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BEHAVIOURAL AND NEUROCHEMICAL CORRELATES OF DRUGS ACTING AT IMIDAZOLINE AND α2-ADRENOCEPTOR BINDING SITES.

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Doctor of Philosophy

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The University of Aston in Birmingham

Behavioural and neurochemical correlates of drugs acting at imidazoline and α2-adrenoceptor binding sites.

Shaun Jordan

Submitted for the degree of Doctor of Philosophy 1993

Summary

A number of agents with differing selectivity profiles for the non-α2-adrenoceptor binding site (NAIBS), imidazoline preferring receptor (IPR) and α2-adrenoceptor were employed in a series of behavioural and neurochemical experiments to determine a functional role for the former two sites.

The highly selective NAIBS ligand RX801077 produced an increase in rat brain extracellular noradrenaline (NA) levels, as determined by the technique of *in vivo* microdialysis, which may underlie its ability to produce a discriminable cue in the same species. This increase in NA may be due to a suggested link between the NAIBS and the monoamine oxidase inhibitor (MAOI) activity of RX801077. For instance, the RX801077 cue was substituted for by the MAOI drugs pargyline and moclobemide, which themselves down regulate NAIBS when administered chronically. RX811059 substituted for the RX801077 cue which may be due its ability to stimulate NA release via its activity as a highly selective α2-adrenoceptor antagonist. An effect upon NA output may also explain the ability of RX801077 to 'mimic' the anti-immobility effect of the antidepressant drug desmethylimipramine (DMI) in the forced swimming test. Further studies are therefore required to examine a possible role for the NAIBS in the treatment of depression.

Discriminable cues were also produced by RX811059 and the α2-adrenoceptor agonist clonidine, probably as a consequence of their respective ability to stimulate and inhibit NA output via their opposing activity at α2-adrenoceptors. The IPR has been suggested to play a role in mediating the hypotensive effect of clonidine, although a precise role was unable to be established for this site in the present studies due to the unavailability of highly selective IPR agents.

KEY-WORDS: Non α2-adrenoceptor idazoxan binding site (NAIBS)
Imidazoline preferring receptor (IPR) *In vivo* brain microdialysis
Porsolt' forced swimming test Drug discrimination

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To Charlotte

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List of abbreviations

NA	Noradrenaline
DA	Dopamine
5-HT	Serotonin
ACh	Acetylcholine
GABA	Gamma-amino-butyric acid
DMI	Desipramine
DD	Drug discrimination
LC	Locus coeruleus
NRL	Nucleus reticularis lateralis
IPR	Imidazoline Preferring Receptor
NAIBS	Non $\alpha 2$-adrenoceptor idazoxan binding site
PAC	Para-amino-clonidine
CDS	Clonidine displacing substance
CNS	Central nervous system
OK	Opossum kidney
1-PP	1-(2-pyrimidinyl)-piperazine
MAOI	Monoamine oxidase inhibitor
ECS	Electro-convulsive shock
REM	Rapid eye-movement
HPLC-ECD	High performance liquid chromatography-electrochemical detection
i.p.	Intraperitoneal
FR	Fixed ratio
OD	Outer diameter
I.D	Internal diameter
S	Saline
D	Drug
T	Test day

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INTRODUCTION

1.1. An introduction to α_2-adrenoceptors and imidazoline specific Sites.

A variety of agents previously thought to be specific for α2-adrenoceptors has since been demonstrated to bind to several distinct imidazoline sites in a variety of central and peripheral cell types (Ernsberger et al, 1987). The possibility therefore exists that at least some of the pharmacological properties of these agents, such as clonidine and idazoxan, may be due to an action at either or both of these imidazoline specific sites.

Two principal subtypes of imidazoline sites (I1 and I2) have been identified on the basis of ligand binding studies (Reis et al, 1992). For the purpose of this thesis these sites will be referred to as the imidazoline preferring receptor (IPR) and the non α2-adrenoceptor idazoxan binding site (NAIBS) respectively. It is currently uncertain whether these sites are only binding sites or receptors linked to a function. Indeed, a functional role as yet remains to be established for these sites. Supposing the IPR and the NAIBS are functional, further research may reveal endogenous ligands for these sites which could have a unique therapeutic profile or serve as useful research tools. This thesis will concentrate on the possible functions of the NAIBS, because as yet there are no agents selective for the IPR.

1.2. α2-adrenoceptors and other adrenoceptor subtypes

α-adrenoceptors mediate the effects of the catecholamines adrenaline and NA in the periphery and CNS. Ahlquist (1948)

initially subclassified adrenoceptors into α- and β-subtypes according to the pharmacological potency profile of NA, adrenaline and isoprenaline at this receptor.

Both α- and β-adrenoceptor subtypes have been further subdivided, on the basis of the rank order of potency of agonists, into α1- and α2- and β1- and β2-adrenoceptors (Lands et al, 1967; Berthelson & Pettinger, 1977). α1-adrenoceptors are situated postsynaptically, whereas α2-adrenoceptors exist presynaptically at various sites over the surface of central noradrenergic neurones such as on the cell body itself or upon its terminals. However, postsynaptic α2-adrenoceptors also exist in central and peripheral tissues (Langer, 1974; Pinder, 1985).

Presynaptic α2-adrenoceptors, or autoreceptors, are involved in the regulation of NA release through a negative feedback mechanism mediated by NA itself (Starke, 1977; Langer, 1981; Raiteri et al, 1983). During NA release elicited by stimulation of the noradrenergic nerve terminal, once the neurotransmitter reaches a threshold concentration in the synaptic cleft, it activates presynaptic α2-adrenoceptors triggering a negative feedback mechanism that inhibits further NA release from the nerve terminal. This inhibitory action of NA may be mimicked by synthetic α2-adrenoceptor agonists, such as clonidine or guanabenz (Langer, 1981). In contrast, α2-adrenoceptor antagonists, such as yohimbine and idazoxan, increase NA release via their blockade of a2-adrenoceptors (Raiteri et al, 1983; Dennis et al, 1987). Extracellular NA levels are also regulated by presynaptic neuronal reuptake systems (Iverson, 1971). Reuptake of synaptic NA into the nerve terminal can be prevented by the tricyclic antidepressant DMI (Langer et al, 1981). An increase in extracellular NA concentration occurs as a consequence of reuptake blockade, this in

turn can activate presynaptic α2-adrenoceptors to produce a greater degree of autoinhibition (Starke, 1987). Thus the extent to which an α2-adrenoceptor agonist or antagonist may influence NA output appears to be dependent upon the concentration of endogenous NA available in the synaptic cleft.

α2-adrenoceptors can also act as heteroreceptors. For instance, they exist on serotoninergic nerve endings where their activation inhibits 5-HT release (Gothert et al, 1981; Raiteri et al, 1983). Auto- and hetero- α2-adrenoceptors appear to differ pharmacologically since NA and clonidine are more potent at the former (Raiteri et al, 1983: Maura et al, 1985) and (-)mianserin and 1-(2-pyrimidinyl)-piperazine (1PP) at the latter (Maura et al, 1985; Bianchi et al, 1988).

Guanine nucleotide regulatory (G) proteins couple hormone and neurotransmitter receptors to second messenger systems. Both ß1- and ß2-adrenoceptors are coupled to the stimulation of adenylate cyclase by Gs, although there are some ß-adrenoceptor actions which are independent of cAMP but still mediated by Gs (see Bylund, 1988). Similarly, α2-adrenoceptors are coupled to the inhibition of adenylate cyclase through Gi. Recent evidence suggests that at least some of the actions of α2-adrenoceptors (eg. Na+/H+ antiporter, Ca++ channels, K+ channels) are not mediated by Gi. The mechanism of action of α1-adrenoceptors is less well understood, although they may be coupled to phosphatidylinositol breakdown through a G protein termed Gx that has not ben specifically identified as yet.

α2-adrenoceptors have been subdivided on the basis of radioligand binding techniques in a variety of tissues and species. The human platelet is the prototype tissue for the α2A-adrenoceptor subtype which has a relatively low affinity for

prazosin and a higher affinity for oxymetazoline, while the opposite is true for the α2B-adrenoceptor subtype (Bylund, 1981; Bylund et al, 1985, 1988). Regan and coworkers (1988) demonstrated the existence of two separate α2-adrenoceptor genes in mammalian cells the products of which display a differential sensitivity to prazosin and oxymetazoline. The C10 and C2/5a clones are believed to encode the α2A- and α2B-adrenoceptor subtypes respectively. These receptors are both believed to consist of a protein chain of 450 amino acids consisting of seven transmembrane segments, and they appear to differ only with respect to the number of amino acids present on the fifth and sixth segments (see Harrison et al, 1991).

The relationship between presynaptic α2-adrenoceptors and the α2A- and α2B subtypes which are both present in rat cerebral cortex (Cheung et al, 1982; Dickinson et al, 1986) in similar proportions (Bylund, 1985) has not yet been clarified. Various studies have reported that the presynaptic α2-auto or heteroreceptors do not belong to the α2A-adrenoceptor subtype, suggesting the modulation of NA and 5-HT may be mediated by the α2B-adrenoceptor subtype (Raiteri et al, 1983; Gothert et al, 1981; Frankhuyzen & Mulder, 1982; Gobbi et al, 1990). The pharmacological characterisation of α2-adrenoceptors in an opossum kidney (O.K) derived cell line suggested the possible existence of a third α2-adrenoceptor subtype (Murphy & Bylund, 1988). This receptor initially appeared to have a pharmacological profile more akin to the α2A-adrenoceptor subtype, although further characterisation suggests it to be as different from both subtypes as they are different from each other. Thus, the α2-adrenoceptor of the O.K cell appears to represent a third subtype termed the α2C-adrenoceptor (Bylund, 1988).

1.3. The Imidazoline Preferring Receptor (IPR)

The location of this site in the brain appears to be restricted to the nucleus reticularis lateralis (NRL) of the ventrolateral medulla (Bousquet et al, 1981). Clonidine acts via the central nervous system (CNS) to reduce sympathetic outflow to the periphery to produce hypotension (see review Kobinger, 1978). However, a site other than the α2-adrenoceptor appears to be responsible for this effect for several reasons. Firstly, neither neurotransmitter depletion with reserpine, nor destruction of nerve terminals with 6-hydroxydopamine substantially attenuates clonidine-induced hypotension which excludes an involvement for presynaptic α2-adrenoceptors (Dollery & Reid, 1973). Secondly, α2-adrenoceptor agonists of imidazoline (eg. clonidine) but not phenylethylamine (eg. noradrenaline; NA) structure induce hypotension following their direct microinjection into the NRL (Bousquet et al, 1984). Moreover, microinjections of the α2-adrenoceptor antagonist idazoxan (imidazolinic), but not yohimbine (non-imidazoline) abolish clonidine-induced hypotension (Tibirica et al, 1988). In addition, compounds with a high affinity for the IPR (Ernsberger et al, 1992) which act as weak α2-adrenoceptor agonists (eg. moxonidine and rilmenidine) have a hypotensive action with less sedation than clonidine (Bricca et al, 1989; Fillastre et al, 1988). Finally, microinjections into the NRL of imidazolines without α2-adrenoceptor agonist activity, such as cirazoline and ST 587, mimic the cardiovascular property of clonidine (Bousquet et al, 1984), although these α1-adrenoceptor agonists have no such effect when adminsitered intravenously or into the vertebral artery (DeJonge et al, 1976). Likewise, histaminergic agents possessing an imidazoline moiety, such as cimetidine, bind to the

IPR (Ernsberger et al, 1987, 1988). [3H]-para-amino-clonidine (PAC) binds to the IPR and is displaced with a rank order of potency of phentolamine > PAC > clonidine > idazoxan > cimetidine > imidazole-4-acetic acid >> cirazoline >> amiloride (Reis et al, 1992). Thus, the IPR is an imidazoline specific site distinct from the α2-adrenoceptor and might be involved in the pathogenesis of hypertension.

1.4. Non α2-Adrenoceptor Idazoxan Binding Sites (NAIBS)

The α2-adrenoceptor antagonist idazoxan binds to a second site distinct from the α2-adrenoceptor and the IPR (Michel & Insel, 1989). This site is the non α2-adrenoceptor idazoxan binding site (NAIBS) which has been identified in a variety of central and peripheral cell types (see Michel & Insel, 1989). It has been defined as that proportion of idazoxan binding which is not displaced by catecholamines such as NA (Boyajian et al, 1987). NAIBS identified in rabbit renal basolateral membranes have a high affinity for a number of imidazolines (eg. phentolamine and clonidine), guanidino compounds (eg. guanabenz) and for the diuretic amiloride (Coupry et al, 1987). However, NAIBS localized in renal tissue of rat, pig, hamster and human display a high affinity for some imidazolines (eg. tolazoline and UK-14,304) but not for others (eg. phentolamine and clonidine). The NAIBS can be distinguished from the IPR in that the NAIBS has a low affinity for amiloride and a high affinity for guanidino (eg. guanabenz) and benzazepine (eg. SK&F 86466) compounds. The latter two classes of compounds are not recognised by the IPR (Michel et al, 1989, Coupry et al, 1987, MacKinnon et al, 1989). Unlike the IPR, NAIBS have been identified in chromaffin cells and astrocytes and dense populations exist in rat brain area

postrema, interpeduncular nucleus, arcuate nucleus, ependyma, nucleus tractus solitarius, frontal cortex, hypothalamus and the anterior olfactory nucleus (Hudson et al, 1991).

A solubilized form of the NAIBS, retaining the above pharmacological profile, has been isolated from guinea-pig cerebral cortex (Wikberg & Uhlen, 1990). This form of NAIBS differs from the IPR in having a low affinity for the histaminergic-imidazolinic compounds histamine, cimetidine and imidazole-4-acetic acid, further supporting a separate identity for this site.

A function as yet remains to be established for the NAIBS, although several roles have been proposed. For instance, NAIBS may be involved in the regulation of prolactin release from the anterior pituitary, as idazoxan and yohimbine (an $\alpha 2$-adrenoceptor antagonist inactive at the NAIBS) decrease and increase prolactin release respectively (Krulich et al, 1989). Furthermore, the NAIBS has been proposed to be involved in hyperphagic mechanisms, as idazoxan-induced hyperphagia was not produced by the highly selective $\alpha 2$-adrenoceptor antagonist RX811059 which has minimal NAIBS affinity (Jackson et al, 1991). A hyperphagic role for the NAIBS is supported by the location of these sites in brain areas concerned with appetite such as the area postrema and hypothalamus (Hudson et al, 1991). The NAIBS might be responsible for the proposed neuroprotective actions of idazoxan with respect to global and local cerebral ischaemia (Gustafson & Wieloch, 1992).

Supposing the IPR and/or the NAIBS are functional, further research may reveal an endogenous ligand for these sites which may have a unique therapeutic profile or serve as a useful research tool. Indeed, a compound isolated from bovine calf brain may be the endogenous agent for these imidazoline sites. This chemically unidentified compound displaces labelled clonidine from the IPR

(Atlas & Burstein, 1984). This clonidine displacing substance (CDS) binds with high affinity to the IPR and NAIBS as well as to $\alpha 2$-adrenoceptors (Ernsberger et al, 1988; Reis et al, 1992). The presence of CDS in synaptosomes and its regional localisation in the brain (Meeley et al, 1989) suggest it may be a neurotransmitter, while its presence in serum and its potent ability to stimulate catecholamine release from gastric chromaffin cells suggest hormonal properties (Reis et al, 1992).

1.5. Central noradrenergic anatomy

Various histochemical and biochemical techniques have been used to determine the distribution of NA in the CNS. A number of noradrenergic cell groups have been detected which reside exclusively in the pons and medulla oblongata. The rat brain stem contains about 4700 NA cells on either side of the brain (Swanson & Hartman, 1975). These groups form three major cell systems (Dahlstrom & Fuxe, 1965; Moore & Bloom, 1979): the locus coeruleus (LC) complex; the lateral tegmental cell system; and the dorsomedullary cell group (Figure 1.1).

1.5.1. Locus coeruleus (A6).

The largest cell group is the LC (A6 cell group of Dahlstrom & Fuxe, 1965) in the dorsolateral pons, whose projections extend to almost the entire neuraxis. It is composed almost exclusively of NA neurons (Dahlstrom & Fuxe, 1965), which represent about 45% of all NA cell bodies in the mammalian brain. LC cell bodies extend rostrally in the dorsolateral part of the central gray and dorsolaterally along the medial aspect of the superior cerebellar peduncle into the roof of the fourth ventricle (A4 cell group, which is prominent in primates and humans; Demirjian et al, 1976).

All noradrenergic projections to the cerebellum, neocortex and hippocampus originate in the LC (Mason & Fibiger, 1979; Room et al, 1981; Ungerstedt, 1971; Morrison et al 1981). In addition, the LC provides major inputs to the thalamus, septum, amygdala, and to a lesser extent the hypothalamus (Sawchenko & Swanson, 1981). The LC appears to exert a global infleunce over brainstem function, particularly with regard to its motor and integrative functions (Fritzschy & Grzanna, 1990b). A descending LC projection passes through the spinal cord within layers I and II terminating in the dorsal horn and intermediate zone (Fritschy & Grzanna, 1990a).

Figure 1.1 The NA innervation of the CNS (From Kruk & Pycock, 1992).
The nerve-cell bodies are concentrated in the brainstem and locus ceoruleus; the distribution of axon terminals is shown.

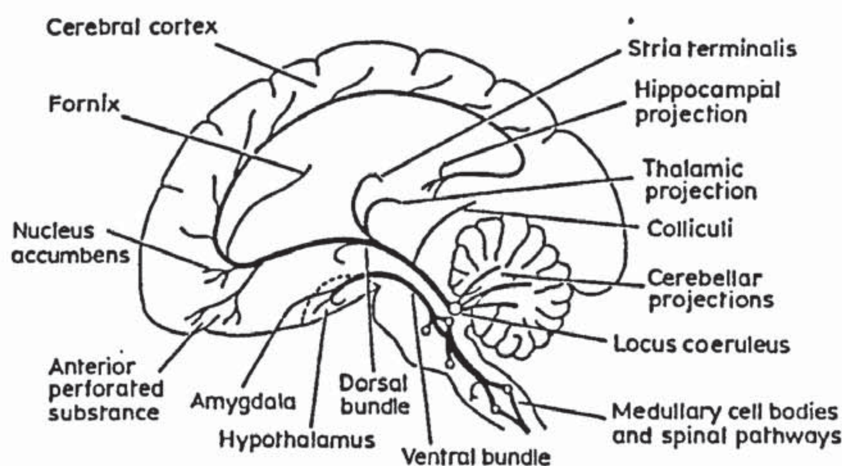

Figure 3.5 The noradrenaline innervation of the CNS. The nerve-cell bodies and dentrites are concentrated in the brainstem and locus coeruleus; the distribution of axon terminals is shown.

1.5.2. Ventrolateral tegmental (A1, A5 & A7) cell groups.

The NA cells of the lateral tegmental cell system are located in the ventrolateral tegmentum, and they extend from the caudal pole of the medulla oblongata to the level of the motor nucleus of the trigeminal nerve in the pons (Blessing et al, 1980; Dahlstrom & Fuxe, 1965). The medullary part of this system consists of the A1 and A7 groups. These medullary cells extend from the pyramidal decussation up to the level of the rostral part of the inferior olivary nucleus. They are mainly scattered around the lateral reticular nucleus, and in the rat some cell bodies are found within this nucleus (Blessing et al, 1980); these cells form the A1 group. The A5 cell group is located in the pontine part of the lateral tegmental cell system, and these cells are distributed from the rostral part of the facial nucleus up to the trigeminal motor nucleus. Caudally they are caudal and medial to the outgoing fibres of the facial nerve, close to the superior olivary complex. Further rostrally these cells extend into the area between the ventrolateral border of the superior cerebellar peduncle and the lateral lemniscus, forming the A7 cell group. The border between the A7 and subcoeruleus groups is not well defined (Bjorklund & LIndvall, 1986).

1.5.3. Dorsal medullary (A2) cell group.

This group is located in the posterior aspect of the nucleus of the solitary tract and in the commissural nucleus (Blessing et al, 1980; Dahlstrom & Fuxe, 1965; Swanson & Hartman, 1975).

1.5.4. Major noradrenergic projection systems.

There are three major noradrenergic projection systems, the dorsal, medial and ventral noradrenergic bundles (Ungertsedt, 1971), which consist of ascending and descending projections from

NA cells in and around the LC (Figure 1.1). The ventral noradrenergic bundle arises ventrally and caudally to the LC. This ascending system terminates in the hypothalamus and sub-cortical sites. The dorsal noradrenergic bundle originates from pontine LC cells. Its ascending projections innervate the thalamus, the dorsal hypothalamus, the limbic system, the hippocampus and the neocortex. Ascending medullary projections consisting of short axons project to the nucleus tractus solitarius and also the dorsal vagal nucleus. In addition, three projections from the medulla descend to the dorsal and ventral horns and the lateral column of the spinal cord.

1.6. Possible roles of NA in the CNS.

Noradrenergic projections innervate almost the entire neuraxis which would suggest an involvement for these cells in a variety of behaviours and physiological systems.

1.6.1. NA and stress.

One of the first roles suggested for NA was an involvement in stress. In early studies, cold and electric foot-shocks decreased brain and adrenal NA levels in rats (Barchas & Freedman, 1963; Maynert & Levi, 1964). Similar results are seen with aggregation stress (Bliss et al, 1968), hypoxic stress (Koob & Annau, 1974), muscular exertion (Gordon et al, 1966), and exhaustion stress (Stone, 1973). Increases in NA synthesis and its major metabolites accompanied these stress-induced decreases in brain NA (Gordon et al, 1966; Stone, 1973; Stolk et al, 1974; Stone, 1975). Thus stress

appears to activate central NA neurones, which in turn, may dampen the organism's response to stressors. For example, the increased noradrenergic activity which accompanies epileptic seizures may in turn suppress seizure activity (Calderini et al, 1978).

1.6.2. Depression.

Depression is a disorder in which the major change is in mood or 'affect'. The symptoms include depressed mood, low self-esteem, motor retardation or agitation, and other physiological symptoms such as early wakening from sleep, loss of appetite, loss of libido and general tiredness. A depressed patient may express some if not all of these symptoms. Depression with physical symptoms and no apparent cause is termed 'endogenous' and is generally more severe than 'reactive' depression which may be precipitated by external events and is frequently assocated with anxiety. The endogenous type responds better to drug therapy than the reactive type, although this may reflect the inability of these drugs to treat the primary defect in the latter case (see Nutt & Glue, 1989).

A reduced monoaminergic (noradrenergic and/or serotoninergic) function has been postulated in the aetiology of endogenous depression (Schildkraut, 1965; Weiss et al, 1985; Cheetham et al, 1991). As presynaptic α2-adrenoceptors play an important physiological role in the regulation of synaptic NA levels in the CNS (Langer et al, 1981), elevated autoreceptor activity, accompanied by decreased NA release, could be involved in the aetiology of depression (Dickinson, 1991). This is supported by the finding that the density and affinity of α2-adrenoceptors are increased in the brain of depressed suicides (Meana et al, 1992).

Moreover, selective and specific $\alpha 2$-antagonists may be of value in depression (Dickinson, 1991).

