would potentiate already existing levels of analgesia.

However, although the β carbolines inhibit MAO-A the related indole alkaloid ibogaine does not (Nelson et al, 1979). Ibogaine shows dose dependent, significant and at the highest dose - full substitution for the 2-BFI cue (Chapter 7). As mentioned in chapter 7 ibogaine or endabuse™ has recently been patented as an anti-addictive drug. Behavioural models of addiction indicate ibogaine modulates addictive symptoms (Szumlinski et al, 2000; Glick et al, 1994; Szumlinski et al, 1999). This is particularly interesting because I_2 ligands such, 2-BFI, BU224 and agmatine potentiate opioid analgesia or reduce the symptoms of withdrawal in behavioural models of addiction (Kolesnikov et al, 1996; Li et al 1999; Hudson et al, 1999b; Sanchez-Blazquez et al, 2000). The method in which ibogaine modulates these effects has yet to be clarified (Szumlinski et al, 2001). The acute administration of ibogaine results in a wide range of pharmacological responses, i.e, increases in dynorphin release, substance P immuno-reactivity and extraneuronal levels of 5-HT, but decreases in extraneuronal levels of dopamine (Glick et al, 1994; Wei et al, 1998; Alburges and Hanson, 1999; Alburges et al, 2000). However, in spite of these wide ranging effects ibogaine has a low micromolar affinity for most receptors, except for the $sigma_2$ receptor (Nelson et al, 1979; Bowen et al, 1995 Mach et al, 1995; Codd, 1995; Mah et al, 1998; Glennon et al, 2000). However, in chapter 7 we administered the $sigma_2$ ligand SKF10,047 and this failed to elicit levels of 2-BFI appropriate lever pressing over that of saline vehicle. 2-BFI has

been extensively studied and shows a low affinity for all receptors examined within the CNS except the I_2 site (Nutt et al, 1995). In chapter 7 based on the strong association that exists between ibogaine administration and increases in extraneuronal levels of 5-HT a number of SSRI's were administered. All the SSRI's administered, paroxetine, citalopram and clomipramine showed significant levels of 2-BFI responding. In a further study the potent 5-HT releaser fenfluramine was administered and consistent with these results fenfluramine significantly substituted for 2-BFI at the highest dose tested. This is particularly surprising as extensive studies utilising the technique of *in vivo* microdialysis have failed to show I_2 ligands increase extraneuronal levels of 5-HT (M. Lalies, personal communication; Jordan, 1993; Lalies and Nutt, 1993; Hudson et al, 1999b).

One of the main aims of this thesis was to look at the regional distribution of [^{3}H]2-BFI I_2 sites and look at factors or drugs that may reduce I_2 site density. Hence utilising the technique of autoradiography chapter 8 looked at the regional distribution of [^{3}H]2-BFI I_2 sites in the rat brain. The results generated compared favourably to those previously published for [^{3}H]2-BFI and [^{3}H]idazoxan I_2 sites (Lione et al, 1998) i.e, [^{3}H]2-BFI labels the lining of the ventricles and a number of circumventricular organs (Chapter 8). The data presented in chapter 8 indicates [^{3}H]2-BFI I_2 binding shows a similar autoradiographical distribution to that of MAO-B protein and mRNA (see chapter 1, chapter 8, figure 10.1; Saura et al, 1993; Luque et al, 1995).

The relationship between MAO and I_2 sites was explored further in chapter 9. Data within chapter 9 indicated short term experiments (acute or pre-incubation experiments) failed to alter levels of [^{3}H]2-BFI within the arcuate nucelus, interpeduncular nucleus, area postrema and pineal gland. This data was consistent with the hypothesis which suggests I_2 ligands bind to a site distinct to that of the classical acetylenic MAO inhibitors deprenyl and pargyline. However, the acute administration of tranylcypromine significantly increased levels of [^{3}H]2-BFI specific binding, suggesting that tranylcypromine induces a conformational change in MAO and consequently exposes more [^{3}H]2-BFI binding sites. Chronic treatment (moderate doses over 5 days) with tranylcypromine and deprenyl led to significant reductions