1.6.3. Arousal and attention.

Studies monitoring LC discharge patterns under various conditions in freely moving and restrained rats support a role for the LC system in arousal and attention (Aston-Jones & Bloom, 1981a,b). Discharge rates decrease during sleep, grooming, and sweet-water consumption. In contrast, bursting activity of LC neurons accompanies arousing stimuli (eg. awakening stimuli) which interrupt these behaviours. The LC system has been suggested to facilitate changes between behavioural states. Thus it may be able to signal that something important may be going on, causing awakening from sleep, arousal from drowsiness, or orientation of attention towards relevant stimuli (Aston-Jones & Bloom, 1981b). Selective lesions of the ascending noradrenergic system elicit certain behaviours including an inability to extinguish previously acquired reinforced behaviours. This resistance to extinction has been interpreted as an inability to filter out irrelevant stimuli. According to Mason and Iverson (1979) the LC "appears to be telling the forebrain when to attend incoming stimuli and when to ignore them". This supports the suggestion that the LC helps filter out irrelevant stimuli and increase the signal-to-noise ratio of behaviourally significant stimuli (Segal & Bloom, 1976).

1.6.4. Learning.

Increases in learning and memory processes have been ascribed to compounds able to increase catecholamine levels (Dunn, 1980). Moreover, drugs able to reduce catecholamine output impair

the performance of well-learned responses (Hunter et al, 1977). Studies to assess the role of the LC in learning suggest these neurones may not necessarily be implicated in all types of learning, but they may be preferentially involved in learning contingencies regarding challenging or noxious events. For instance, rat neurochemical studies and pigeon brain stimulation studies indicate an active role for LC neurones in aversive, but not appetitive, conditioning (Welsh and Gold, 1985). Yohimbine, has been reported to enhance learning behaviour in rats, although this may be due to increased arousal and/or attention rather than direct actions on memory and recognition systems (Huang et al, 1987).

1.6.5. Noradrenergic systems and anxiety

1.6.5.1. Pharmacological evidence.

Clonidine, like diazepam, abolishes the anxiogenic actions of yohimbine in humans (Charney et al, 1983). Similarly, increases in arousal and anxiety have been reported for idazoxan and yohimbine in human (Charney et al, 1983) and rat (Handley & Mithani, 1984; Pellow et al, 1987) studies. However, anxiolytic-like actions have been detected in the punished component of the Geller-Seifter conflict model associated with increased responding (Kruse et al, 1981). Yohimbine produces a discriminative cue in rat DD studies, which may be due to its anxiogenic action (Winter et al, 1978; Browne, 1981; Colpaert, 1984; Colpaert & Janssen, 1985). This cue may be substituted for by piperoxane, which like yohimbine is an $\alpha 2$-antagonist with an anxiogenic profile in humans (Browne et al, 1981). Moreover, the yohimbine cue can be blocked by clinical anxiolytics ,such as diazepam, which supports an anxiogenic action

for this agent (Browne et al, 1981).

1.6.5.2. Physiological evidence.

Low intensity LC stimulation in conscious restrained stump-tailed monkeys produces behaviours similar to those following direct threatening confrontation by humans (Redmond et al, 1976). LC lesions decrease the natural occurence of these behaviours in a social situation and in response to similar direct threatening confrontation by humans (Redmond & Huang, 1979). Furthermore, drugs which modulate LC firing produce behaviours consistent with the electrical stimulation or lesion effects (Redmond, 1977; Redmond & Huang, 1979).

1.6.6. Locomotor activity.

Noradrenergic denervation of the spinal cord by 6-OHDA, or α-adrenoceptor blockade by phenoxybenzamine, causes no profound impairment of locomotion (Morgan & Huffman,1980; Stevens et al, 1982), although the stimulus threshold of the mescencephalic motor region required to elicit locomotion may be elevated (Stevens et al, 1982). Thus, the noradrenergic system may regulate, or set, the threshold to induce locomotion by other parallel descending systems (Grillner, 1981). This is consistent with electrophysiological observations that NA, like serotonin (5HT) increases the general excitability of motorneurons to excitatory inputs (Vandermaelen & Aghajanian, 1980). The α2-adrenoceptor agonist clonidine produces various signs of behavioural suppression, such as hypoactivity (Heal et al, 1989), a loss of the righting reflex (Harsing et al, 1989), and it reduces the potentiated startle response (Davis et al, 1977). In contrast, α2-adrenoceptor antagonists act as general stimulants (Huang et al, 1987). For, example, yohimbine and idazoxan produce increases in

locomotion and startle responses in animals (Handley & Thomas, 1979; Anden et al, 1982; Chopin et al, 1986; Dickinson et al, 1990).

1.6.7. Feeding.

Central noradrenergic systems may be involved in the regulation of feeding. Injections of NA into the paraventricular nucleus of the hypothalamus elicit feeding in satiated rats (Grossman, 1960; Leibowitz, 1970). However, the mechanism by which this occurs is unclear, as is its relationship to the normal homeostatic feeding mechanism. A number of α2-adrenoceptor agonists, such as clonidine, elicit feeding (Sanger et al, 1983; McCabe et al, 1984; Goldman et al, 1985). Idazoxan produces hyperphagia in rats, which may reflect its affinity for the NAIBS, as α2-adrenoceptor antagonists with a minimal affinity for this site (eg. RX811059 and RX821002) are inactive (Jackson et al, 1991). The NAIBS may play a role in appetite since they are concentrated in brain areas such as the hypothalamus and area postrema, which are themselves involved in appetite control (Hudson et al, 1991; Jackson et al, 1991).

1.6.8. Sexual behaviour.

Sexual behaviour may be influenced by central NA systems. For example, NA injections lead to changes in sexual behaviour, and ventral noraderenergic bundle lesions virtually eliminate the female lordotic response and the analgesia associated with vaginal stimulation (Everitt et al, 1975; Crowley et al,1977). α2-adrenoceptor antagonists have been implicated in sexual behaviour. For instance, yohimbine increases sexual activity in the male rat (Clark et al, 1984) and may be used to treat male human impotence (Goldberg & Robertson, 1983).

1.6.9. Physiological and behavioural properties associated with α2-adrenoceptor agents.

The behavioural and physiological properties of α2-adrenoceptor agonists, such as clonidine, UK-14,304, guanabenz, are well documented (see Fielding & Lal, 1981; Cambridge et al, 1981; Langin et al, 1990; Heal et al, 1989). These compounds are most well known for their antihypertensive action (Kobinger, 1978). Other effects associated with this class of drugs in rodents include sedation, an impairment of the righting reflex, abnormal gait, body sag, analgesia and ptosis (Marley & Nistico, 1975; Drew et al, 1979; Paalzow & Paalzow, 1976; Heal et al, 1983; Harsing et al, 1989). Furthermore, α2-adrenoceptor agonists reduce locomotor activity, and produce mydriasis, hypothermia and a suppression of the acoustic startle reflex (Bugajski et al, 1980; Davis & Astrachan, 1981; Heal et al, 1989). It is possible to define clonidine's effects at α2-adrenoceptors by blockade with selective α2-adrenoceptor antagonists, such as fluparoxan and idazoxan (Doxey et al, 1983, 1984; Sanger, 1988; Heal, 1990; Halliday et al, 1991). However, clonidine also interacts with sites other than the α2-adrenoceptor. For instance, it binds with high affinity to the IPR and the NAIBS in the brain and periphery respectively (Ernsberger et al, 1990; Coupry et al, 1987). Moreover, clonidine may act as a partial agonist at α1-adrenoceptors (Bradshaw et al, 1982).

α2-adrenoceptor antagonists, such as yohimbine, idazoxan and RX811059, produce a variety of subjective and objective effects in humans and animals (Chopin et al, 1986; MacDonald et al, 1988; Goldberg & Robertson, 1983; Montgomery, 1988;

Dickinson et al, 1990). However, some of these effects may be due to interactions with sites other than the α2-adrenoceptor. For example, yohimbine inhibits locomotor activity in rats, which is believed to be due to its ability to inhibit DA receptors (Chopin et al, 1986). However, the more selective α2-adrenoceptor antagonists idazoxan and RX811059 stimulate locomotor activity in the same species (Dickinson et al, 1990). A functional interaction between NA and DA within the limbic system has been proposed to underlie this effect, by which α2-adrenoceptor antagonism leads to increased DA activity which stimulates locomotor activity (see Dickinson et al, 1990).

Nevertheless, idazoxan binds to sites other than the α2-adrenoceptor. It demonstrates equally high affinities for both the IPR and the NAIBS in brain (Ernsberger et al, 1990; Boyajian et al, 1987; Michel & Insel, 1989). In addition to this, it may act as an agonist or antagonist at α1-adrenoceptors in peripheral tissues. In contrast, RX811059 is a highly selective α2-adrenoceptor antagonist at concentrations at which it displays minimal NAIBS and α1-adrenoceptor affinities (Mallard et al, 1991; Doxey et al, 1983). On the other hand, RX801077 is highly selective for the NAIBS over the α2-adrenoceptor (A. Hudson - personal communication). However, the affinity of RX811059 and RX801077 for the IPR is uncertain, although the former compound contains an imidazoline moeity and as such it might be expected to bind to this site.

1.7. Selectivity of drugs acting at α-adrenoceptors, the IPR and the NAIBS

A variety of drugs are available with differing selectivities for α-adrenoceptors, IPR and the NAIBS. Clonidine was originally considered to be a selective agonist at α2-adrenoceptors (Fielding & Lal, 1981), and it mimics the action of NA in a number of experimental situations (Heal, 1990; MacDonald et al, 1988). It is possible to define clonidine's effects at α2-adrenoceptors by blockade with selective α2-adrenoceptor antagonists such as fluparoxan (reviewed in Halliday et al, 1991), and RX811059 (Mallard et al, 1991). Clonidine may also act as a partial agonist at α1-adrenoceptors (Bradshaw et al, 1982). The more selective (between α1-adrenoceptors and α2-adrenoceptors) α2-adrenoceptor agonists UK-14,304 (Cambridge et al, 1981), which binds to the IPR, and guanabenz which does not (Ernsberger et al, 1990; Gomez et al, 1991) are useful to distinguish between α2-adrenoceptor and IPR associated effects.

A variety of α2-adrenoceptor antagonists are available with differing selectivity profiles. For example, yohimbine interacts with α1-adrenoceptors, dopamine (DA) and 5-HT receptors (Scatton et al, 1980; Goldberg & Robertson, 1983). Rauwolscine, is a yohimban diastereoisomer with an α2-/α1-adrenoceptor selectivity ratio 50 times that for yohimbine (Convents et al, 1989a). Rauwolscine is inactive at the IPR and NAIBS but it binds to 5-HT_{1A} receptors (Convents et al, 1989b). Idazoxan is a more selective α2-adrenoceptor antagonist than the yohimbans (Doxey et al, 1983), however, it has been reported to act as an α1-adrenoceptor agonist or antagonist in peripheral tissues (Doxey et

al, 1983; Paciorek & Shepperson, 1983). Furthermore, unlike yohimbine, idazoxan binds with similar affinity to the IPR and NAIBS (Ernsberger et al, 1990; Boyajian et al, 1987; Michel & Insel, 1989). A series of idazoxan analogues have been developed with high α2-adrenoceptor affinity and minimal IPR and NAIBS affinities. These include RX821002 (2-methoxy-idazoxan) and RX811059 (2-ethoxy-idazoxan) (Doxey et al, 1984; Langin et al, 1990; Mallard et al, 1991). Other highly selective α2-adrenoceptor antagonists with unidentified IPR and NAIBS affinities include fluparoxan (Halliday et al, 1991), atipamezole (Scheinin et al, 1988) and 1-(2-pyrimidinyl)-piperazine (1-PP; Bianchi et al, 1988).

In contrast, a series of compounds exist which demonstrate a high affinity for the NAIBS over the α2-adrenoceptor. These include RX801023 (Mallard et al, 1991), RX801029 (Hudson et al, 1992), and the highly selective agents RX801077 (Ki=1nmol at NAIBS, Ki at α2-adrenoceptors = 3μmol; A. Hudson - personal communication) and B910101. These compounds would be expected to be inactive at the IPR as they do not possess an imidazoline moeity in their chemical structure, but this has not yet been measured.

1.8. Aims of the project.

The aim of the project was to detect a functional consequence of a drug binding to the NAIBS which was not accompanied by any effect at α2-adrenoecptors. Detection of an effect of a ligand binding to the NAIBS would establish that these binding sites have a functional role and this would provide a basis for further work to elucidate the nature of that role. Because binding to the IPR has not been widely characterised; it was accepted that non-adrenoceptor effects of such agents might not

be unequivocally assignable to the NAIBS rather than the IPR.

α2-adrenoecptor ligands have many behavioural and biochemical effects, for example effects on NA release, locomotion, sedation, feeding, depression and anxiety have been described in this Introduction. Historically it has been assumed that these effects are all mediated by α2-adrenoecptors. However, the discovery of the NAIBS and, indeed, the IPR have both rendered this assumption unsafe and have generated a starting point for the search for a function for the NAIBS. From amongst these effects, three have been selected for investigation here. However, in order not to miss an obvious function through using selected paradigms, an initial behavioural screen of several ligands with varying selectivity for the NAIBS and α2-adrenoecptors was performed.

1.9. Introduction to the experimental techniques.

1.9.1. Drug discrimination (DD). The technique of DD has been used to classify and compare the subjective effects of psychoactive compounds in animals (see Stolerman et al, 1989). Typically, animals are trained to respond in one manner following a drug treatment and in another after receiving vehicle, with correct responding being rewarded. The effects of a drug serve as a 'cue' to the subject to respond in the drug-associated manner and the absence of this cue triggers a vehicle-associated behaviour. The ability of one compound to substitute (i.e. elicit drug-associated responding) for the cue of another suggests a functional similarity between the two. The rate of responding may also be determined, although choice behaviour is the dependent variable.

In a commonly used method of DD animals are trained to

respond on one of two levers positioned either side of a reward hatch in an operant conditioning unit (Skinner box). The most commonly used subjects for DD are rats, pigeons, and monkeys. Hunger, thirst and the desire for sweetened compounds are used to maintain responding and lever pressing results in the delivery of the appropriate reward (eg. food or water). In addition, the reinforcing properties of certain drugs may be used to sustain responding (Brady & Fischman, 1985), i.e. lever presses can be rewarded by delivery of these drugs. Most studies employ one treatment-test interval although certain drugs exert different time-dependent effects. Schecter and Lovano (1982) have suggested ethanol to produce qualitatively different stimulus effects at different pretreatment times. Progressively fewer drug-appropriate responses occur with decreasing dosage of the training drug.

DD learning is related to the phenomenon of state-dependent learning (i.e., a behaviour learned in one state is not easily retrievable unless the state present during the initial learning episode is reinstated). Some believe these phenomena to differ (Colpaert, 1977), while others (eg. Jarbe, 1987) assume both to basically reflect the same properties of drugs.

1.9.2. Animal models of depression

The monoamines NA and 5-HT have been closely identified with the causes of depression since the discovery that reserpine induced depression in a small number of patients prescribed the drug as an antihypertensive (Schildkraut, 1965). In rodents, the reversal of the behavioural and physiological effects of reserpine with antidepressants was the first animal model of depression (Costa et al, 1960). There are currently more than 20 experimental procedures used to model depression in animals. The methods

employed to induce depression include stress, social separation, pharmacological treatments and other miscellaneous techniques (Table 1.1; Willner, 1991).

The central feature of these models is that various behavioural abnormalities are reversed by clinically effective antidepressant drugs. For example, the most commonly used model is learned helplessness (for review see Garber et al, 1979). This involves exposing an animal to an uncontrollable stressor which produces performance deficits in subsequent learning tasks, which are not seen in subjects able to control the identical stressor. Such deficits are reversible with a variety of antidepressants (eg. Sherman et al, 1982). The most frequently studied behaviour in all models is a general decrease in locomotor activity. Other behaviours include: increased locomotor activity, decreased or increased motivation and persistence, decreased social contact, and anhedonia (for reviews see Borsini & Meli, 1988; Willner, 1991). Anhedonia, the decreased ability to enjoy pleasure, is one of the core symptoms of depression proposed to reflect subsensitive brain reward mechanisms (Klein, 1974).

Table 1.1. Some animal models of depression.

Stress models

- Learned helplessness
- "Behavioural despair" and variants (eg. tail suspension)
- Failure to adapt to stress
- Chronic unpredictable stress
- Chronic unpredictable mild stress
- Amphetamine withdrawal

Separation models

- Primate separation models (infant and adolescent)
- Distress calling in separated chicks
- Separation in pair-bonded hamsters
- Social isolation in rats

Pharmacological models

- Reserpine reversal
- Amphetamine potentiation
- 5-Hydroxytryptophan-induced behavioural depression

Miscellaneous

- Olfactory bulbectomy
- Muricide
- Waiting behaviour
- Differential reinforcement of low rate responding
- Circadian rhythm models

1.9.2.1. Porsolt's forced swimming test.

This stress model is used routinely in the screening of antidepressant compounds (Willner, 1984; Borsini & Meli, 1988). Animals forced to swim out of their own depth make an initial frenzied attempt to escape followed by an immobile posture. This state has been named "behavioural despair"; it is assumed that the animal has "given up hope of escaping" (Porsolt et al, 1977a, 1978).

The total immobility time in a second forced swim can be

reduced by pretreatment with a variety of antidepressant therapies including tricyclics (eg. imipramine, amitriptyline or desipramine (DMI)), monoamine oxidase inhibitors (MAOI's; eg. clorgyline or deprenyl), atypical antidepressants (eg. iprindole or mianserin), electro-convulsive shock (ECS) and rapid eye-movement (REM) sleep deprivation (Borsini et al, 1981; Porsolt et al, 1977b; Porsolt et al, 1978; Finnegan et al, 1987; Porsolt, 1981; Kitada et al, 1981).

This test successfully discriminates antidepressants from neuroleptics and anxiolytics (Porsolt et al, 1977a,b), although false positives have been reported for stimulants, convulsants, anticholinergics, antihistamines, pentobarbital, opiates and other neuropeptides, and a number of other drugs (Betin et al, 1982; Browne, 1979; Kastin et al, 1978; Porsolt, 1981; Schecter & Chance, 1979; Wallach & Hedley, 1979). However, some effects of non-antidepressant agents are non-specific, for instance stimulants and anticholinergics reduce immobility by an indiscriminate stimulation of locomotor activity, rather than by delaying the onset of immobility (Kitada et al, 1981). The effectiveness of acute antidepressant treatments does not correspond to their time course of clinical action. Nevertheless, the effects of these treatments are potentiated (i.e. lower immobility times are recorded) when a chronic dosing schedule is used (Kitada et al, 1981; Porsolt, 1981). The forced swim test appears to be suitable for detecting antidepressant activity in rats but not in mice, as opposing behavioural responses to drugs exist between these species. For instance, tricyclic antidepressant compounds produce decreases and increases in immobility time in rats and mice respectively (see Borsini & Meli, 1988). Nevertheless, it is used in mice as a primary screen. A more detailed evaluation of drug effects in this model is to be found in

the Introduction to Chapter 7.

The forced swim test will be employed in this study to screen a variety of compounds, with different binding profiles for the α2-adrenoceptor, IPR and the NAIBS, for their ability to reduce immobility time. This may indicate a functional role for these sites in the modulation of depression.

1.9.3. *In vivo* brain microdialysis.

In vivo microdialysis allows studies to be performed on the extracellular environment of the brain and other bodily tissues. The earliest techniques used to approach the extracellular environment of the brain were developed by Gaddum (1961) using push-pull cannulae. However, the introduction of a dialysis membrane into the tissue in the form of a dialysis sac was first performed by Bito and coworkers (1966). This introduced the idea of a "compartment" surrounded by a dialysis membrane which equilibrates to the extracellular environment.

The purpose of microdialysis is to mimic the passive function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue. The compounds in the perfusate thus reflect the composition of the extracellular fluid due to the diffusion of substances back and forth across the membrane. Microdialysis is particularly useful in studying brain tissue as it allows local samples to be collected in freely moving or anaesthetised animals for several hours or days. In addition, endogenous and exogenous substances can be introduced and/or recovered with minimal damage to the blood-brain barrier (Benveniste et al, 1984; Tossman & Ungerstedt, 1986).

Brain dialysis was first applied to estimate the extracellular

concentration of amino acids (Bito et al, 1966; Delgado et al, 1972). However, the first systematic study utilizing this technique concentrated on brain monoamines, and, in particular on DA (Zetterstrom et al, 1983; Zetterstrom & Ungerstedt, 1984). Other neurotransmitters studied include NA (L'Hereux et al, 1986), 5-HT (Carboni & Di Chiara, 1989), acetylcholine (ACh; Consolo et al, 1987), gamma-amino-butyric-acid (GABA; Tossman et al, 1985), and to a lesser extent other amino acids, amines and peptides (Tossman & Ungerstedt, 1986). The endogenous neurotransmitters collected by this technique are thought to reflect their synaptic concentration, although not in absolute terms. The dialysis probe itself is much greater in size than a single nerve terminal, thus individual samples represent release from the vicinity of many terminals.

Three different types of probes are used in *in vivo* microdialysis studies, they include the transcerebral, U-shaped and concentric probe designs. The procedure adopted in the present work involved the use of the concentric dialysis probe. This probe consists of one tube inserted inside another, with a dialysis membrane attached to the end of the outer tube. The dialysis medium is pumped down through the inner tube which allows it to pass around the inner surface of the dialysis membrane, while the outer membrane surface is in direct contact with the extracellular tissue. Provided no osmotic pressure gradients or electrical potentials exist across the membrane then solutes can diffuse across the membrane provided a concentration gradient exists. The resultant dialysate moves up via the outer tube to exit the probe for collection

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METHODS

1. Animals. Studies of ongoing behaviour were performed in drug naive male outbred MF1 mice (25 - 30g; Aston University). Drug discrimination experiments were performed on drug naive male Lister Hooded rats (Harlan Olac Limited, Bicester, U.K) which weighed 200 - 250g. However, male Wistar rats (270-300g, Bantin & Kingman, Hull, U.K) were used in the *in vivo* microdialysis and forced swim tests. All animals were housed in a laboratory at an ambient temperature of 23±2°C on a dark / light cycle (lights on at 7.00 hours) with free access to a standard 41B cube diet supplied by Pilsbury Limited with tap water ad libitum. Animals were maintained under these conditions at all times except during experimentation when they were briefly removed to the test apparatus which was located in the same or an adjacent room.

2. General experimental conditions. All animals were held in the same room used for experimentation for 2 weeks prior to testing, except those used in DD studies which were transfered to an adjacent room during testing. Animals used in the drug discrimination studies were housed individually, whilst those used for other studies were housed in groups of 8, however those used for *in vivo* microdialysis were stored in pairs. Experiments were performed between 8.00 and 17.00 hours.

3. Injection technique. All injections were administered via the intraperitoneal (i.p) route. Injections were made by inserting a 26G (mice) or 25G (rats) needle into the abdominal wall towards the diaphragm, taking care not too penetrate too deeply so as to avoid internal organ damage. All drugs were delivered in 0.9% saline using a dose volume of 1ml/kg (rats) and 10ml/kg (mice).

Doses of all drugs are expressed as the hydrochloride salt except for UK-14,304 which was expressed as the tartrate form, and moclobemide which was suspended in 0.3% (v/v) Tween-80 in distilled water.

4. Behavioural pharmacological screening.

A comprehensive assessment of the behavioural and physiological effects of drugs in mice were examined using a technique adopted from Irwin (1968). Animals were randomly divided into control and experimental groups (4 mice per group), and 20 minutes after injection were placed into the appropriate section of an observation arena (dimensions 30x30x20 cm either side of dividing wall). The behaviours and states listed in Table 2.1 were systematically assessed using the individual observational methods described by Irwin (1968). A subjective rating scale of 0 (identical to control) 2, 4, 6 and 8 (maximum difference from control) was used throughout. Animals identical to the control group were awarded a score of 0, while increases and decreases in behaviours received scores up to +8 (increased) and -8 (decreased) respectively.

Table 2.1. Behavioural and physiological drug screening procedure. For details of methods see Irwin, 1968.

1. Vocalisation
2. Restlessness
3. Increased or decreased alertness
4. Stereotyped behaviour
5. Straubtail
6. Increased or decreased respiration rate
7. Tremor
8. Writhing
9. Convulsions
10. Head twitches (frequency)
11. Exophthalmos
12. Ptosis
13. Fearfulness
14. Abnormal gait
15. Increases or decreases in locomotor activity
16. Increases or decreases in frequency of grooming behaviour
17. Increased or decreased startle reflex (to air puff)
18. Increased or decreased responding to gentle touch
19. Increased or decreased reactivity to a new environment
20. Increased or decreased passivity to being picked up
21. Increased or decreased grip strength (to wire grid)
22. Body sag (on vertical grid)
23. Increased or decreased body position in the home cage
24. Increased or decreased body position on a bar
25. Increased or decreased pinna reflex intensity
26. Increased or decreased corneal reflex
27. Piloerection
28. Lachrymation
29. Salivation
30. Vasodilation (pinkness of bare skin)
31. Cyanosis (bluish grey colouration of bare skin)
32. Hypothermia or hyperthermia (as judged when held)
33. Catalepsy (forepaws placed on 4cm bar)
34. Increased or decreased ipsilateral flexor reflex (to light toe pinch)
35. Increased or decreased pain response (to tail pinch)
36. Righting reflex loss (degree of clumsiness on landing)
37. Diarrhoea

5. Drug discrimination studies.

5.1 Experimental procedure. The experiments were performed in standard two lever operant conditioning units (Campden Instruments Limited), controlled by an Apple IIe computer using the program 'Operant Program for the Neurosciences' (Emmett-Oglesby et al, 1982). Each of the 8 units contained a delivery hatch positioned equidistant between two response levers. Upon completion of various fixed-ratio (F.R) lever-pressing schedules a dipper presented a reward of sweetened condensed milk (Fussells Limited - diluted 1 part to 2 parts tap water) to the hatch. All sessions began with the presentation of a free reward, and were performed during 9.00 to 12.00 hours each weekday.

5.2 Preliminary training. A reward was presented each time either of the levers was pressed (i.e. a fixed ratio or FR = 1 schedule with both levers operative), this was then increased in stages to an FR = 10 through FR = 2, 4 and 7 with both levers operative. Discrimination training commenced when all rats were responding consistently on the FR = 10 schedule (i.e. without any bias for either lever being noted in 10 consecutive sessions).

5.3 Discrimination training. In each training session, a reward was delivered after 10 consecutive presses on the correct lever. Injections of the training drug or 0.9% saline were given 20-minutes prior to each session in the daily (except weekends) sequence of SDDSSDSSDD (D = drug, S = saline). To avoid any possible olfactory stimuli leading to lever preferences, the rats were divided randomly into two groups. Following training drug treatment, one group was rewarded after completing 10 presses on the left-hand lever, while the other group was rewarded after

completing 10 correct responses on the right-hand lever. Following saline treatment, rats were rewarded for each 10 correct responses on the opposite lever. Each session was terminated after 15 minutes.

The criterion for entry into the test sessions (below) was the completion of 10 consecutive daily error-free sessions. An error occured when less than 8 of the first 10 responses were made on the correct lever, or when less than 90 % of the total responses during the 15-minute session occurred on the correct lever.

5.4 Test sessions: substitution and antagonism studies. The test schedule consisted of two test days (T) per week in the daily sequence (weekdays only) of STDTSDTSTD. Test injections were administered once only to each rat, and the order of testing of individual drugs and doses was identical for each rat. Testing began 20 minutes after injection of each test compound, except for moclobemide and pargyline which were given 60 minutes prior to testing. Each test (i.e. S,T or D) session was terminated, without the provision of a reward, upon 10 responses being made on either the training drug or the saline-associated levers, the lever on which this occurred was regarded as the selected lever. The number of responses by each rat on the training drug-associated lever was expressed as a percentage of the total responses made on both levers. The mean of these percentages represented the ability of a drug to substitute for the training drug-induced cue. For the antagonism studies each drug was given 30-minutes prior to the training drug and tests began 20-minutes later.

The time for lever selection to occur (i.e. 10 correct responses) enabled a response rate to be calculated after each saline control (S) or test drug (T) session. The mean response rate for each test drug session was expressed as a percentage of the

mean control response rate in the previous 0.9% saline control training session.

6. Forced swimming test.

6.1 Immobility measurement. The procedure was essentially the same as described elsewhere (Porsolt et al, 1978). Each animal was placed in a plexiglass cylinder (40cm height, 18 cm internal diameter) containing 24 cm of water at 23±2°C thus avoiding the learning effect of rats which buoy themselves by touching the bottom of the cylinder with their tails or hind legs. Rats were exposed to two 8 minute swims 24 hours apart after each of which they were dried off under a warm lamp. A sub-chronic dosing schedule was used with injections being administered 0.5, 19 and 23 hours after swim 1. To avoid the influence of pheromones such as 'alarm substance' (Abel, 1991) upon swimming activity fresh water was used for each swim. Video recordings of each rat's second swim were made, upon play-back blind observations were made of the total period each rat remained immobile during three different time slices of swim 2 (i.e. during 0-8, 0-5 and 3-8 minutes), to allow for time-dependent drug effects. A rat was judged to be immobile when it remained floating in the water making only the necessary movements to keep its head above water.

7. *In vivo* brain microdialysis studies

7.1. Probe design. Concentric microdialysis probes were used in both the anaesthetised and conscious rat studies, as shown in Figures 2.1 and 2.2 respectively. Extra design features of probes

used for the conscious studies are detailed at the end of this section. Stainless steel tubing (23 gauge; 1.3cm for frontal cortex and 2.5cm for the hippocampus x 635μm O.D., Coopers Needle Works Ltd., Aston, U.K) formed the main body of all probes (Figures 2.1 & 2.2). A length of polyethylene tubing (400μm I.D., 800μm O.D., Duval Plastics and Engineering Limited, Australia) was attached to one end of the the steel tubing. Silica glass tubing (50mm x 170μm O.D., Scientific Glass Engineering, Australia) was introduced into the open end of the stainless steel and pushed through a small hole made with a needle in the polyethylene tubing at the opposite end. The polyethylene tubing was secured to the stainless steel and silica glass tubing with araldite adhesive so that 3.5mm of silica glass tubing protruded from the opposite end of the probe. A 5 mm length of polyacrylonitrile dialysis membrane (200μm I.D., 300μm O.D., molecular weight cutoff 40 000, Hospal Medical, New Jersey, USA) was sealed at one end using araldite adhesive, and fitted over the silica glass tubing protruding from the bottom of the probe. Araldite adhesive was used to ensure a leak proof seal between the stainless steel tubing and the dialysis membrane.

The following details of probe construction apply to chronically indwelling probes used in the conscious rat microdialysis studies (Figure 2.2). A 4cm length of polyethylene tubing (200μm I.D., 500μm O.D., Duval Plastics and Engineering Limited, Australia) was placed over the exposed silica glass tubing and araldite fixative provided a leak proof seal for the dialysate to exit the probe for subsequent collection and analysis.

Figure 2.1. Design and construction of the concentric dialysis probe as used in the anaesthetised rat microdialysis studies.

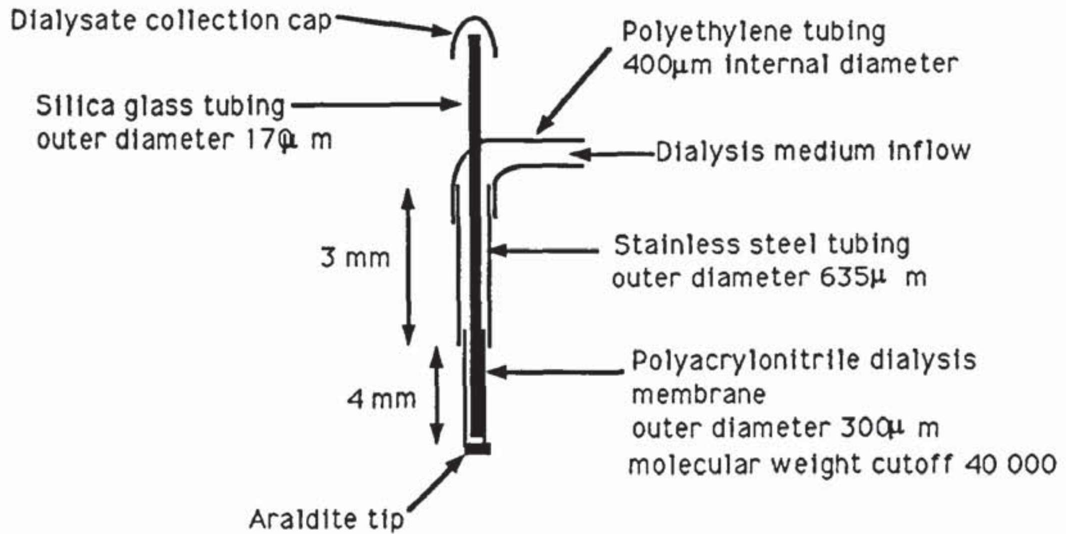
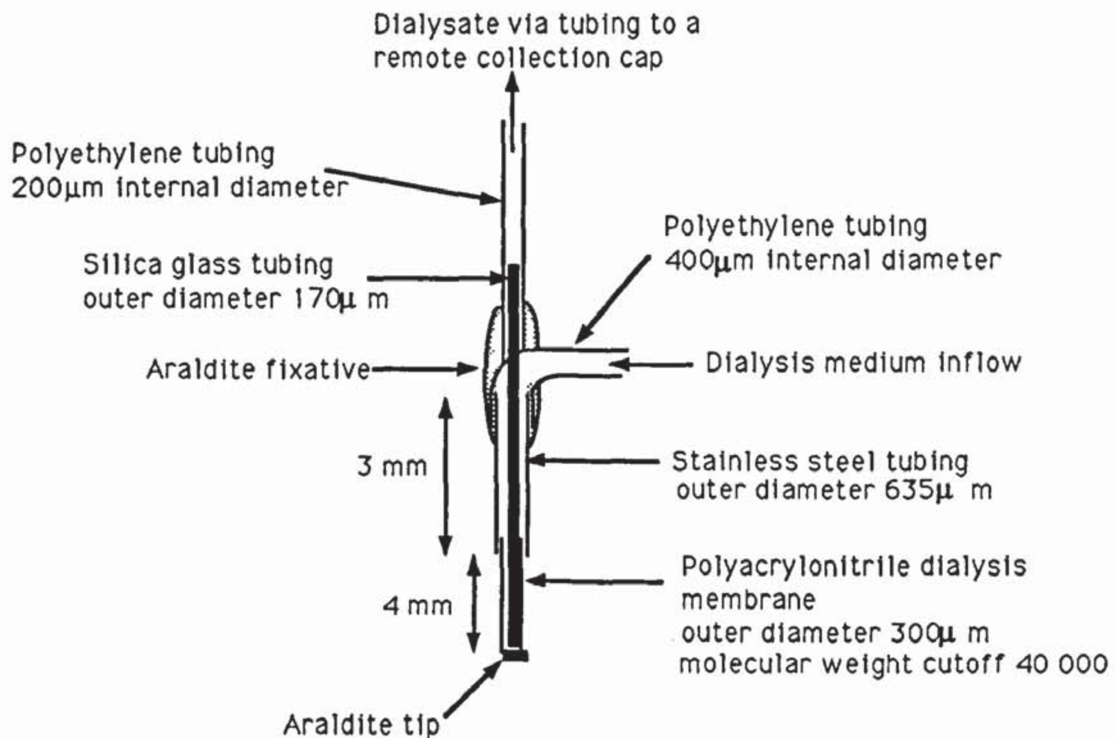


Figure 2.2. Design and construction of the chronically indwelling concentric dialysis probe used in the conscious rat microdialysis studies.



7.2. Dialysis medium. The dialysis medium used in the anaesthetised and conscious studies was continually infused via the probe using a microlitre syringe pump (Harvard apparatus Limited, Edenbridge, Kent, U.K) at a constant rate of 2.34µl/min resulting in recovery volumes of 35µl for each 15 minute sample. The ionic composition and osmotic potential of the dialysis medium are intended to be in equilibrium with the extracellular fluid surrounding the probe. Neurotransmitters may then diffuse down a concentration gradient across the dialysis membrane into the *in vitro* space of the probe. The resultant dialysate moves up the silica glass tubing exiting the probe for collection in a plastic cap.

The dialysis medium recipe used throughout these studies was 147 mM NaCl, 3mM KCl, 1mM $MgCl_2$ and 1.3mM $CaCl_2$, which was set to pH 7.4 with NaH_2PO4 and Na_2HPO4 (final osmolarity 1.5mM).

7.3. Surgical techniques. Male Wistar rats (270-300g, Bantin & Kingman, Hull, U.K) were anaesthetised with chloral hydrate (400mg/kg; 100mg/ml, i.p) and supplemental injections (90mg/kg) were given upon re-establishment of the corneal reflex. Each animal was placed in a stereotaxic frame (Kopf, USA) and 37°C core temperatures were maintained throughout surgery using a homeothermic blanket control unit (Harvard Apparatus Limited, Edenbridge, Kent, U.K). The skull was exposed by an anterior-posterior incision in the skin. Probe implantation coordinates were calculated from intra-aural line coordinates according to the atlas of Paxinos and Watson (1976), and these coordinates were verified against those relative to bregma (Table 2.2) once the skull was exposed.

TABLE 2.2. Stereotaxic coordinates for probe placement.

Brain region	Coordinates relative to bregma (cm)		
	Anterior-posterior	***Lateral***	***Depth from dura***
Frontal cortex (FR1)	+0.32	-0.26	-0.35
Hippocampus	-0.58	-0.50	-0.60

The dialysis probe was lowered to the surface of the exposed skull and the area directly beneath the probe was marked. A small burr hole was drilled through this point on the skull to expose the brain. The dura surrounding the brain was pierced and retracted from the region of the burr hole and the dialysis probe was lowered until it was resting on the brain surface. Final depth measurements were calculated from this position and the dialysis probe was accurately lowered to its final position over a period of several minutes to minimise disturbance to the brain.

For animals used in the conscious studies layers of dental fixative were built up around the probe to hold it firmly in position during and after surgery. In addition, a small screw was implanted into the skull surface, which was lightly pitted with the drill, to anchor the fixative to the skull. Finally, the probe inlet and outlet were sealed with araldite adhesive and the animal was left to recover in its home cage for 24 or 48 hours before collection of samples began.

7.4 High Performance Liquid Chromatography - Electrochemical Detection (HPLC-ECD). The HPLC apparatus consisted of a high performance two-piston pump (HPLC Pump 420, Kontron Instruments, U.K) with a built in pressure gauge. An

external pulse dampener was fitted after the pump to minimise 'pump noise'. Manual injections of the dialysis samples were performed using a Rheodyne 7125 (Cocati, CA, USA) fitted with a 50μl sample loop. A 3μ ODS analytical column (15cm x 4.5 mm I.D; HiChrom Ltd., Reading, U.K) was used throughout. The analytical cell (model 5011; Environmental Sciences Associates (ESA), Bedford, MA, USA) was controlled by an ESA Coulochem 5100A controller. The final output was to a dual pen chart recorder (Kipp and Zonen BD41, Labdata, U.K).

7.5. Conditions for the NA assay. The recipe for the mobile phase was as follows: sodium acetate 24.0mM, citric acid 14.8mM, SOS 2.6mM and EDTA 207μM. 12% methanol was added before buffering to pH 5.0 (using 10M NaOH), and then the final mobile phase was filtered and degassed. Detector 1 of the analytical cell was set at a reduction potential of -250mV and an oxidation potential of +350mV was used for detector 2. All traces were recorded from detector 2. Prior to detection samples passed through a conditioning cell set at -400mV. The flow rate was set at 1ml/min and analysis of the dialysate was complete in 8 minutes.

7.6 NA standards and calculation of unknowns. Aliquots of a standard NA stock solution (1mg/ml in 0.4M PCA) were stored for periods of less than two weeks at -70°C and diluted in dialysis medium to give standard concentrations of 5, 10, 50 and 100 pg in a 40 µl volume. HPLC traces for the dialysis medium, a 5 pg NA standard, and typical basal samples from the frontal cortex and hippocampus are shown in Figures 2.3a-d respectively. Data points were fitted to a straight line using a linear regression program written on the Lotus 123 spreadsheet package to generate a standard curve described by the equation :

$$y = mx \pm c \qquad \text{(Equation 1)}$$

where y = concentration of NA in sample (pg/sample).

x = peak height (mm).

c = intercept on y-axis, assumed to be zero.

The NA content of each sample was calculated by substituting the peak height into equation (1) or using a spreadsheet package written on Lotus 123.

Figure 2.3. HPLC-ECD chromatograms.

A. Dialysis medium alone.

B. 5 pg NA standard in dialysis medium.

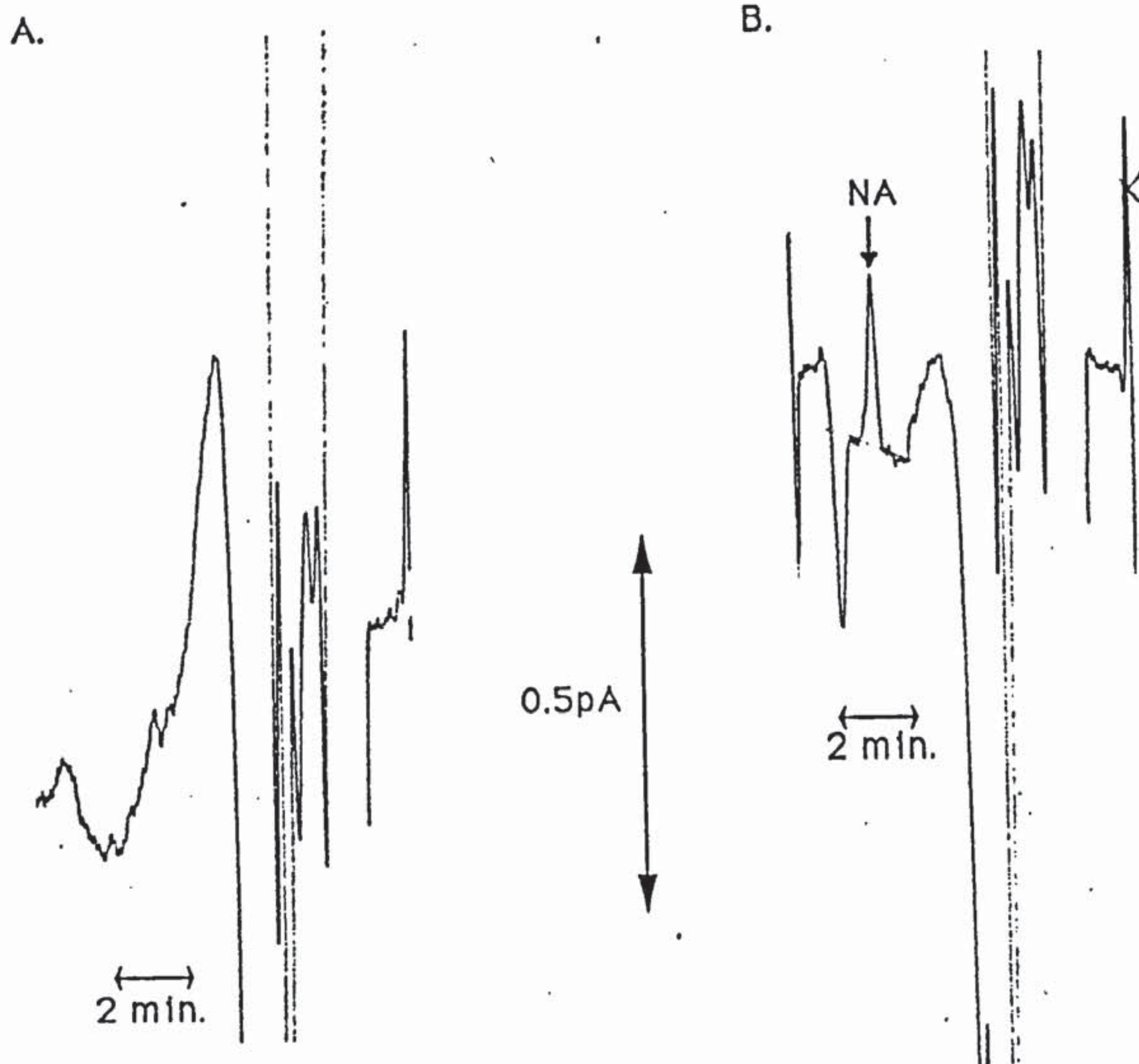
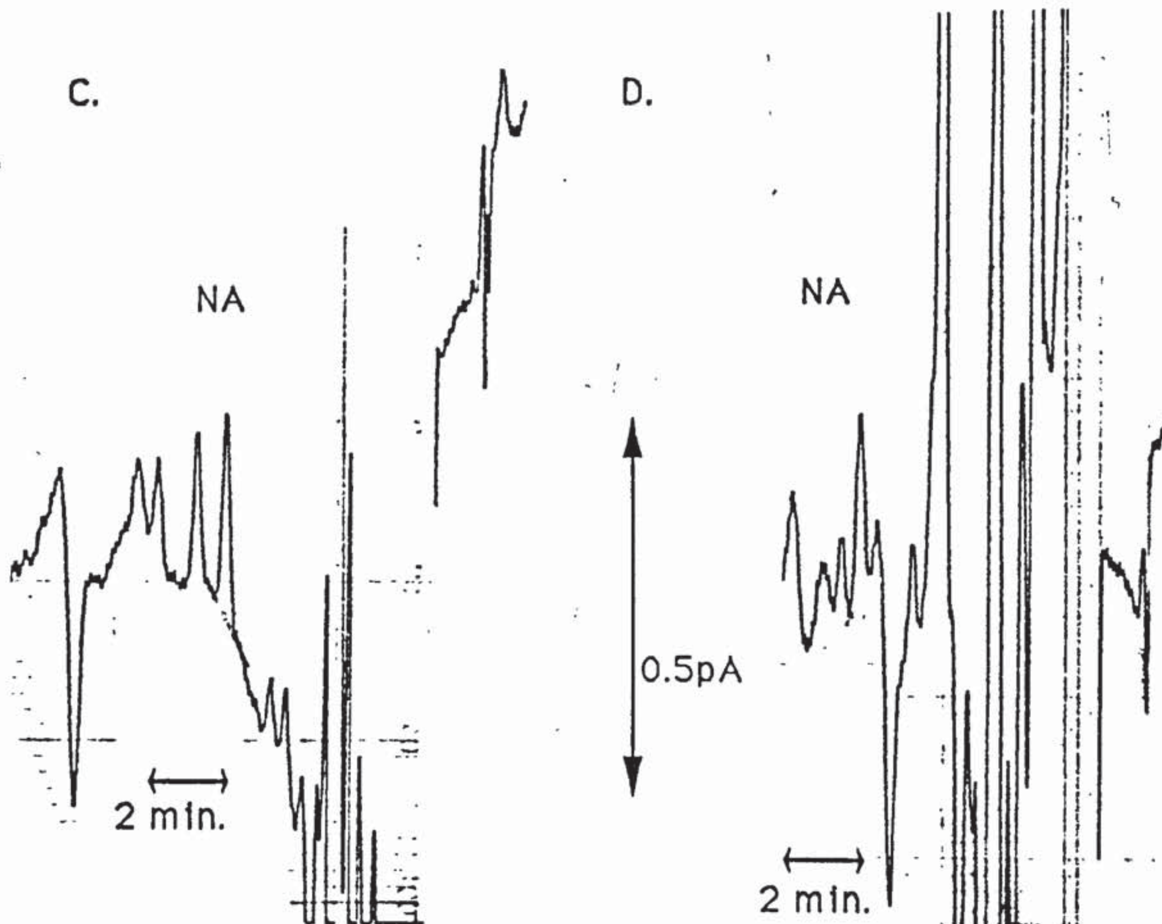


Figure 2.3. HPLC-ECD chromatograms.