in the specific binding of [^{3}H]2-BFI to all four nuclei previously mentioned (Chapter 9). In an identical experimental design we administered the irreversible MAO inhibitor pargyline. Surprisingly chronic treatment only resulted in significant reductions in [^{3}H]2-BFI specific binding in two of the nuclei mentioned previously, the arcuate nucleus and area postrema. It is unclear if the data generated reflects an actual loss of the enzyme or only a loss of function/binding site availibility. However, the failure of the short term experiments to reduce [^{3}H]2-BFI specific binding suggests the latter may be the case, i.e., due to the differing binding sites utilised by I_2 ligands and MAO inhibitors a reduction in I_2 MAO sites may only manifest after the enzyme has been be metabolised by the cell. Furthermore the failure of pargyline to reduce levels of [^{3}H]2-BFI specific binding in some of the nuclei assessed suggests subpopulations of I_2 sites exist that are insensitive to deactivation by pargyline.

The aim of chapter 10 was two fold. Firstly to look at the distribution of [^{3}H]2-BFI I_2 sites within the mouse brain and secondly to compare these levels of binding to those found within transgenic litter mates that over-express MAO-B (Richards et al, 1998). The results suggest that in control C57/B mice 2-BFI labels similar sites to that found within the rat i.e., the circumventricular organs and lining of the ventricles. In contrast to the results from the rat the mouse shows low levels of specific binding within the SFO and very high levels in the dorsal raphe and paraventricular thalamus (Chapter 10).

Comparison of this data to that found in transgenic MAO-B over-expressers indicated areas of low (<20 fmol/mg tissue) [^{3}H]2-BFI specific binding within controls showed large % increases in specific binding in transgenics. However, control brain areas that contained moderate or high levels of [^{3}H]2-BFI specific binding showed very low percentage increases when compared with the same region in transgenic over-expressers. This data suggests that two components of [^{3}H]2-BFI binding appear to exist. A number of radiochemical binding studies have suggested that two I_2 sites exist that differ with respect to their affinity for I_2 ligands (Olmos et al, 1993; Alemany et al, 1997; Mallard et al 1992; Alemany et al, 1995b; Alemany et al, 1995a; Lione et al, 1998). Based on autoradiographical data presented in

chapter 8 it is plausible to suggest that two populations exist. The first population of [^{3}H]2-BFI I_2 sites are regionalised to discrete nuclei, i.e, arcuate nucelus and area postrema, whilst the second population is found at low levels diffusely throughout the brain. It is important to remember these autoradiography assays are conducted below the K_D and hence the resulting autoradiograms will show less than 50% of available I_2 sites. The transgenic data suggests that all regions that show low levels of (<20 fmol/mg tissue) [^{3}H]2-BFI binding, i.e., visual cortex, motor cortex or sensory cortex, also show large % increases (>390% increase) when compared with those levels within transgenic MAO-B over expressers. However, areas which show high levels of (>50 fmol/mg tissue) [^{3}H]2-BFI binding, i.e., aqueduct, dorsal 3rd ventricle, lateral ventricle and lateral 4th ventricle, show very low percentage increases when compared with those levels within transgenic MAO-B over expressers. It is therefore tempting to suggest that the second diffuse, low affinity, population is MAO-B. Hence, areas which show low levels of [^{3}H]2-BFI binding within control brain tissue would be increased greatly in transgenics through the over-expression of MAO-B. Conversely, within a brain region that shows high levels of specific binding this increase in 'background' MAO-B would not increase sufficiently to go above the levels of [^{3}H]2-BFI bound by the possible non MAO-B, high affinity component. It is unclear if this component is MAO-A or a possible non MAO I_2 site.

The levels of [^{3}H]2-BFI specific binding reported in rats and mice (Chapter 8 and 10) are considerably lower than that reported for [^{3}H]Ro19-6327 within the same species (Saura et al, 1992; Saura et al, 1994). Generally speaking autoradiographical data derived for MAO is measured in the low pico moles (Saura et al, 1992), whilst data for the [^{3}H]2-BFI I_2 site is measured in the low femto moles (Chapter 8 and Chapter 9). In the rat brain the K_D has been reported as 1.71 nM (Lione et al, 1998). As mentioned in chapter ten the K_D of the [^{3}H]2-BFI I_2 site has not been assessed, but has been suggested to be between 2 and 4 nM (Alan Hudson, personal correspondence). The autoradiographical results of Saura et al, (1994) and (1992) within the mouse and rat are based on a much higher concentration of 15 nM which is the K_D reported for the rat (Cesura et al, 1989b). Hence, as previously

mentioned the studies reported here are using concentrations below the K_D it is unlikely that [^{3}H]2-BFI is binding to 50% of available I_2 sites. This is in contrast to the MAO-B studies which use the K_D and thus will image 50% of [^{3}H]Ro19-6327 labelled MAO-B sites. However, this alone cannot account for the considerable differences between levels of [^{3}H]Ro19-6327 and [^{3}H]2-BFI specific binding.