C. Basal release in hippocampus.

D. Basal release in frontal cortex.



7.7 General experimental procedure for anaesthetised studies. Local tissue damage occurs due to probe implantation which leads to abnormally high levels of neurotransmitter release, therefore sample collection was delayed until one hour after implantation after which a consistent baseline is re-established. Samples were collected every 15 minutes to a final volume of 35µl. Basal samples were collected until the NA content of three consecutive samples did not significantly differ from one another. Drug treatments were then given and sample collection continued for 2 hours onwards to observe any drug effects.

7.8 General experimental procedure for conscious studies. Following recovery from surgery (i.e. 24 or 48 hours after implantation) the probe inlet and outlet seals were cut and connected to the polyethylene delivery and collection tubes. A swivel (Instech Laboratories, Plymouth Meeting, PA., USA) was used to allow the animal to move freely about its home cage without tangling the connecting tubing.

7.9 Verification of dialysis probe placement. At the end of each study animals were decapitated and the brains were carefully removed from the skull. A Jacobowitz (Zivic Miller, PA, USA) rat brain slicer was then used to visually assess anterior-posterior, depth and lateral positioning.

7.10. Dialysis probe histology. Histological brain examinations were performed on brains which had been rapidly frozen using isopentane cooled over dry ice and then stored at -70°C until sectioning. For the frontal cortex (Plate 1) the sections were cut coronally whilst in the hippocampus (Plate 2) sections were cut

saggitally. Care was taken to cut the sections parallel to the plane of the stereotaxic atlas (Paxinos & Watson, 1976) by adjusting the cutting edge. 20μm sections were cut on a cryostat and every 5th section was mounted on a slide for staining.

Sections were stained using cresyl violet for 15 - 30 minutes. This was followed by dehydration of the sections using 70%, 95%, 100% alcohol and xylene for 2 - 3 minutes. Finally cover slips were applied before microscopic examination of the sections was performed.

Plate 1 Evidence of correct dialysis probe implantation into rat frontal cortex (saggital section).

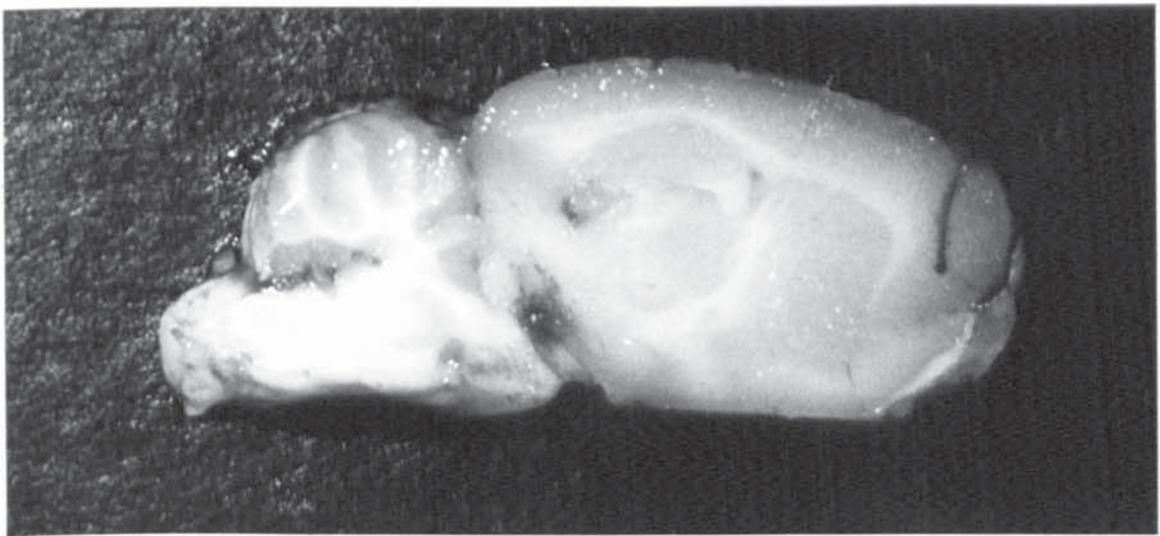
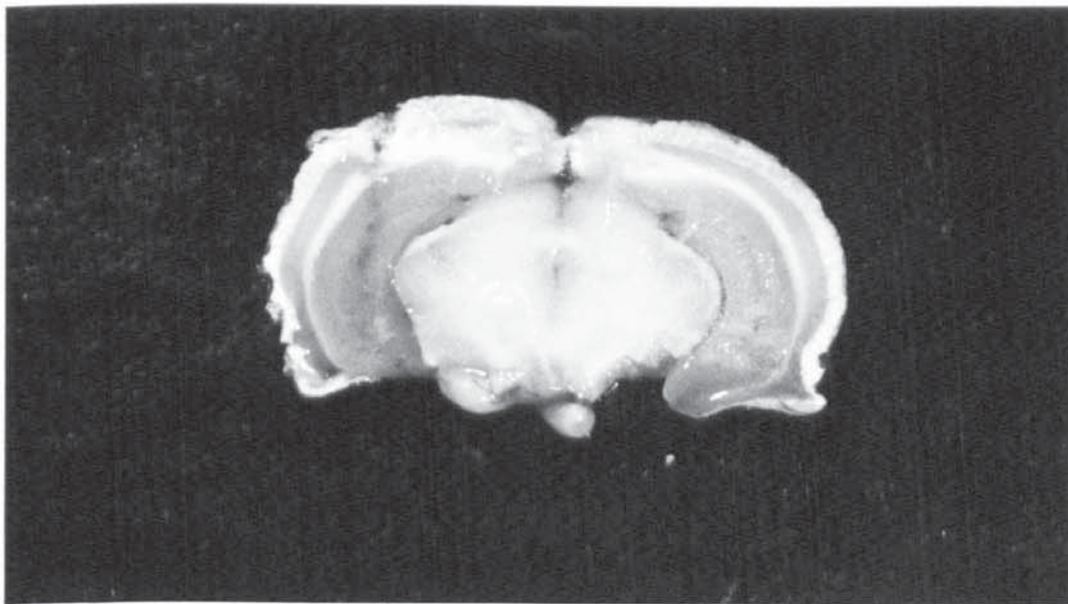


Plate 2 Evidence of correct dialysis probe implantation into rat hippocampus (coronal section).



8. Drugs and suppliers.

Compound	Supplier
B910101†	Reckitt & Colman, Hull, U.K.
RX801077†	Reckitt & Colman, Hull, U.K.
Idazoxan	Reckitt & Colman, Hull, U.K.
Clonidine	Boehringer Ingelheim , Germany.
RX811059 (2-ethoxy idazoxan)	Reckitt & Colman, Hull, U.K.
Fluparoxan	Glaxo, Ware, Herts. U.K.
L659-066*	Merck, Sharp & Dohme
Desmethylimipramine (DMI)	Sigma Chemials Ltd., Poole, U.K.
Yohimbine	Sigma Chemials Ltd., Poole, U.K.
Atipamezole	Farmos Group Ltd., Finland.
Cirazoline	Synthelabo, France.
1-(2-pyrimidinyl)-piperazine (1-PP)	Aldrich Ltd., Dorste, U.K.
2-phenyl-2-imidazoline	Aldrich Ltd., Dorset, U.K.
Phenylephrine	Sigma Chemials Ltd., Poole, U.K.
UK-14,304¥	Pfizer, Sandwich, U.K.
Guanabenz	Wyeth Laboratories
Moclobemide	Roche, Switzerland.
Pargyline	Sigma Chemicals Ltd., Poole,U.K.
Diazepam	Roche, Switzerland.
Tween-80	Sigma Chemicals Ltd., Poole,U.K.

†Chemical structure not available.

* ((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 (hydrochloride salt)

¥ (5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline tartrate

9. Reagents used in the HPLC-ECD assay

Chloral hydrate	Fluka Chemicals Ltd, Glossop, U.K.
±Noradrenaline (free-base)	Sigma Chemials Ltd., Poole, U.K.
Sodium chloride (sterile)	BDH Limited, Poole, U.K.
Calcium chloride	BDH Limited, Poole, U.K.
Magnesium chloride	BDH Limited, Poole, U.K.
Pottasium chloride	BDH Limited, Poole, U.K.
Magnesium chloride	BDH Limited, Poole, U.K.
Sodium dihydrogen phosphate buffer	BDH Limited, Poole, U.K
Di-sodium hydrogen phosphate buffer	BDH Limited, Poole, U.K
Sodium acetate	Fluka Chemicals Limited, Glossop, U.K.
Citric acid	Fluka Chemicals Limited, Glossop, U.K.
Sodium hydroxide	Fluka Chemicals Limited, Glossop, U.K.
SOS *	Fluka Chemicals Limited, Glossop, U.K.
EDTA **	Boehringer Mannheim GmbH, Germany.
Methanol	BDH Limited, Poole, U.K.
Sodium hydroxide	BDH Limited, Poole, U.K.

*SOS (1-Octanesulphonic acid sodium salt monohydrate)

**EDTA ((Ethylenedinitrilo) tetra acetic acid disodium salt)

CHAPTER 3. Survey of the effects of clonidine, idazoxan, RX811059, RX801077 and B910101 upon a variety of behavioural and physiological states in mice.

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Chapter 3

1. Introduction.

The techniques described by Irwin (1968), provide a method to comprehensively assess the behavioural and physiological effects of drugs in mice. With this technique, the pattern profile of various classes of pharmacological compounds can be identified and differentiated, and the relative specificity of their actions may be defined. A method adopted from Irwin (see Chapter 2, section 2.4) was used to survey a variety of drugs with differing selectivity profiles for the α2-adrenoceptor, the IPR and NAIBS (viz. clonidine, RX811059, idazoxan, RX801077 and B910101), for their effects upon the behavioural and physiological states listed previously (see Table 2.1). Individual drug-induced effects detected would then be studied in more detail using more objective techniques, in an attempt to establish functional role for the NAIBS.

2. Results.

The ability of each drug to affect each of the behavioural and physiological states listed in Table 2.1 were examined, although only significant effects are reported here. Tests upon body temperature and pupil diameter were abandoned due to preliminary studies which revealed problems in detecting differences from the control state.

2.1. Locomotor activity. A significant dose dependent inhibition of locomotor activity was produced by the α2-adrenoceptor agonist clonidine (0.02 & 0.04 mg/kg; Figure 3.1). In contrast, the α2-adrenoceptor antagonists RX811059 (1, 5 & 10 mg/kg; Figure 3.6) and idazoxan (3 & 10 mg/kg; Figure 3.10) significantly stimulated locomotor activity in a non dose dependent manner. However, the highly selective NAIBS agents RX801077 and B910101 were both unable to influence locomotor activity at doses up to 10 mg/kg.

2.2. Spontaneous grooming behaviour. The frequency of spontaneous grooming behaviour was significantly increased by both RX811059 (0.1, 1 & 5 mg/kg; Figure 3.7) and to a lesser extent with idazoxan (3 & 10 mg/kg; Figure 3.12). In contrast, the NAIBS selective agent RX801077 significantly reduced the frequency of grooming episodes 65 minutes after its injection, albeit at only one of the doses tested (7 mg/kg; Figure 3.14). However, no change in spontaneous grooming behaviour was produced by B910101 or clonidine at doses up to 10 and 0.04 mg/kg respectively.

2.3. Spontaneous head twitches. A significant increase in spontaneous head twitching was produced by both RX811059 (0.1, 1 & 5 mg/kg; Figure 3.5) and idazoxan (1, 3 & 10 mg/kg; Figure 3.11). However, this behaviour was unaffected by clonidine, RX801077 and B910101 at doses up to 0.04, 10 and 10 mg/kg respectively.

2.4. Pinna reflex. Light tactile stimulation of the external auditory meatus with a hypodermic needle stylet induced a reflex retraction of the ear. The intensity of this response was increased by both RX811059 (Figure 3.9) and idazoxan (Figure 3.13) at doses up to 10 mg/kg in each case. However, this reflex was not affected by RX801077, B910101 and clonidine at doses up to 10, 10 and 0.04 mg/kg respectively.

2.5. Startle reflex. A significant increase in the magnitude of the startle reflex (i.e. sudden body jerking in response to a finger 'snap') was produced by RX811059 at doses up to 10 mg/kg (Figure 3.8). However, this reflex was unaffected by idazoxan (1, 3 & 10 mg/kg), clonidine (0.01, 0.02 & 0.04 mg/kg), RX801077 (1, 3, 7 & 10 mg/kg) and B910101 (1, 3 & 10 mg/kg).

2.6. Loss of righting reflex. Clonidine dose dependently impaired the righting reflex at doses up to 0.04 mg/kg (Figure 3.3), although RX811059, idazoxan, RX801077 and B910101 were inactive at doses up to 10 mg/kg in each case.

2.7. Tail pinch reponse. The response produced by pinching of the tail with forceps was significantly reduced (i.e slower in onset and intensity) by 0.02 and 0.04 mg/kg clonidine 35 and 80 minutes after injection respectively (Figure 3.4). This response

was unaffected by RX811059, idazoxan, RX801077 and B910101 at doses up to 10 mg/kg for each drug.

2.8. Response to touch. Stroking along the sides of the thorax and abdomen produced a withdrawal response. The speed and intensity of this withdrawal response were reduced following clonidine treatment (0.01, 0.02 & 0.04 mg/kg; Figure 3.2), although RX811059, idazoxan, RX801077 and B910101 were unable to affect this response using doses up to 10 mg/kg in each case.

None of the other behavioural and physiological measures listed in table 2.1 was significantly altered by any of the agents used in this study.

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3. Discussion.

The present survey successfully identified some of the behavioural effects previously reported for the α2-adrenoceptor agonist clonidine in mice which included: locomotor inhibition; an impairment of the righting reflex, and reduced responses to both touch and the tail pinch (Fielding & Lal, 1981; Heal et al, 1989; Drew et al, 1979). The α2-adrenoceptor antagonists RX811059 and idazoxan both produced several signs of behavioural stimulation, such as increases in the frequency of both spontaneous grooming and head twitch behaviours. These agents also increased locomotor activity, which has been demonstrated elsewhere in rats (Dickinson et al, 1990). In addition to this, RX811059 and idazoxan both increased the intensity of the pinna reflex, and the former compound increased the magnitude of the startle reflex. The present survey failed to establish a definite functional role for the NAIBS, although the frequency of spontaneous grooming behaviour was significantly reduced 65 minutes after one of the doses of the selective NAIBS agent RX801077.

The present survey was unable to identify certain effects normally associated with clonidine. For example, clonidine has been demonstrated elsewhere to inhibit the startle reflex (Davis & Astrachan, 1981) and produce ptosis (Harsing et al, 1989), both of which were not detected in the present study. Furthermore, clonidine-induced mydriasis and thermoregulation (Heal et al, 1989), were unable to be detected using the manual techniques described by Irwin (1968). For instance, hypothermia would normally be detected using a rectal probe to detect core body temperature, rather than determining superficial body

temperature by hand (Irwin, 1968). Nevertheless, the present survey was employed as a preliminary system to identify gross drug-induced effects which could be examined in greater detail using more specific and reliable techniques.

The sedative effects of the α2-adrenoceptor agonist clonidine may be assessed by an increase in passivity, and decreases in alertness, body position and touch response (Drew et al, 1979), although only the latter effect was observed in the present studies. Nevertheless, clonidine produced other signs of behavioural inhibition, such as an impairment of the righting reflex and a reduction in locomotor activity which are characteristic α2-adrenoceptor agonist effects (Heal et al, 1990). However, its inhibition of the tail pinch response may be related to its analgesic effect (Paalzow & Paalzow, 1976), rather than behavioural suppression.

RX811059 appeared to produce more profound increases in both spontaneous grooming behaviour frequency and pinna reflex intensity when compared with idazoxan. The weaker and less consistent effects observed for idazoxan might possibly be due to its ability to interact with the NAIBS and/or the IPR (Ernsberger et al, 1990; Boyajian et al, 1987; Michel & Insel, 1989). Indeed, the NAIBS selective agent RX801077 reduced the frequency of spontaneous grooming behaviour in the present study, although this was produced by one dose only. Furthermore, the magnitude of the startle reflex was increased by RX811059 but not by idazoxan which could perhaps be due to the latter drug's effects at non-α2-adrenoceptor sites. However, both drugs increased the frequency of spontaneous head twitches. This is in agreement with a previous report which demonstrated their ability to block the inhibitory effect of 8-OHDPAT upon

DOI-induced spontaneous head twitches (Handley & Dursun, 1992). Moreover, α2-adrenoceptor agonists reduce the frequency of spontaneous head twitches (Handley & Brown, 1982).

In summary, the present survey identified a variety of behavioural and physiological effects for clonidine, idazoxan and RX811059; some of which confirm previous findings for these drugs. A single inhibitory effect upon grooming behaviour frequency was observed for the NAIBS selective agent RX801077, although the significance of this at present is unknown. These experiments therefore did not generate any indication for further detailed work with RX801077 or B910101, and they suggest a lack of involvement for the NAIBS in mediating the effects observed for clonidine and idazoxan in the present study.

Figure 3.1. Effect of clonidine upon locomotor activity in mice, in relation to a 0.9% saline control group. Values are means where n=4

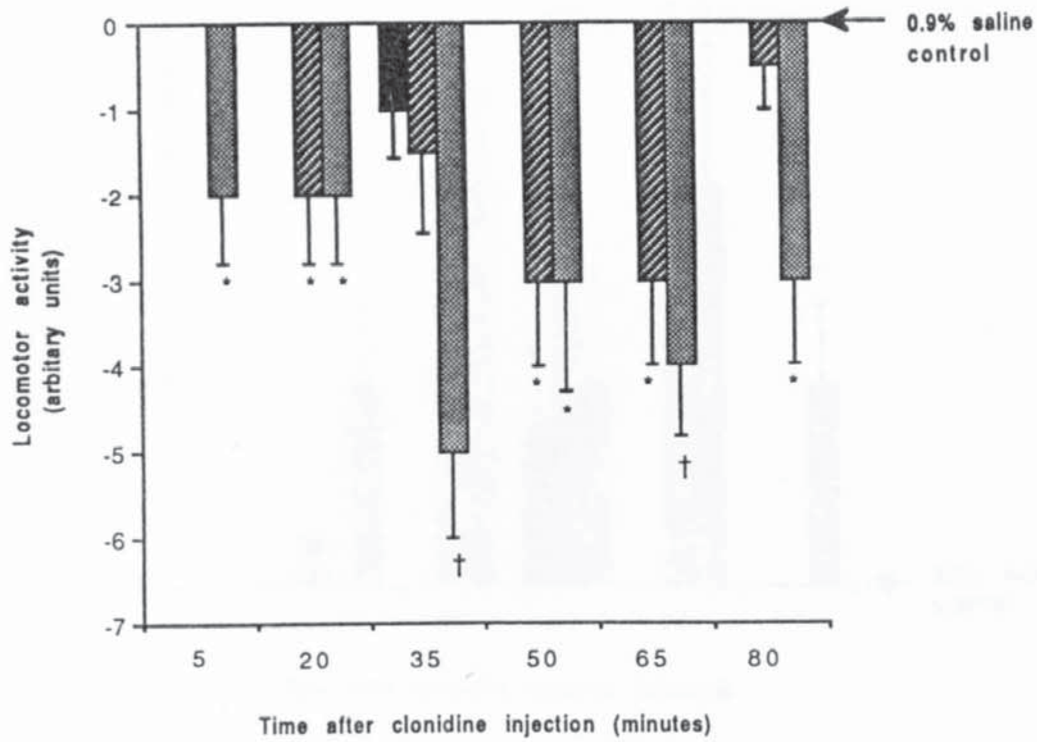
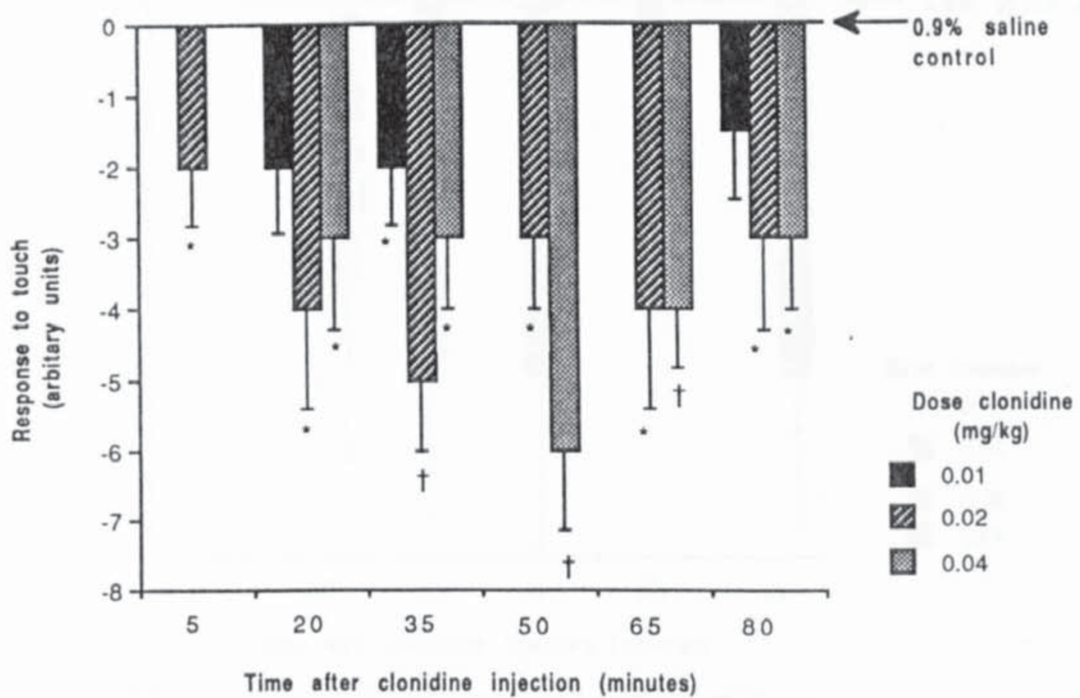


Figure 3.2. Effect of clonidine upon the touch response in mice, in relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05, †P<0.005 versus 0.9% saline control (Mann-Whitney 'U'-test)

Figure 3.3. Effect of clonidine on loss of the righting reflex in mice, in relation to a 0.9% saline control group. Values are means where n=4.