As mentioned above the regionalisation of [^{3}H]2-BFI distribution and the relationship this distribution has to % increases in specific binding when comparing controls to transgenic MAO-B over-express ers suggests that a non MA0-B imidazoline site may exist. In addition to MAO-B [^{3}H]2-BFI distribution is similar to that of angiotensin II receptors (Hauser et al, 1998). Iodinated angiotensin II labels the area postrema, arcuate nucelus, inferior olives, locus coeruleus, solitary tract and as mentioned in chapter 10 binds to discrete subnuclei within the interpeduncular nucleus, an ability it shares with [^{3}H]2-BFI (Hauser et al, 1998). This finding is of particular interest for a number of reasons. Firstly, in contrast to previous rat brain autoradiographical studies which use [^{3}H]2-BFI (Lione et al, 1998) data within chapter 8 indicate the rat subfornical organ (SFO) shows high levels of [^{3}H]2-BFI specific binding Secondly, the injection of angiotensin II into the rat subfornical organ increases blood pressure (Mangiapane and Simpson, 1980) and this hypertensive response can be modulated by the application of some I_2 ligands which include idazoxan to the hypothalamus (Arrais et al, 1997; Saad et al, 1998; Araujo-Almeida et al, 1999). It has been firmly established that α_2-adrenoceptor agonists that contain an imidazoline moiety reduce blood pressure and it has been proposed that this effect is mediated through I_1 sites, not α_2-adrenoceptors (Ernsberger, 1998 & 1999, Van Zwieten & Peters, 1999). Hence, a number of lines of evidence associate I_2 sites and I_2 ligands with fluid balance and blood pressure. It may be the case that I_2 ligands activate angiotensin II directly, the affinity of angiotensin II for I_2 sites and conversely the affinity of I_2 ligands for angiotensin II receptors has not been established. Alternately I_2 sites may be heteroreceptors located elsewhere on the cell that regulate neuronal firing. *In vivo* studies

have indicated that the release of 5-HT from pineal gland cells is under noradrenergic control and mediated by α_1-adrenocetors (Aloyo and Walker, 1988). It is possible that I_2 heteroreceptors depolarise noradrenergic neurons which then release NA onto post synaptic α_1-adrenoceptors, which would then induce 5-HT release from the pineal gland. This could account for the ability of both methoxamine and fenfluramine to significantly substitute for 2-BFI (Chapter 5 and 7).

Alternately the inhibition of MAO-A or MAO-B by I_2 ligands may be sufficient to increase concentrations of 5-HT and catecholamine neurotransmitters such that they activate postsynaptic receptors. Hence, the I_2 MAO site could be located pre-synaptically to α_1 adrenoceptors. The inhibition of MAO situated in these terminals may result in an extracellular increase in NA, in a manner similar to amphetamine (a drug which has been shown to significantly substitute for 2-BFI; Chapter 5), NA would then stimulate whatever receptors were situated postsynaptically, possible α_1-adrenoceptors. The type of postsynaptic receptor stimulated would be further dependent on the substrate available for the I_2 MAO site. Hence, it may be the case that a subset of I_2 MAO sites are located in serotonergic neurons and the inhibition of these enzymes would then lead to the accumulation of 5-HT and subsequent activation of postsynaptic 5-HT receptors.