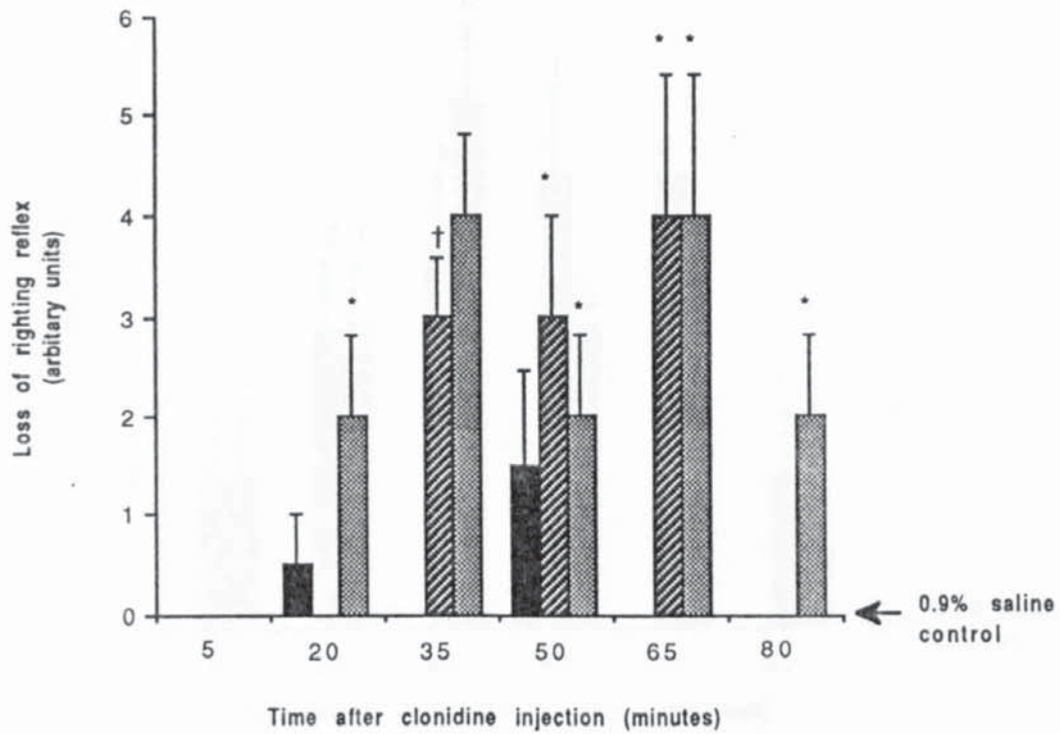
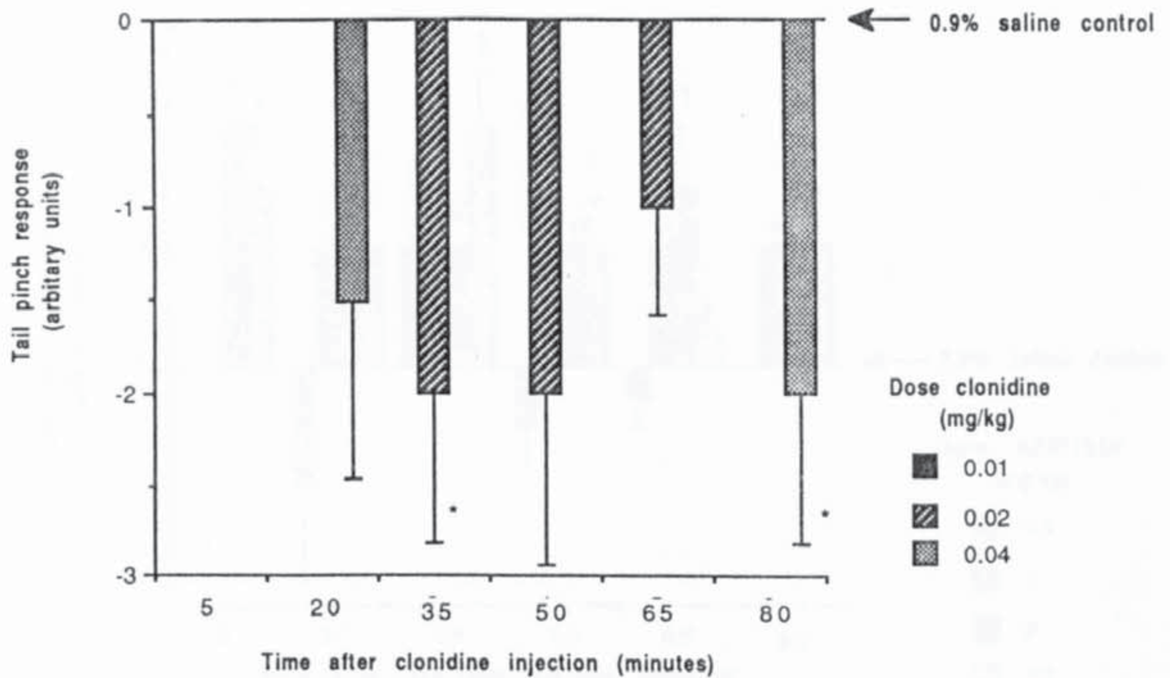


Figure 3.4. Effect of clondine upon the tail pinch response in mice, in relation to a 0.9% saline control group. Values are means where n=4.



*$P<0.05$, †$P<0.005$ versus 0.9% saline control group (Mann-Whitney 'U'-test)

Figure 3.5. Effect of RX811059 upon the frequency of spontaneous head twitches in mice, in relation to a 0.9% saline control group. Values are means where n=4.

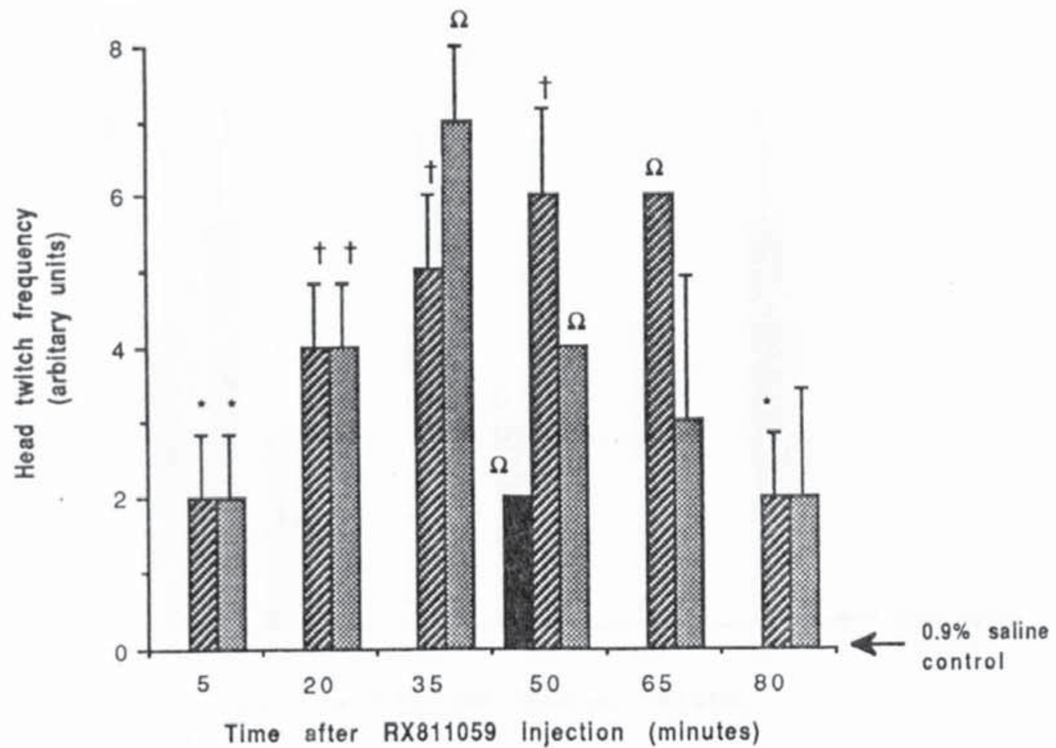
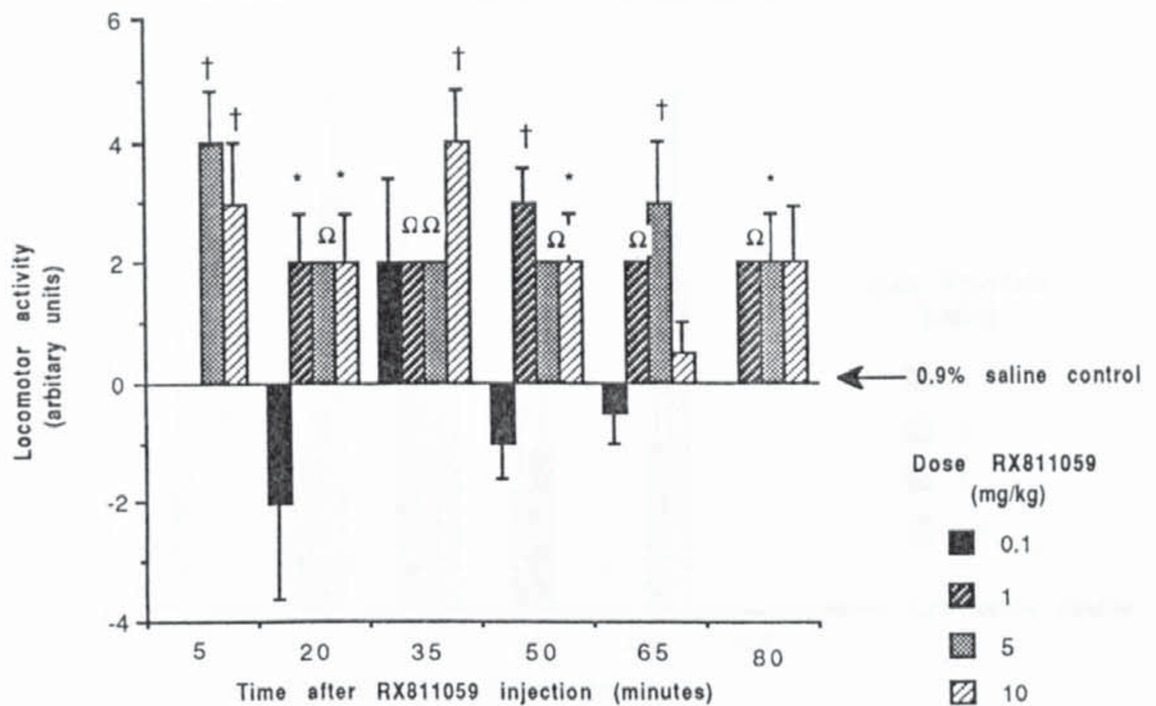


Figure 3.6. Effect of RX811059 upon locomotor activity in mice, in relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05, †P<0.005, ΩP<0.0005 versus 0.9% saline control group (Mann-Whitney 'U'-test)

Figure 3.7. Effect of RX811059 upon the frequency of spontaneous grooming behaviour in mice in relation to a 0.9% saline control group. Values are means where n=4.

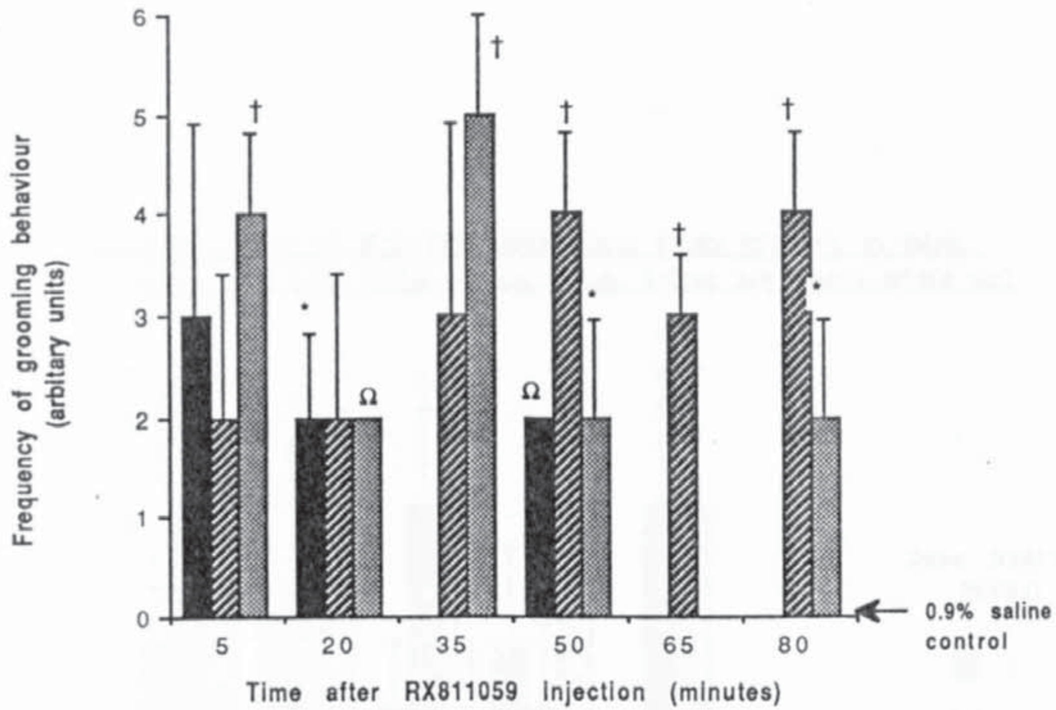
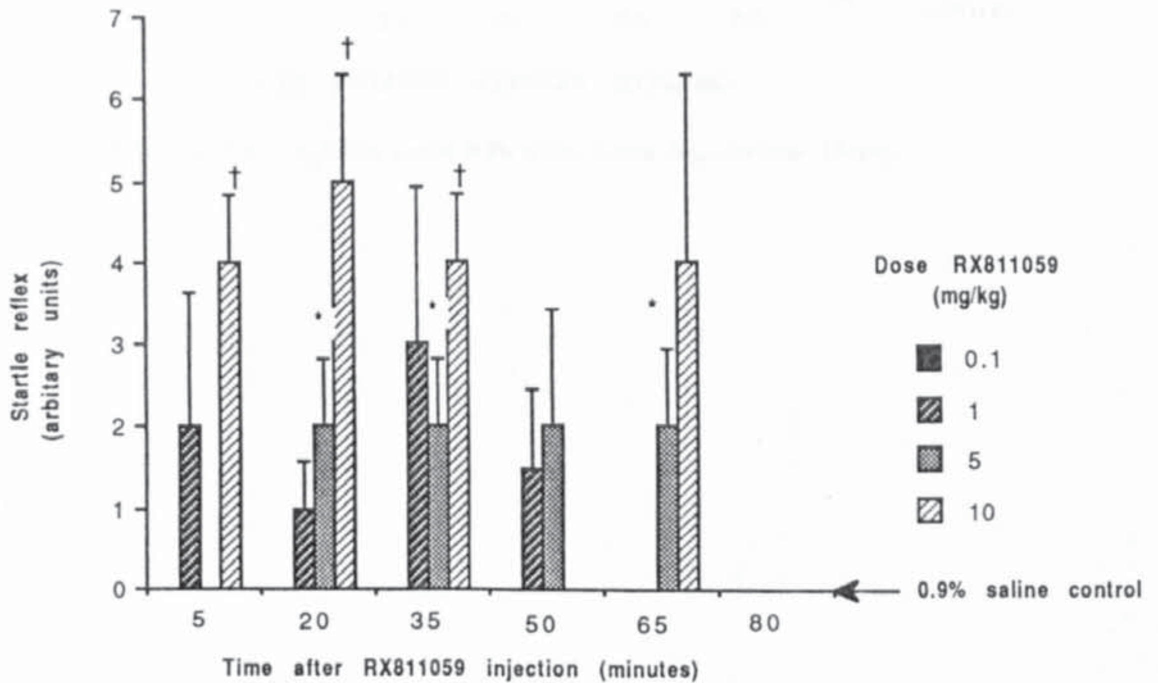
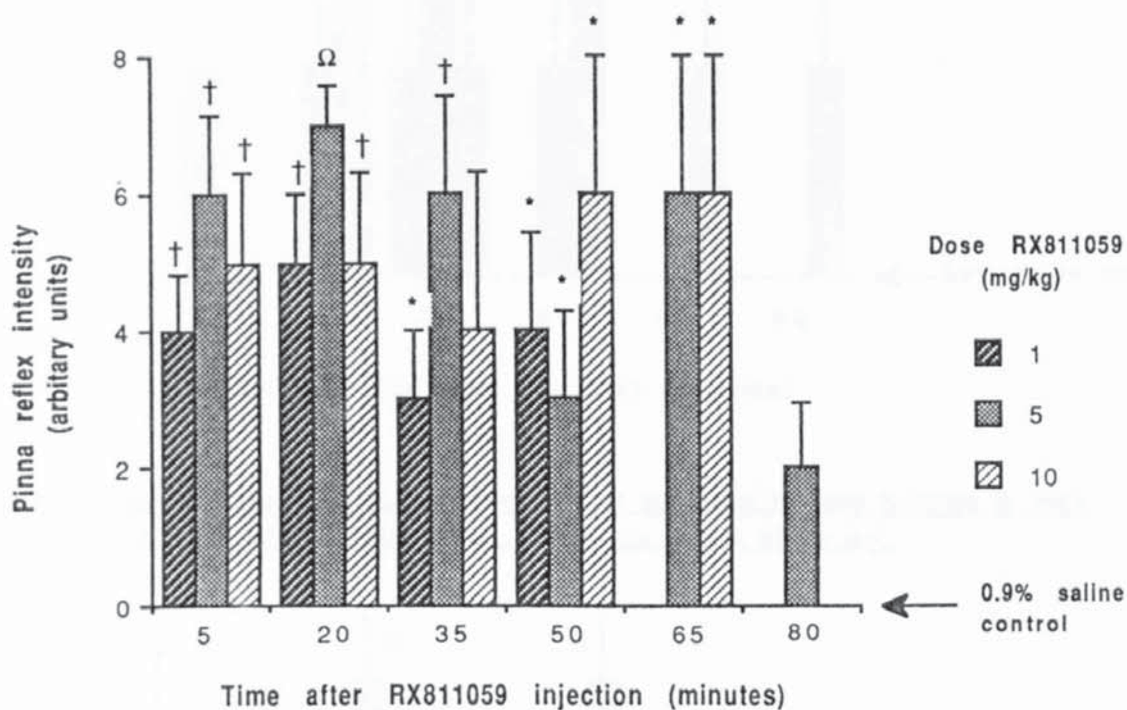


Figure 3.8. Effect of RX811059 upon startle reflex magnitude in mice. In relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05, †P<0.005, ΩP<0.0005 versus 0.9% saline control (Mann-Whitney 'U'-test)

Figure 3.9. Effect of RX811059 upon pinna reflex intensity in mice, in relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05, †P<0.005, ΩP<0.0005 versus 0.9% saline control (Mann-Whitney 'U'-test)

Figure 3.10. Effect of idazoxan upon locomotor activity in mice, in relation to a 0.9% saline control group. Values are means where n=4.

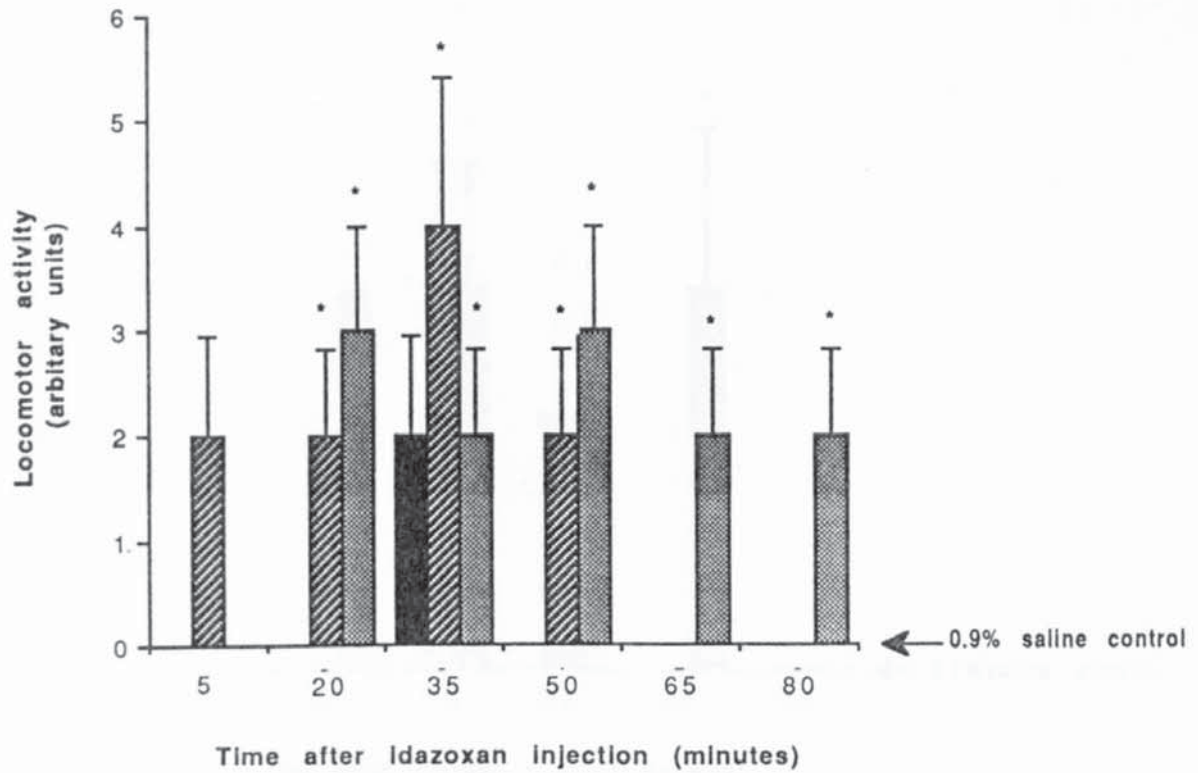
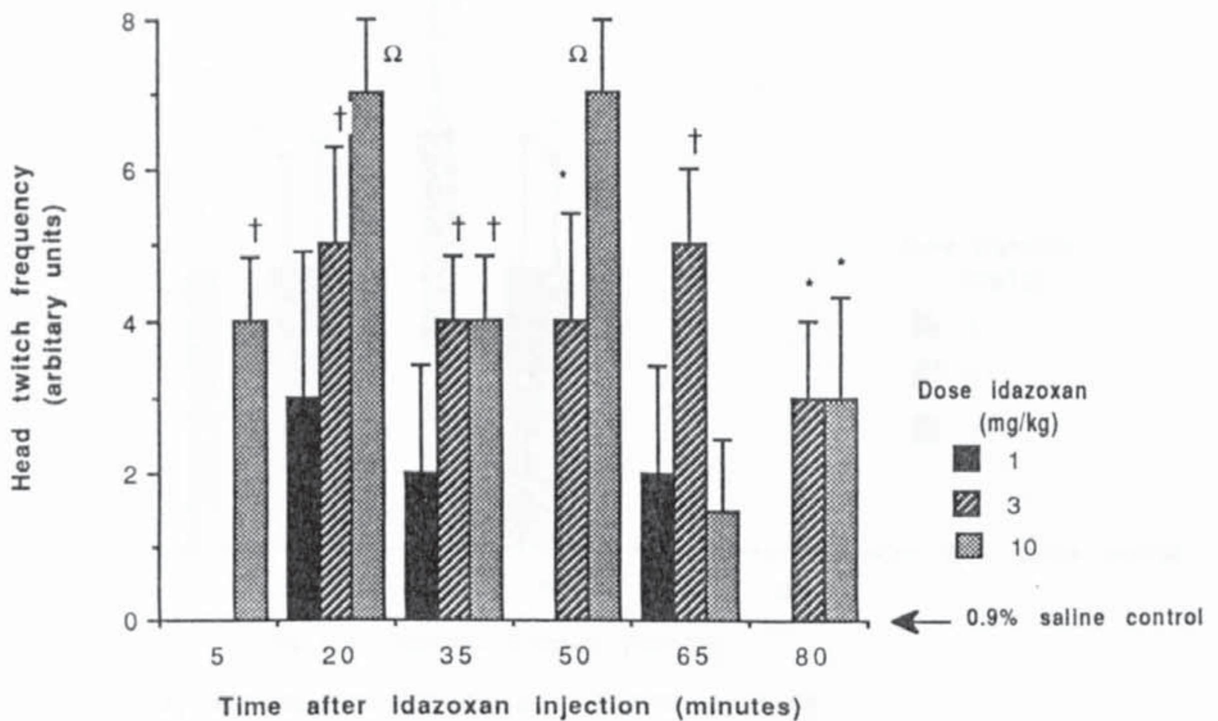


Figure 3.11. Effect of idazoxan upon the frequency of spontaneous head twitches in mice, in relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05, †P<0.005, ΩP<0.0005 versus 0.9% saline control (Mann-Whitney 'U'-test)

Figure 3.12. Effect of idazoxan upon frequency of spontaneous grooming behaviour in mice, in relation to a 0.9% saline control group. Values are means where n=4.

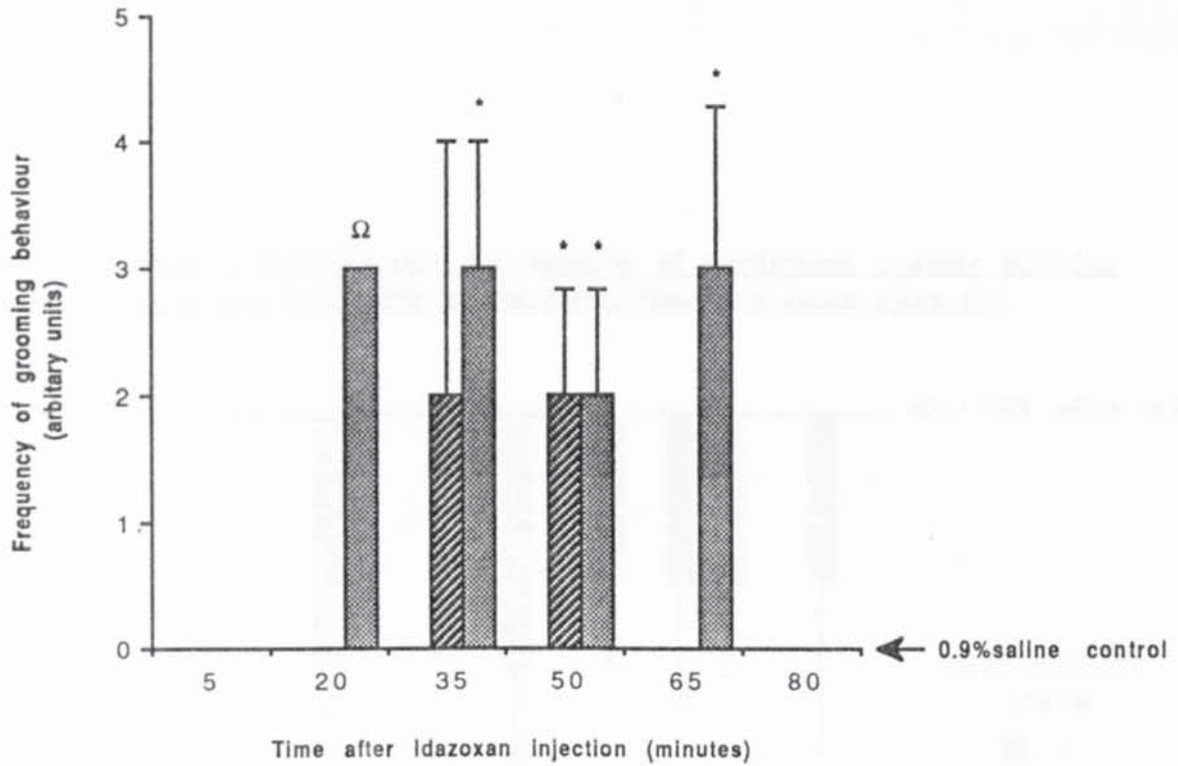
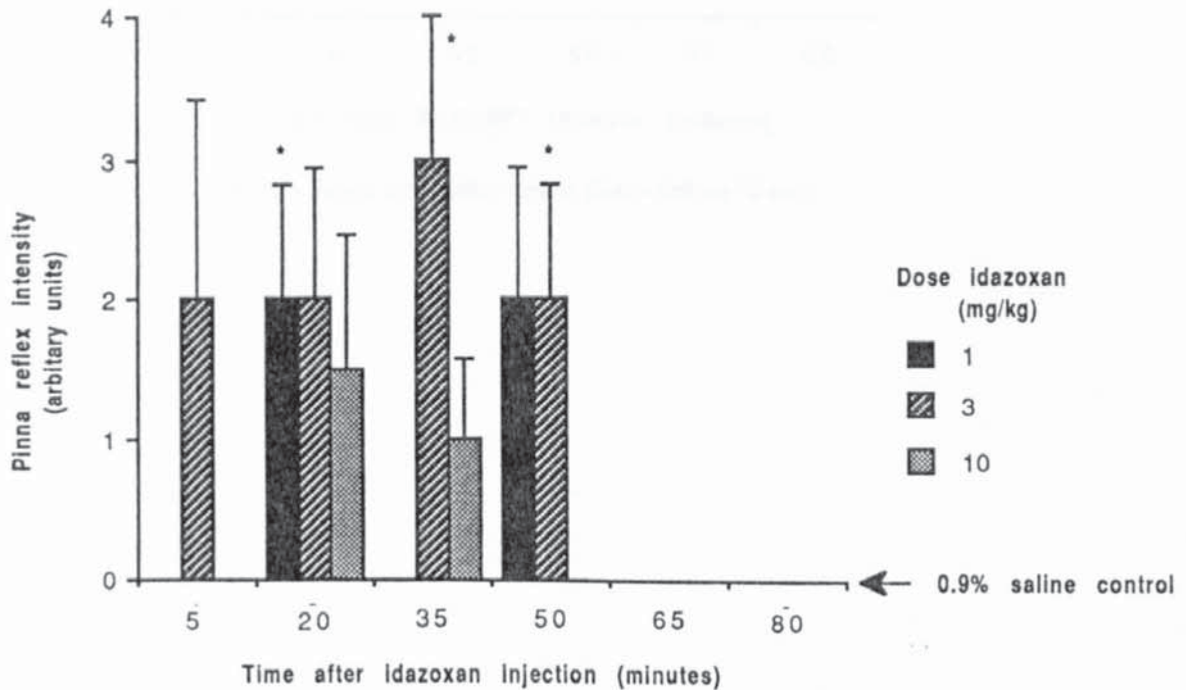
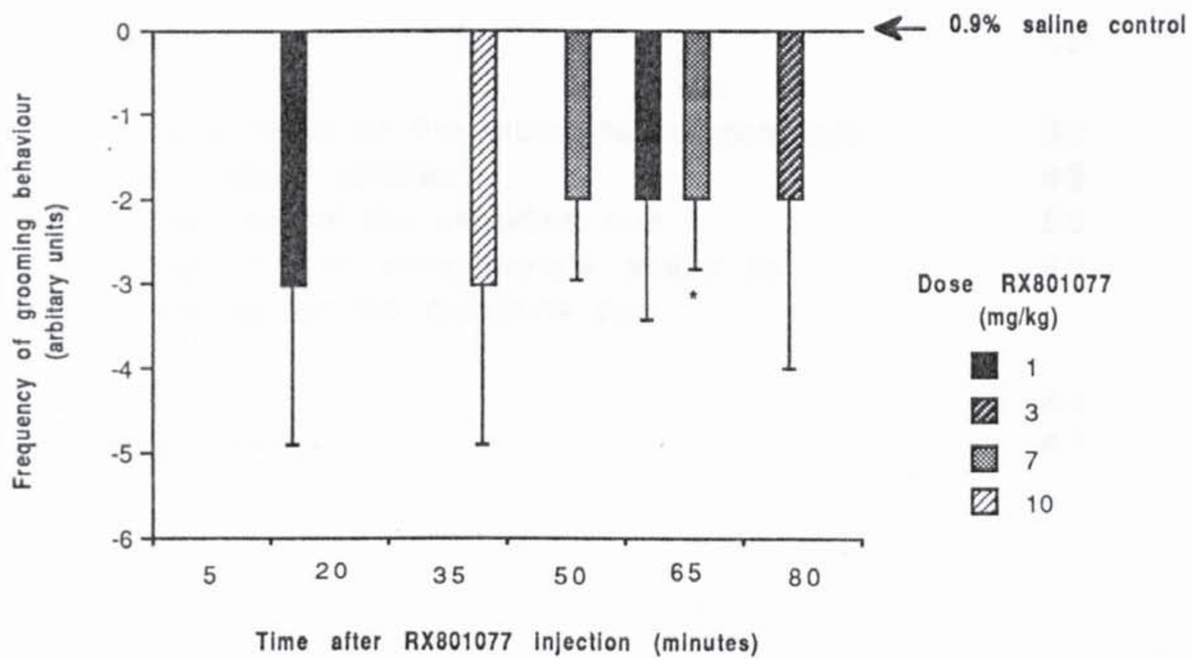


Figure 3.13. Effect of idazoxan upon pinna reflex intensity in mice, in relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05, ΩP<0.0005 versus 0.9% saline control (Mann-Whitney 'U'-test)

Figure 3.14. Effect of RX801077 upon the frequency of spontaneous grooming behaviour in mice in relation to a 0.9% saline control group. Values are means where n=4.



*P<0.05 versus 0.9% saline control (Mann-Whitney 'U'-test)

Chapter 4. The ability of the α2-adrenoceptor agonist clonidine to produce a cue in a rat drug discrimination paradigm.

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Chapter 4

1. Introduction

Clonidine produces a reliable cue in rat drug discrimination studies which has been attibuted to α2-adrenoceptor stimulation (eg. Bennett & Lal, 1982; Lal & Yaden, 1985). However, the exact nature of this cue remains to be determined because of the discovery of the IPR and the NAIBS, the above studies did not attempt to distinguish whether these sites could contribute to the clonidine cue.

Because α2-adrenoceptors are located both in the brain and periphery, the clonidine-induced drug discrimination cue could be mediated through either or both of these sites. It is possible to distinguish between these two possibilities by using the peripherally selective α2-adrenoceptor antagonist L659,066 (Clineschmidt et al, 1988; Jackson et al, 1991).

As described in the Introduction, a hypotensive effect of clonidine can be produced by direct injections into the ventrolateral medulla. This hypotensive effect may be due to an action at the IPR (see Introduction) and it could contribute to the clonidine cue. Other potential agonists at this site include rilmenidine, an antihypertensive which is less sedating than clonidine (Fillastre et al, 1988). Receptor binding studies in human brain have shown that rilmenidine binds with high affinity to the IPR, being 2.5 times more selective for this site than for α2-adrenoceptors when compared to clonidine (Bricca et al, 1989). Not all antihypertensive α2-adrenoceptor agonists also act at the IPR, for instance guanabenz has a high micromolar affinity at the IPR (Ernsberger et al, 1990; Gomez et al, 1991).

The aim of this study was to investigate the roles of the α2-adrenoceptor, the NAIBS and the IPR in the clonidine-induced discriminative cue by using selective agonists and antagonists for these sites. All drug doses were selected upon the basis of their activity in previous drug discrimination and other behavioural studies.

2. Results

2.1. Establishment of the clonidine-induced cue. All six rats learned to discriminate 0.02 mg/kg of clonidine from 0.9% saline in an average of 40 (±5) training sessions. Following training the clonidine-associated lever was "selected" (i.e. the first lever to receive ten presses to complete the session) by all animals after receiving 0.02 mg/kg of clonidine, and no responses occured on the saline-associated lever, i.e. 100% substitution for the clonidine-induced cue occurred (Table 4.1; Figure 4.1).

2.2. Substitution studies. The α2-adrenoceptor agonists guanabenz (0.32 mg/kg), UK-14,304 (0.16 mg/kg), rilmenidine (1.25 mg/kg) and clonidine (0.02 mg/kg) itself, completely substituted (i.e. over 80 % of responding occured on the clonidine-associated lever) for the clondine cue under test conditions (Table 4.1; Figures 4.1 - 4.4). On the other hand, 2-phenyl-2-imidazoline (3 to 10 mg/kg) failed to substitute for the clonidine cue (i.e. less than 20 % of responding occured on the clonidine-associated lever), as did the α2-adrenoceptor antagonists RX811059 (1 & 2.5 mg/kg) and fluparoxan (1 & 3 mg/kg) (Table 4.2; Figures 4.6 - 4.8). The α2-adrenoceptor agonists guanabenz, UK-14,304, rilmenidine and clonidine dose dependently inhibited the rate of responding at doses upto 0.32, 0.16, 1.25 and 0.02 mg/kg respectively (Table 4.1; Figures 4.1 - 4.4). In contrast, RX811059 and fluparoxan produced non-significant increases in response rate (Table 4.2; Figures 4.7 & 4.8). Finally, the NAIBS selective ligand RX801077 partially substituted for the clonidine cue (i.e. 20 to 80% of responding occured on the clonidine-associated lever) at doses up to 10 mg/kg, although it was unable to significantly influence the rate of

responding at any dose (Table 4.2; Figure 4.5). Attempts to achieve complete substitution for the clonidine cue using doses of RX801077 above 10 mg/kg were avoided due to the possibility of toxicity since the rats were required for the studies below.

2.3. Antagonism of the clonidine cue. When 0.02 mg/kg clonidine was given in combination with 30 minute pretreatments with the α2-adrenoceptor antagonists RX811059 (0.02 - 2.5 mg/kg) and fluparoxan (0.5 - 3 mg/kg) the clonidine-induced cue was dose-dependently inhibited (Table 4.3; Figures 4.9 & 4.10). However, the peripheral α2-adrenoceptor antagonist L659,066 (0.1 - 5 mg/kg) had no effect on the clonidine-induced cue (Table 4.3; Figure 4.11). The clonidine-induced inhibition of response rate (Table 4.1; Figure 4.1), was dose-dependently antagonised by pretreatment with RX811059 or fluparoxan but not by L659,066 using doses upto 2.5, 3.0 and 5.0 mg/kg respectively (Table 4.3; Figures 4.9 - 4.11).

2.4. Antagonism of rilmenidine's ability to substitute for the clonidine cue. The ability of rilmenidine (1.25 mg/kg) to substitute for the clonidine-induced cue (Table 4.1; Figure 4.4) was antagonised when given in combination with a 1.5 mg/kg pretreatment of RX811059 (produced only 7.69% substitution for the clonidine cue). Similarly, this property of rilmenidine was blocked by a 3 mg/kg fluparoxan pretreatment (3.0% substitution for the clonidine cue). Moreover, the significant reponse rate inhibition observed for rilmenidine (1.25 mg/kg; Table 4.1; Figure 4.4) was antagonised by both 1.25 mg/kg RX811059 (97.6% control response rate) and 3 mg/kg fluparoxan (98.3% control response rate).

4. DISCUSSION

This study demonstrates that the α2-adrenoceptor agonist clonidine produces a discriminable stimulus or "cue" in rats after about 40 training sessions. Similar findings have been reported by other workers (Bennett & Lal, 1982; Lal & Yaden, 1985). The present findings suggest that the cue is mediated by an action at α2-adrenoceptors for three reasons.

Firstly, it was completely substituted for by the more selective α2-adrenoceptor agonists UK-14,304 (Cambridge et al, 1981; Langin et al, 1990), which binds to the IPR, and guanabenz which does not (Ernsberger et al, 1990; Gomez et al, 1991). Secondly, the cue was blocked, but not substituted for, by the selective α2-adrenoceptor antagonists fluparoxan and RX811059. Although the affinity of these agents for the IPR has not been determined, fluparoxan would not be expected to bind to it as it does not contain an imidazoline moiety.

The pharmacological activity of 2-phenyl-2-imidazoline at the α2-adrenoceptor, IPR and the NAIBS is currently uncertain. This compound possesses an imidazoline moeity and as such it would be expected to bind to the IPR, and its inability to substitute for the clonidine cue would suggest it to be inactive at α2-adrenoceptors.

Of interest was the finding that the clonidine cue was not blocked by L659,066 at pharmacologically active doses (Jackson et al, 1991). Since L659,066 only poorly penetrates the blood-brain barrier (Clineschmidt et al, 1988), this would suggest that the cue by which rats discriminate clonidine is located in the brain.

The oxazoline rilmenidine completely substituted for the clonidine cue. This compound is an α2-adrenoceptor agonist (Koenig-Berard et al, 1988) which also binds to the IPR (Bricca et

al, 1989; Gomez et al, 1991). However, its ability to substitute for the clonidine cue is unlikely to be mediated by the IPR, as its ability to substitute was inhibited by both RX811059 and the non-imidazoline fluparoxan.

Clonidine has been suggested to act as a partial α1-adrenoceptor agonist, however the α2-adrenoceptor agonists which substituted for its cue do not appear to possess this action (Bradshaw et al, 1982).

The ability of the NAIBS selective ligand RX801077 to partially substitute for the clonidine cue was surprising. However, clonidine possesses low and high affinities for NAIBS in the CNS and periphery respectively (Michel & Insel, 1989; Coupry et al, 1987). Therefore, the possibility exists that a component of the clonidine cue may be mediated via an interaction with NAIBS in the periphery. If this is the case it may indicate that RX801077 has an agonist action at the NAIBS. Idazoxan also possesses a high affinity at the NAIBS in the periphery (Coupry et al, 1987; Boyajian et al, 1987), which may underlie its ability to elicit feeding in rats (Sleight et al, 1988; Jackson et al, 1991). Idazoxan did not, however, substitute for clonidine.

What other mechanisms might be responsible for the clonidine-induced cue? One possibility is that response rate inhibition may cue the rats to respond in the clonidine-associated manner. In this study the response rate (i.e. time for ten presses to occur on the drug or saline associated lever to terminate each session) was dose-dependently reduced by the α2-adrenoceptor agonists clonidine, guanabenz and UK-14,304 which may have been due to the sedative or hypolocomotor effects reported for these compounds (Marley & Nistico, 1975; Drew et al, 1979; Heal et al, 1989). This inhibition was dose-dependently reversed by RX811059

and fluparoxan, suggesting it to be mediated by α2-adrenoceptors. However this is unlikely because in a similar study a variety of non-α2-adrenoceptor agents (eg. diazepam and prazosin) and α2-adrenoceptor agonists (eg. guanabenz, prazosin and lofexidine) suppressed responding. However, substitution for a clonidine cue was only observed with the α2-adrenoceptor compounds (Bennett & Lal, 1982).

Another possibility is that the cue may reflect clonidine's effects on blood pressure. For instance the decrease in blood pressure produced by increasing doses of clonidine correlates with its ability to produce a cue in spontaneously hypertensive rats (Lal & Yaden, 1985). On the other hand, the clonidine cue does not appear to be associated with changes in blood pressure in normotensive rats (Bennett & Lal, 1982). Also the centrally mediated hypotensive effects of clonidine appear to be due to actions at the IPR and the present study has indicated that the IPR is not likely to be involved. The clonidine cue may be due to a combination of its many reported effects (Fielding & Lal, 1981) acting together to produce a compound cue. It is possible that peripheral NAIBS contributed to this.

In conclusion, the clonidine-induced cue appears to be mediated by an interaction with central α2-adrenoceptors as opposed to the IPR. However, a variety of pharmacological effects may contribute to the clonidine cue, some of which may be mediated by an interaction with NAIBS in the periphery. Finally, the property of this drug serving as its discriminable cue remains to be identified.

Table 4.1. The substitution of imidazoline (I) and non-imidazoline (NI) α2-adrenoceptor agonists for a clonidine (0.02 mg/kg, i.p) induced cue.

Test drug	Dose (mg/kg)	Mean % substitution for a clonidine cue ±SEM	[1]n / N	Mean response rate (responses/min. ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	2.8±0.10**	0 / 6	15.8±0.6	15.5±1.3	98.3
Clonidine (I)	0.005	18.7±9.8*	1 / 6	14.0±1.6	11.6±2.1	83.0
Clonidine	0.01	59.7±7.5	4 / 6	14.4±1.4	9.8±2.0	68.4
Clonidine	0.02	100	6 / 6	13.8±1.8	8.4±2.3	61.1¥
Guanabenz (NI)	0.02	2.8±2.5**	0 / 6	16.4±1.1	15.7±3.3	95.5
Guanabenz	0.04	25.7±5.3*	1 / 6	15.6±0.8	15.0±2.5	96.2
Guanabenz	0.08	48.3±16.1	3 / 6	15.0±1.7	10.9±2.7	72.8
Guanabenz	0.16	69.4±15.4	4 / 6	15.4±3.9	9.6±2.7	62.3
Guanabenz	0.32	100	6 / 6	15.8±0.9	8.6±1.1	54.5¥
UK-14,304 (I)	0.01	18.7±9.8*	1 / 6	12.2±2.4	9.1±1.9	74.4
UK-14,304	0.04	43.8±17.2	3 / 6	15.3±2.8	11.0±3.1	72.1
UK-14,304	0.08	52.6±16.1	4 / 6	14.4±2.5	8.9±4.6	61.7
UK-14,304	0.16	91.9±3.7	6 / 6	11.7±1.9	6.4±3.8	54.5¥
Rilmenidine (NI)	0.25	38.2±11.7	2 / 6	13.6±1.5	12.9±2.9	95.2
Rilmenidine	0.50	60.0±17.7	4 / 6	14.4±2.7	13.3±1.8	92.4
Rilmenidine	1.25	80.6±13.3	5 / 6	14.3±0.9	12.7±4.3	88.6

All values are means of N = 6 observations. *P<0.05; **P<0.01 versus previous 0.02 mg/kg clonidine training session (Student's paired t-test), i.e inability to substitute for a clonidine cue. ¥P<0.05 versus response rate in previous 0.9% saline control training session (Student's paired t-test). [1]n / N = Number of rats selecting clonidine-associated lever / N observations.