Hence, the question that fundamentally needs to be answered is does this inhibition of MAO lead to the accumulation of sufficient 5-HT and catecholamines to active receptor systems. It has been suggested that this is probably not possible (J.G. Richards, personal Communication). However, the reversible MAO-A inhibitors moclobemide and Ro41-1049 do show full substitution in 2-BFI trained rats (Chapter 6). Consistent with this a number of beta carbolines, that have a high potency for inhibiting MAO-A show full substitution for the 2-BFI cue (Chapter 7). It would be interesting to see if rats could be trained with one of these reversible inhibitors. Alternately, using *in vivo* microdialysis, it would be interesting to compare 5-HT, catecholamine and associated metabolite concentrations after the

administration of 7mg/kg 2-BFI, 9mg/kg Ro41-1049 and 20mg/kg moclobemide. If moclobemide for example, fails to increases NA overflow to a similar extent as 7mg/kg 2-BFI then this would suggest that the administration of reversible MAO inhibitors cannot solely account for the 2-BFI cue. Conversely, if the administration of 2-BFI, moclobemide and Ro 41-1049 generated similar concentrations of NA or 5-HT etc, then this would be more correlational data suggesting the cue or psychopharmacological effects of 2-BFI administration are mediated by the inhibition of MAO.

A number of experimental findings within this thesis link I_2 sites and I_2 ligands with MAO. The autoradiographical distribution of [^{3}H]2-BFI I_2 sites bears a closer resemblance to that of MAO-B rather than MAO-A (Chapter 8). Chronic treatment with the irreversible MAO-B inhibitors deprenyl and tranylcypromine reduce [^{3}H]2-BFI I_2 specific binding within the arcuate nucleus, area postrema, pineal gland and interpeduncular nucleus. However, experiments carried out under the same conditions with the irreversible MAO-B inhibitor pargyline significantly reduced binding only within the arcuate nucleus and area postrema (Chapter 9). These findings, and those of others, would associate the distribution of I_2 binding sites with the sites for MAO-B (Raddatz et al, 1997). However, there is a marked disparity between the density of the I_2 sites compared to either subtype of MAO; the density of the I_2 sites is around 100-fold lower which compromises the conclusion that they are associated (Saura et al, 1992; Saura et al, 1994; Chapter 8; Chapter 9; Chapter 10).

In contrast, the behavioural studies reported in this thesis indicate that it is substances that reversibly inhibit MAO-A that substitute for the 2-BFI cue. This was the case both for specific reversible MAO-A inhibitors and for the β-carbolines, which are also reversible inhibitors of MAO-A (Chapter 6; Chapter 7). The specific MAO-B inhibitors examined showed very low levels of substitution (Chapter 6). Therefore, at this time, it is not possible to rationalise the behavioural studies with those of biochemical investigations.

Agmatine has a moderate affinity for α_2-adrenoceptors, I_1 sites and depending on the tissue I_2 sites (Li et al, 1994; Piletz et al, 1995; Lione et al,

1996; Hudson et al, 1996; Regunathan et al, 1996; Lione et al 1998). The results presented in chapter 4 indicate agmatine significantly substitutes for 2-BFI. This is an important finding as it indicates agmatine is bio-active and raises the possibility of training rats for drug discrimination with agmatine. Following a similar method to that used in the drug discrimination NA chapter (chapter 5) i.e., by administration of specific agonists from each subtype of receptor, the psychopharmacology of agmatine could be probed.

In conclusion the data presented in this thesis indicates that 2-BFI is an excellent ligand for exploring the psychopharmacology of I_2 sites *in vivo*. Drug discrimination studies further indicate the quinoline (BU224 and BU216) and isoquinoline (BU226) analogues of 2-BFI are potent I_2 ligands *in vivo* as they are in vitro. Data presented in Chapter 5 indicated that the stimulation of α_1-, but not β- adrenoceptors contributes to the 2-BFI cue. Data in chapter 7 indicates in addition to the noradrenergic component the 2-BFI cue contains a significant serotonergic component. It is possible that levels of 5-HT and NA are increased via the inhibition of MAO. Consistent with this theory the administration of reversible MAO-A, but not MAO-B inhibitors leads to full substitution for the 2-BFI cue. Further studies using the β carbolines, compounds which have recently been shown to have a high affinity in vitro for the I_2 site and inhibit MAO, indicate that the 2-BFI cue is heavily associated with the inhibition of MAO. The autoradiographical data presented in Chapter 8, 9 and 10 further links I_2 sites with MAO. Combined the data presented in this thesis suggests that the behavioural pharmacology of I_2 ligands is heavily associated with MAO. However, this data taken in conjunction with the autoradiographical data presented within this thesis suggests the I_2 MAO site maybe located on a subpopulation of both MAO-A and MAO-B.

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