Table 4.2. The substitution of imidazoline (I) and non-imidazoline (NI) agents for a clonidine (0.02 mg/kg, i.p) induced cue.

Test drug	Dose (mg/kg)	Mean % substitution for a clonidine cue ±SEM	[1] n / N	Mean response rate (responses/min. ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	2.8±0.10**	0 / 6	15.8±0.6	15.5±1.3	98.3
RX801077 (NI)	0.25	27.8±10.4*	1 / 6	18.4±2.8	18.8±2.1	102.2
RX801077	1.0	34.8±9.0*	1 / 6	16.8±1.2	16.7±3.2	99.4
RX801077	3.0	37.1±8.8	3 / 6	16.4±2.8	17.4±1.5	106.1
RX801077	7.0	51.3±7.9	3 / 6	17.3±1.1	18.9±2.5	109.2
RX801077	10.0	62.6±6.8	4 / 6	16.9±3.6	18.0±3.8	106.5
2-p-2-i† (I)	3.0	4.3±2.6**	0 / 6	18.1±3.2	17.6±1.7	97.4
2-p-2-i†	7.0	5.4±3.5**	0 / 5	16.3±7.9	15.9±2.0	97.8
2-p-2-i†	10.0	11.3±7.0**	0 / 6	15.9±8.6	15.6±4.1	98.1
RX811059 (I)	1.0	5.4±2.6**	0 / 6	16.4±3.7	17.3±2.5	105.3
RX811059	2.5	3.0±1.8**	0 / 6	13.6±4.3	15.9±0.4	117.2
Fluparoxan (NI)	1.0	2.8±1.3**	0 / 5	17.7±4.5	19.9±3.4	112.5
Fluparoxan	3.0	4.3±2.9**	0 / 6	18.4±3.1	21.2±4.5	115.4

†2-phenyl-2-imidazoline. All values are means of N = 6 observations. *$P<0.05$; **$P<0.01$ versus previous 0.02 mg/kg clonidine training session (Student's paired t-test), i.e inability to substitute for a clonidine cue. [1] n / N = Number of rats selecting clonidine-associated lever / N observations.

Table 4.3. The antagonism of a (0.02 mg/kg, i.p) clonidine cue by pretreatment with imidazoline (I) and non-imidazoline (NI) α2-adrenoceptor antagonists.

Test drug pretreatment	Dose (mg/kg)	Mean % substitution for a clonidine cue ± SEM	[1]n / N	Mean response rate (responses/minute ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	100	6 / 6	17.6±1.9	17.0±0.8	96.8
RX811059 (I)	0.02	78.8±14.5	5 / 6	16.6±1.1	10.9±3.7	65.4
RX811059	0.05	47.2±17.3	3 / 6	16.0±2.5	11.5±2.8	72.1
RX811059	0.10	44.2±10.2	3 / 6	17.8±1.6	13.6±2.6	76.5
RX811059	1.0	13.5±2.9**	1 / 6	17.7±0.4	15.2±3.6	85.7
RX811059	2.5	3.0±1.8**	0 / 6	15.9±2.4	14.2±0.9	89.1
Fluparoxan (NI)	0.5	23.5±9.5*	1 / 6	13.6±3.2	9.4±3.3	68.9
Fluparoxan	1.0	10.5±6.8**	0 / 6	15.4±0.7	12.1±2.9	78.7
Fluparoxan	3.0	7.7±3.2**	0 / 6	15.3±1.0	13.2±2.6	86.2
L659,066 (NI)	0.1	100	6 / 6	16.3±1.9	15.5±2.0	94.9
L659,066	1.0	98.3±1.7	6 / 6	15.9±0.8	14.3±1.8	90.0
L659,066	5.0	100	6 / 6	16.4±2.1	15.1±2.2	92.2

All values are means of N = 6 observations. *P<0.05; **P<0.01 versus previous 0.02 mg/kg clonidine training session (Student's paired t test), i.e inability to substitute for a clonidine cue. [1]n / N = Number of rats selecting clonidine-associated lever / N observations.

Figure 4.1. Clonidine stimulus substitution gradient

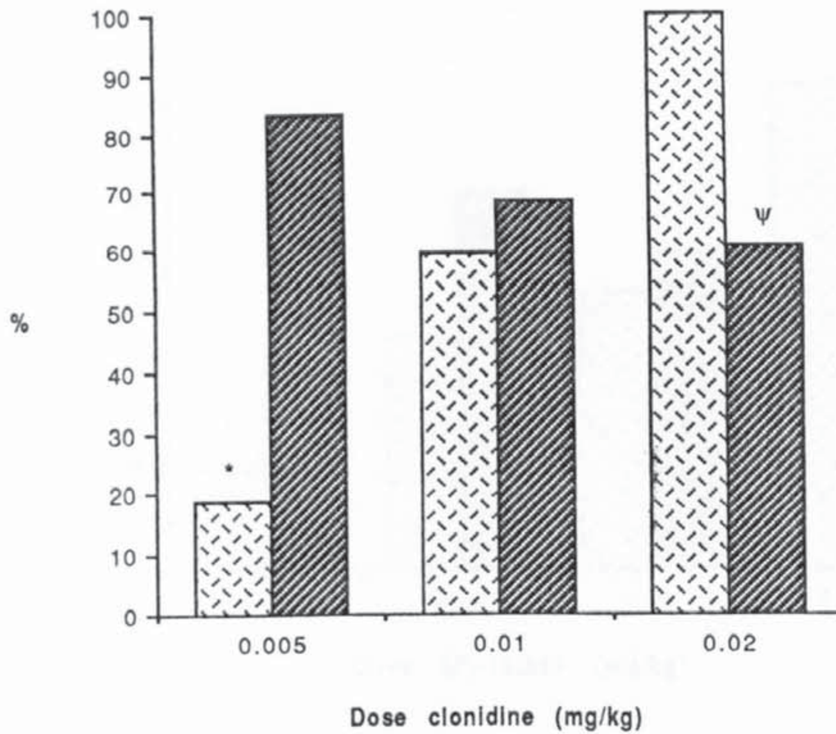
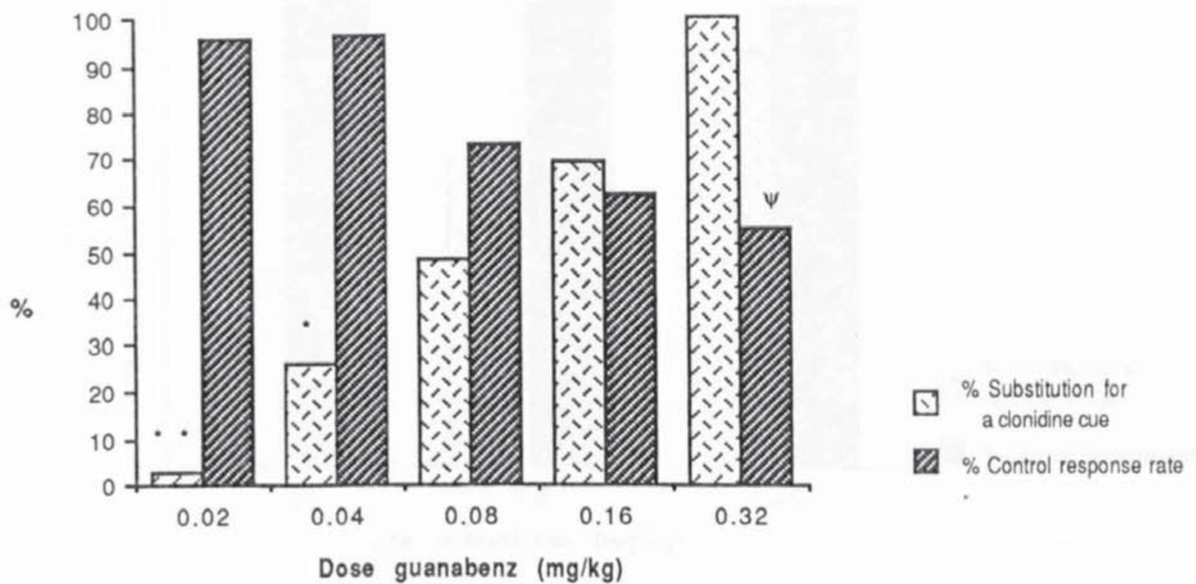


Figure 4.2. The ability of guanabenz to substitute for a 0.02 mg/kg clonidine cue



*P<0.05, **P<0.01 versus previous 0.02 mg/kg clonidine training session, i.e. significant inability to substitute for the clonidine cue (Student's paired t-test). ψ P<0.05 versus previous 0.9% saline control training session, (Student's paired t-test).

Figure 4.3. The ability of UK-14,304 to substitute for a 0.02 mg/kg clonidine cue.

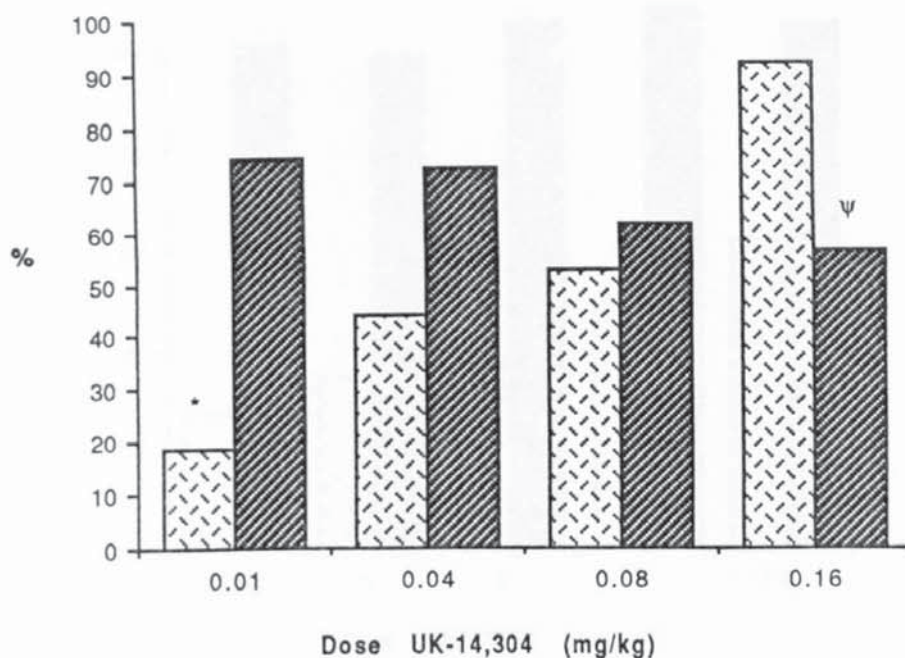
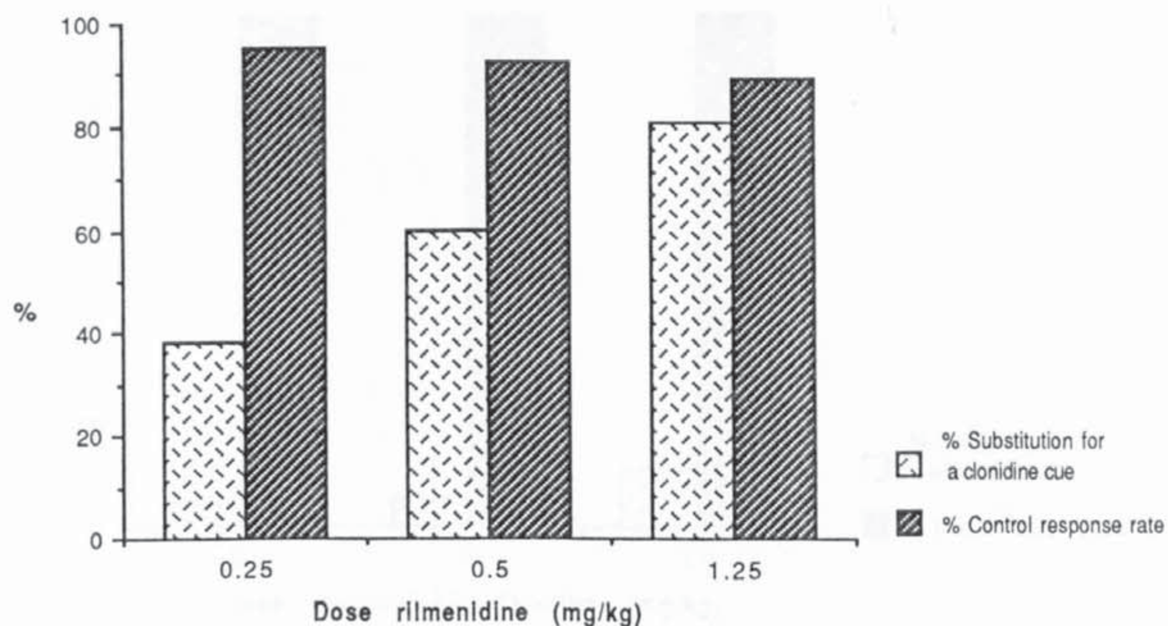


Figure 4.4. The ability of rilmenidine to substitute for a 0.02 mg/kg clonidine cue



*P<0.05 versus previous 0.02 mg/kg clonidine training session (Student's paired t-test), i.e. significant inability to substitute to a clonidine cue ¥ P<0.05 versus response rate in previous 0.9% saline control session (Student's paired t-test).

Figure 4.5. The ability of RX801077 to substitute for a 0.02 mg/kg clonidine cue

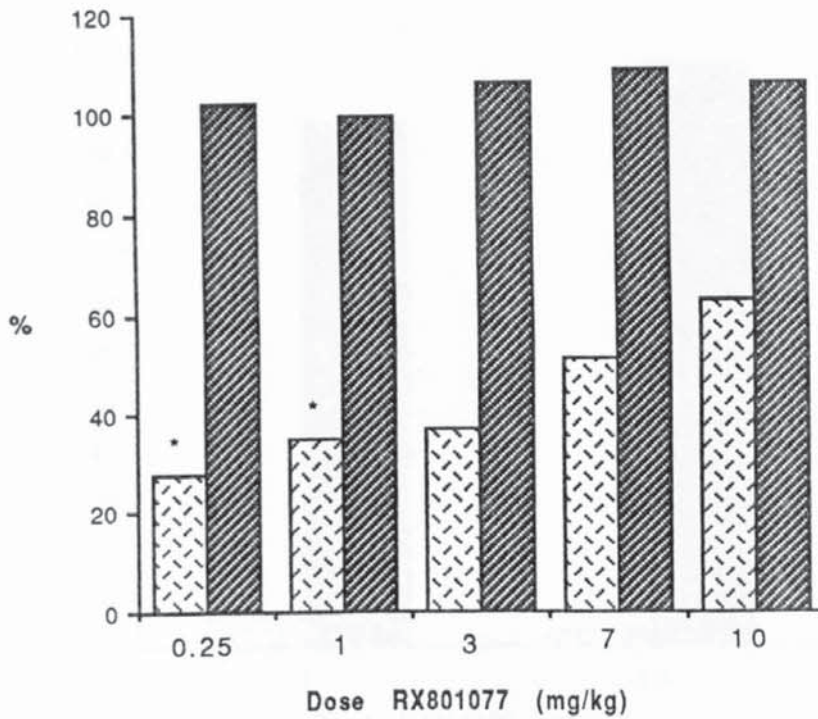
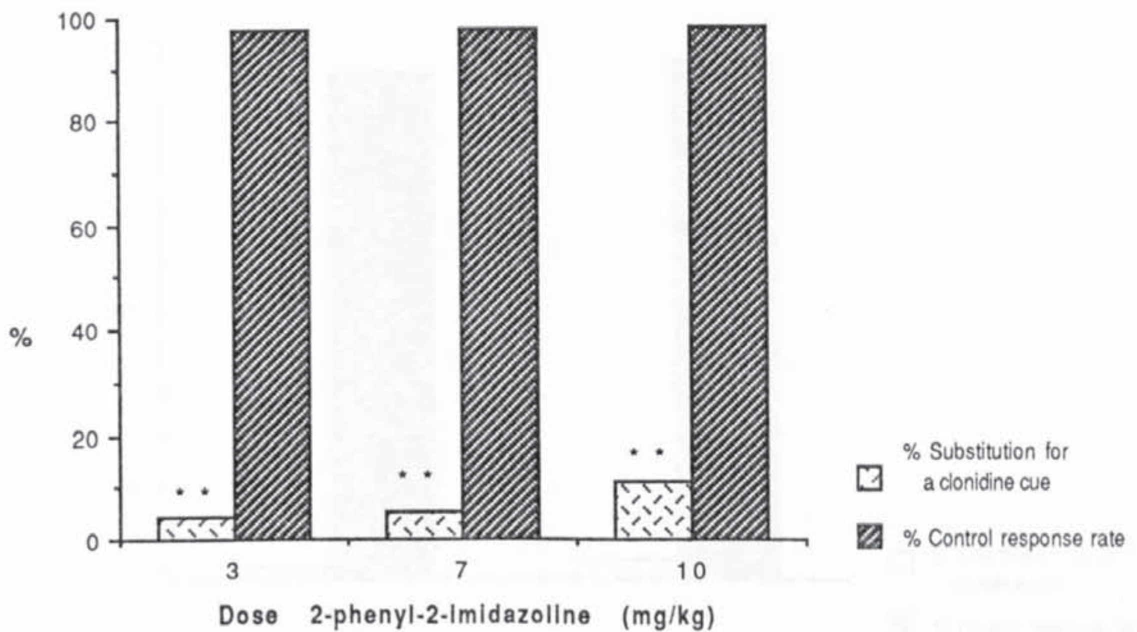


Figure 4.6. The ability of 2-phenyl-2-imidazoline to substitute for a 0.02 mg/kg clonidine cue



*P<0.05, **P<0.01 versus previous 0.02 mg/kg clonidine training session (Student's paired t-test), i.e. significant inability to substitute for a clonidine cue.

Figure 4.7. The ability of RX811059 to substitute for a 0.02 mg/kg clonidine cue

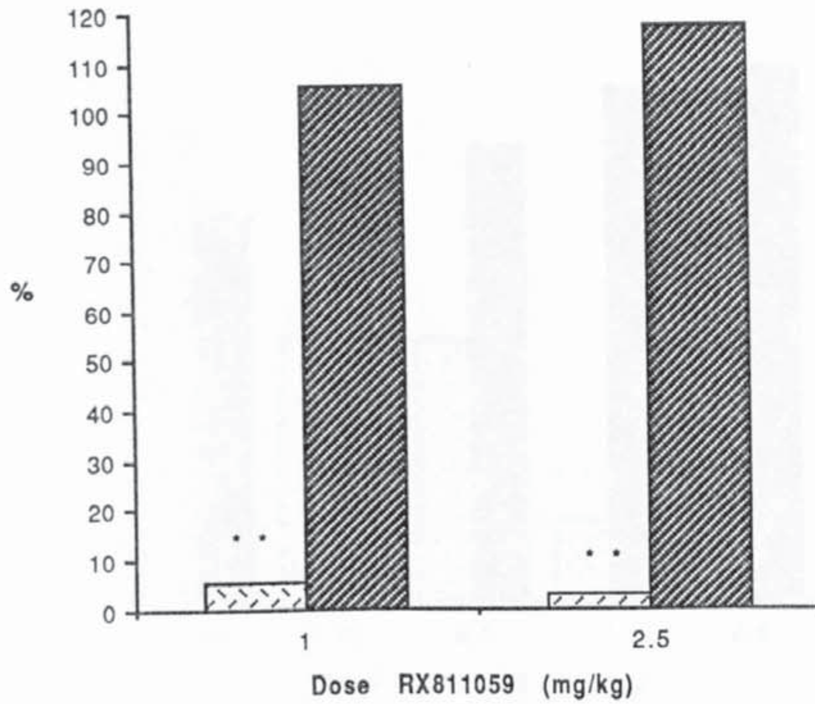
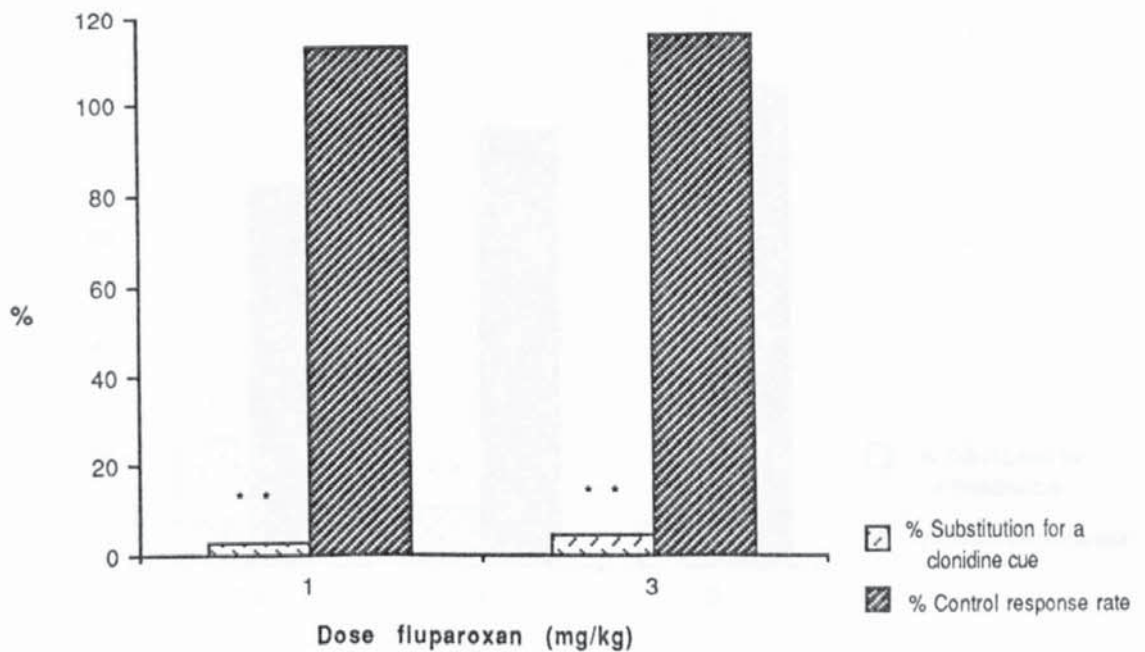


Figure 4.8. The ability of fluparoxan to substitute for a 0.02 mg/kg clonidine cue



**P<0.01 versus previous 0.02 mg/kg clonidine training session (Student's paired t-test), i.e significant inability to substitute for a clonidine cue.

Figure 4.9. The antagonism of a 0.02 mg/kg clonidine cue by pretreatment with RX811059

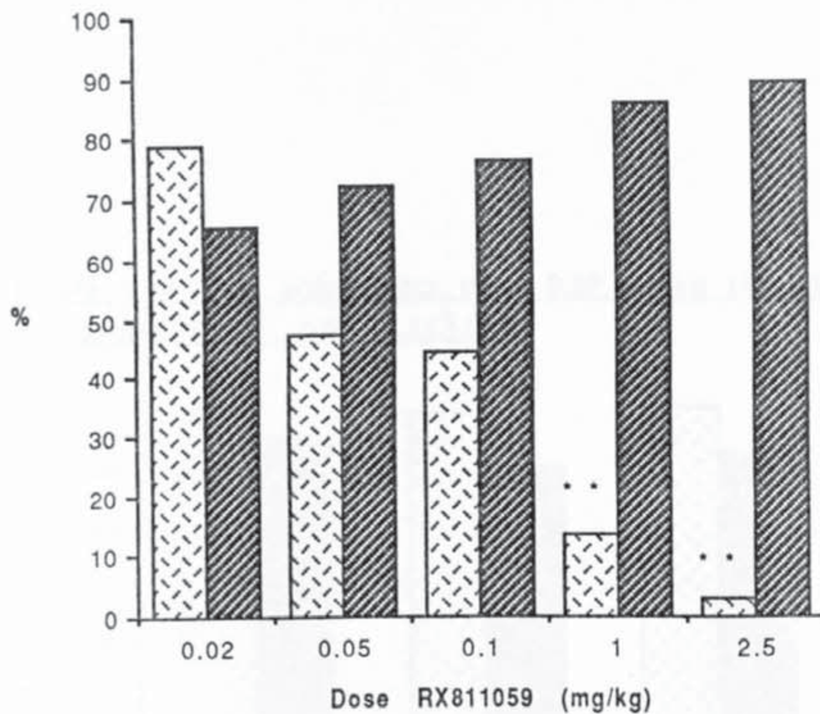
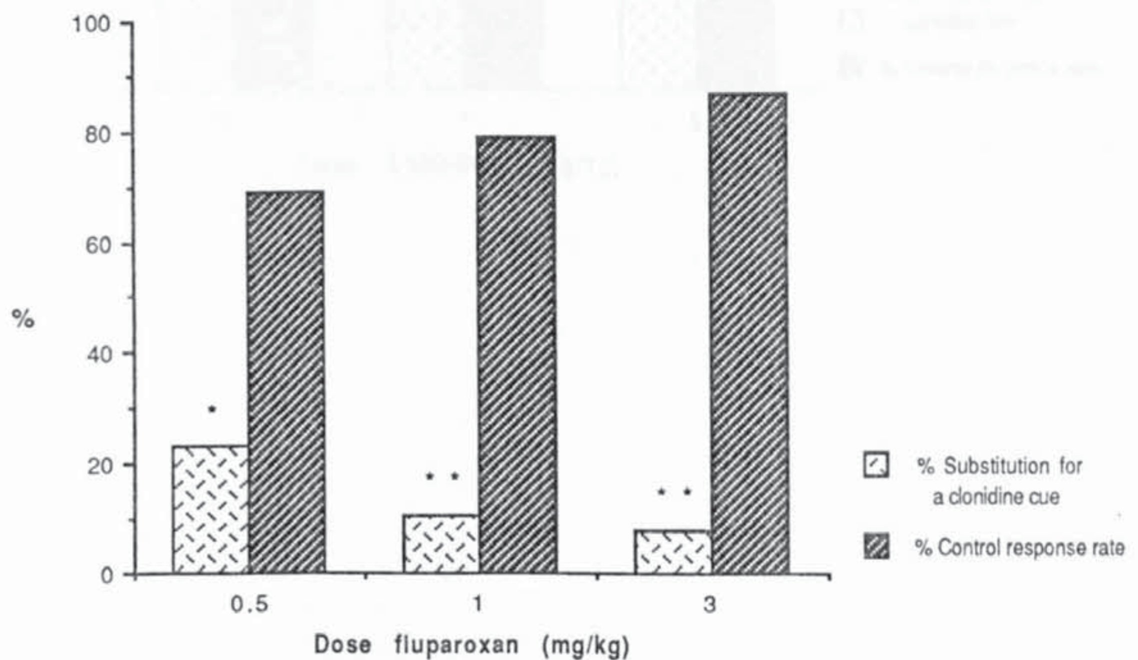
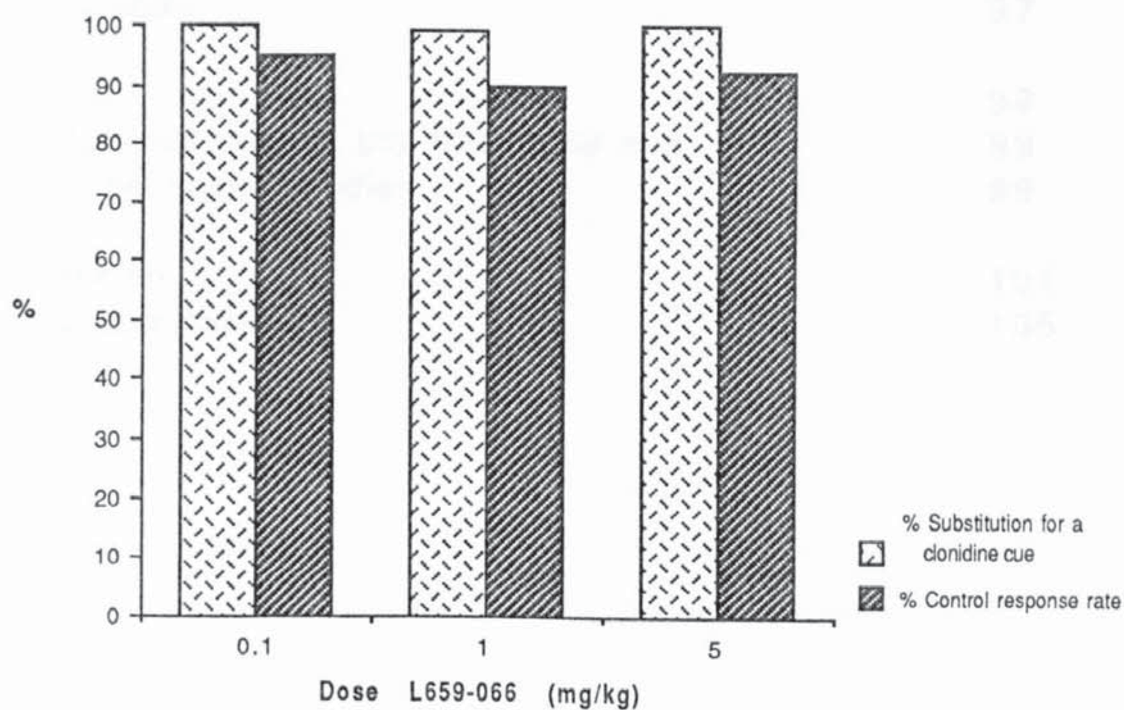


Figure 4.10. The antagonism of a 0.02 mg/kg clonidine cue by pretreatment with fluparoxan



*P<0.05, **P<0.01 versus previous 0.02 mg/kg clonidine training session (Student's paired t-test), i.e. significant inability to substitute for a clonidine cue.

Figure 4.11. The antagonism of a 0.02 mg/kg clonidine cue by pretreatment with L659,066



CHAPTER 5: The ability of the highly selective $\alpha 2$-adrenoceptor antagonist RX811059 to produce a discriminable cue in a rat drug discrimination paradigm.

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Chapter 5

1. Introduction.

Yohimbine and idazoxan have been shown to produce a discriminable cue in rat drug discrimination studies (Winter, 1978; Sanger, 1988; Sanger et al, 1989). However, these α2–adrenoceptor antagonists are known to have limited selectivity for α2–adrenoceptors. For example, yohimbine interacts with α1-adrenoceptors, and with dopamine and serotonin receptors (Scatton et al, 1980; Goldberg & Robertson, 1983). Idazoxan is the most selective α2–adrenoceptor antagonist to be used for this purpose (Sanger et al, 1989), although the exact nature of this cue remains to be determined because of recent findings which reveal that it may act at several different binding sites (Boyajian et al, 1987; Brown et al, 1990; Michel & Insel, 1989). For instance, α1-adrenoceptor agonist and antagonist properties have been reported for idazoxan in peripheral tissues (Doxey et al, 1983; Paciorek & Shepperson, 1983). In addition, it binds with equal affinity to the NAIBS (Boyajian et al, 1987). Moreover, idazoxan binds with a similar low nanomolar affinity to the IPR (Ernsberger et al, 1990; Michel & Insel, 1989). RX811059 (2-ethoxy-idazoxan) and fluparoxan are highly selective α2–adrenoceptor antagonists (Mallard et al, 1991; Halliday et al, 1991) which have only negligable affinity for NAIBS (Mallard et al, 1991; A. Hudson - personal communication). Their affinity for the IPR has not been determined yet, however RX811059 contains an imidazoline moeity and as such it might be expected to bind to this site, whereas fluparoxan is a non-imidazoline which would not be expected to bind to the IPR. Similarly, the α2–adrenoceptor antagonists atipamezole (Scheinin et al, 1988), an imidazoline might be expected to bind to the NAIBS whereas the non-

imidazoline 1-(2-pyrimidinyl)-piperazine (1-PP) (Bianchi et al, 1988) is less likely to exhibit such binding.

The aim of this study was to determine whether the highly selective α2-adrenoceptor antagonist RX811059 could produce a discriminable cue in animals, and to test whether such a cue was due to α2-adrenoceptor antagonism by examining its ability to be substituted for by a variety of α2-adrenoceptor antagonists including idazoxan, fluparoxan, yohimbine, atipamezole and 1-PP (Doxey et al, 1983; Halliday et al, 1991; Goldberg & Robertson, 1983; Scheinin et al, 1988; Bianchi et al, 1988). In addition, the highly selective NAIBS ligand RX801077 was examined for its ability to substitute for the RX811059 cue, as were 2-phenyl-2-imidazoline and the α1-adrenoceptor agonist cirazoline (Cavero et al, 1982); these agents all have nanomolar affinities for NAIBS in receptor binding studies (A. Hudson - personal communication; Wikberg & Uhlen, 1990). Finally, the peripherally-selective α2-adrenoceptor antagonist L659,066 (Clineschmidt et al, 1988; Jackson et al, 1991) was used to examine whether the cue induced by RX811059 involved activity at central or peripheral α2-adrenoceptors. All drug doses were selected upon the basis of their activity in drug discrimination and other behavioural experiments.

2. Results.

2.1. Establishment of the RX811059 cue. All 6 rats learned to discriminate 2.5 mg/kg of RX811059 from 0.9% saline in an average of 44 (S.E.M±2) training sessions. Following training the RX811059-associated lever was 'selected' (i.e. the first lever to receive 10 presses to complete the session) by all animals receiving 2.5 mg/kg of RX811059, and a total of 2 responses occured on the saline-associated lever, i.e. 97% substitution for the RX811059-induced cue occured (Table 5.1; Figure 5.1).

2.2. Substitution studies. The α2–adrenoceptor antagonists idazoxan (10 mg/kg), fluparoxan (3 mg/kg), 1-PP (3 mg/kg) and RX811059 (2.5 mg/kg) itself, completely substituted (i.e. over 80 % of responding occurred on the RX811059-associated lever) for the RX811059 cue under test conditions (Table 5.1; Figures 5.1 - 5.4). Partial substitution (i.e. 20 to 80 % of responding occured on the RX811059-associated lever) for the RX811059 cue was achieved with yohimbine (0.025 - 2 mg/kg), atipamezole (0.25 - 5 mg/kg) and RX801077 (0.5 - 10 mg/kg) (Tables 5.2 & 5.3; Figures 5.5, 5.6 & 5.11). The highest dose of yohimbine tested was 2 mg/kg as its solubility prevented the delivery of higher doses in a 1ml/kg dose volume. Atipamezole was purchased in a 5 mg/kg dissolved form which prevented its ability to be tested at higher doses in a 1ml/kg dose volume. Attempts to achieve complete substitution for the RX811059 cue with doses of RX801077 above 10 mg/kg were avoided due to the possibility of toxicity. In contrast, L659,066 failed to substitute (less than 20 % of responding occured on the RX811059-associated lever) for the RX811059-induced cue at doses up to 8 mg/kg (Table 5.2; Figure 5.7).

Moreover, cirazoline, clonidine and 2-phenyl-2-imidazoline did not substitute for the RX811059 cue at doses up to 0.25, 0.04 and 7 mg/kg respectively (Table 5.3; Figures 5.8 - 5.10). However, partial substitution was achieved with a 10 mg/kg dose of 2-phenyl-2-imidazoline (Table 5.3; Figure 5.9).

RX811059, idazoxan, fluparoxan and 1-PP dose dependently increased the rate of responding above that for saline at doses up to 2.5, 10, 3 and 3 mg/kg respectively (Table 5.1; Figures 5.1 - 5.4). In contrast, cirazoline dose-dependently inhibited the rate of responding such that at 0.25 mg/kg all 6 rats failed to respond on either lever (Table 5.3; Figure 5.8). Likewise, clonidine dose dependently reduced the rate of responding at doses up to 0.04 mg/kg (Table 5.3; Figure 5.10). However, yohimbine, atipamezole, L659,066, 2-phenyl-2-imidazoline and RX801077 did not affect the response rate at doses up to 2, 5, 8,10 and 10 mg/kg respectively (Tables 5.2 & 5.3; Figures 5.5 - 5.7, 5.9 & 5.11).

3. Discussion.

This study demonstrates that the highly selective α2-adrenoceptor antagonist RX811059 produces a discriminable stimulus or 'cue' in rats after about 44 training sessions. A similar finding has been reported for idazoxan, the parent compound of RX811059 (Sanger, 1989). The present findings suggest the RX811059-induced cue to be mediated by α2-adrenoceptor blockade. Several lines of evidence support this mechanism to underlie both the RX811059 and idazoxan-induced cues.

Firstly, the RX811059 cue was completely substituted for by the highly selective α2-adrenoceptor antagonist fluparoxan (Halliday et al, 1991). Likewise, the selective α2-adrenoceptor antagonists idazoxan (Doxey et al, 1983) and 1-PP (Bianchi et al, 1988) substituted for the RX811059 cue. However, yohimbine (Goldberg & Robertson, 1983) only partially substituted for the RX811059 cue at doses up to 2 mg/kg, although in the present study a dose dependent effect was not observed. However, complete substitutions for an idazoxan-induced cue have been reported with higher doses of yohimbine (Sanger, 1989). Yohimbine acts at sites other than the α2-adrenoceptor (eg. Goldberg & Robertson, 1983), and it is possible that such interactions may inhibit its ability to substitute for the RX811059-induced cue. However, this partial substitution effect was also observed with atipamezole (Scheinin et al, 1988). The inability of this highly selective α2-adrenoceptor antagonist to completely substitute for the RX811059 cue is unclear, although it is possible that doses up to 5 mg/kg were insufficient, but figure 5.6 suggests this is unlikely and that a maximum effect is obtained with 0.25 mg/kg. In contrast, cirazoline did not substitute for the RX811059-induced

cue despite the suggestion that it may act concomitantly at adrenoceptors as an α1-agonist and α2-antagonist (Wikberg et al, 1990). Likewise, the α2-adrenoceptor agonist clonidine (Fielding et al, 1981) and the non-α2-adrenoceptor acting agent 2-phenyl-2-imidazoline (A. Hudson - personal communication) failed to substitute for the RX811059 cue, supporting its mediation by α2-adrenoceptor blockade. RX811059, unlike idazoxan, has minimal affinity for the NAIBS (Michel & Insel, 1989), and this was confirmed by the inability of cirazoline (K_D = 1nM at NAIBS; Wikberg & Uhlen, 1990) and 2-phenyl-2-imidazoline (Ki = 181nM at NAIBS; A. Hudson - personal communication) to mimic the RX811059 cue. However, the highly selective NAIBS compound RX801077 partially substituted for the RX811059 cue which might reflect the possibility that, despite its high selectivity for the NAIBS it might interact with α2-adrenoceptors at the doses used. Alternatively, an indirect stimulation of α2-adrenoceptors linked to NAIBS is a possibility. Moreover, RX801077 might act as an agonist at the NAIBS whereas cirazoline or 2-phenyl-2-imidazoline may act as antagonists or vice-versa.

The ability of fluparoxan to completely substitute for the RX811059 cue at pharmacologically active doses (Jackson et al, 1991) suggests this cue is unlikely to be mediated by imidazoline preferring receptors (Michel & Insel, 1989). Although the affinity of RX811059 and fluparoxan for this site has not been determined, a low affinity would be expected for fluparoxan as it does not contain an imidazoline moiety.

Of interest was the finding that L659,066 was unable to substitute for the RX811059 cue. Since this α2-adrenoceptor antagonist only poorly penetrates the blood-brain barrier (Clineschmidt et al, 1988; Jackson et al, 1991), this would suggest

that the cue by which rats discriminate RX811059 is mediated by the blockade of α2-adrenoceptors located in the brain.

What are the mechanisms that are responsible for the RX811059-induced cue? In this study the α2-adrenoceptor antagonists RX811059, idazoxan, fluparoxan and 1-PP dose-dependently increased the response rate (i.e. time for 10 presses to occur on the drug or saline-associated lever to terminate each test session), while smaller increases were produced by yohimbine and atipamezole. Similar findings reported for yohimbine and idazoxan have been attributed to increases in arousal (Sanger, 1989). Small increases in locomotor activity have been reported for both idazoxan and RX811059 (Dickinson et al, 1988), however this effect is minimal when compared with psychomotor stimulants such as amphetamine (Dickinson et al, 1990). Other behavioural properties have been demonstrated for RX811059 and idazoxan which may explain this cue's ability to be mimicked by other highly selective α2-adrenoceptor antagonists. For instance, pro-convulsant (Jackson et al, 1990), anxiogenic (Handley & Mithani, 1984), and antidepressant (Montgomery, 1988) effects have been reported. In contrast, cirazoline dose-dependently reduced the response rate, as did the α2-adrenoceptor agonist clonidine which supports a previous finding (Bennett & Lal, 1982). Finally, the hyperphagic property of idazoxan in rats is unlikely to be involved in the RX811059 cue as this α2-adrenoceptor antagonist is inactive with respect to feeding behaviour (Jackson et al, 1991).

In conclusion, the RX811059 cue appears to be mediated by the antagonism of central α2-adrenoceptors, although its ability to be partially substituted for by the NAIBS selective agent RX801077 remains to be explained. However, the property of this drug serving as its discriminable cue remains unidentified, although general

behavioural stimulation may be involved.

Table 5.1. Results of substitution tests for an RX811059 (2.5 mg/kg, i.p) induced cue using a range of α2-adrenoceptor antagonists.

Test drug	Dose (mg/kg)	Mean % substitution for an RX811059 cue ±SEM	[1]n / N	Mean response rate (responses/minute ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	3.0±0.1**	0 / 6	15.9±2.8	15.5±1.3	97.5
RX811059	0.75	48.6±11.9	3 / 6	16.9±2.0	17.2±3.1	101.8
RX811059	1.50	60.0±16.8	4 / 6	18.6±5.8	23.4±1.2	125.8¥
RX811059	2.50	97.0±1.73	6 / 6	16.8±4.2	22.1±0.9	131.5¥
Idazoxan	0.125	31.6±14.4*	1 / 6	15.4±2.7	15.2±1.8	98.7
Idazoxan	0.50	41.2±13.6*	3 / 6	17.5±3.1	18.7±2.6	106.9
Idazoxan	1.25	53.9±11.7	4 / 6	18.2±2.9	18.8±3.1	103.3
Idazoxan	5	72.5±12.1	4 / 6	17.4±1.8	21.2±1.7	121.8¥
Idazoxan	10	83.3±12.4	5 / 6	16.1±2.3	20.1±1.3	124.8¥
Fluparoxan	0.5	25.7±15.2*	1 / 6	13.0±4.1	12.5±1.2	96.2
Fluparoxan	3	88.0±4.0	6 / 6	14.2±0.9	16.6±0.2	116.9¥
1-PP	2	68.2±18.4	4 / 6	15.4±2.6	17.9±3.8	116.2
1-PP	3	83.1±15.2	5 / 6	15.8±0.4	19.8±1.4	125.3¥

All values are means of N = 6 observations. *P<0.05; **P<0.01 versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e inability to substitute for an RX811059 cue. ¥P<0.05 versus response rate in previous 0.9% saline control training session (Student's paired t-test).[1]n / N = Number of rats selecting RX811059-associated lever / N observations.

Table 5.2. Results of substitution tests for an RX811059 (2.5 mg/kg, i.p) induced cue using a range of α2-adrenoceptor antagonists.

Test drug	Dose (mg/kg)	Mean % substitution for an RX811059 cue ±SEM	[1]n / N	Mean response rate (responses/minute ± SEM) Control training session	Test session	% Control response rate
Yohimbine	0.025	40.0±10.7*	2 / 6	16.2±2.7	15.6±1.8	96.2
Yohimbine	0.10	50.2±12.4	3 / 6	16.1±3.1	15.9±0.7	98.8
Yohimbine	0.5	45.9±5.7	3 / 6	16.6±2.3	18.1±2.7	109.0
Yohimbine	2	48.4±10.0	3 / 6	15.4±3.1	15.9±2.1	103.2
Atipamezole	0.25	41.2±12.0*	4 / 6	17.3±3.7	18.4±1.6	106.4
Atipamezole	1	46.8±10.4	4 / 6	16.8±2.4	18.7±3.1	111.3
Atipamezole	2.5	48.3±12.7	3 / 6	16.2±1.8	17.8±2.4	109.9
Atipamezole	5	43.3±15.1	3 / 6	17.3±0.9	19.7±2.6	113.9
L659,066	3	8.3±3.4**	0 / 6	15.8±2.6	15.5±2.2	98.1
L659,066	8	13.5±2.9**	0 / 6	16.0±0.2	15.9±3.8	99.4

All values are means of N = 6 observations. *$P<0.05$; **$P<0.01$ versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e inability to substitute for an RX811059 cue.[1]n / N = Number of rats selecting RX811059-associated lever / N observations.

Table 5.3. Results of tests to substitute for an RX811059 (2.5 mg/kg, i.p) induced cue: cirazoline, 2-phenyl-2-imidazoline†, RX801077 and clonidine.

Test drug	Dose (mg/kg)	Mean % substitution for an RX811059 cue ±SEM	[1]n / N	Mean response rate (responses/minute ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	3.0±0.1**	0 / 6	15.3±0.3	14.9±0.9	97.4
Cirazoline	0.05	2.8±2.7**	0 / 6	18.2±3.4	5.8±1.9	31.9Ω
Cirazoline	0.1	5.6±3.5**	0 / 6	19.6±3.1	4.6±1.7	23.5Ω
Cirazoline	0.25	0	0 / 6	15.8±1.7	0	0
2-p-2-i†	3.0	10.7±3.9**	0 / 6	17.2±6.1	17.4±2.5	101.0
2-p-2-i†	7.0	12.3±5.3**	0 / 6	16.5±2.6	15.7±1.8	95.2
2-p-2-i†	10.0	48.3±13.4*	4 / 6	14.9±3.1	15.4±3.7	103.4
Clonidine	0.01	3.8±1.74**	0 / 6	11.9±2.1	8.8±0.4	73.9¥
Clonidine	0.02	1.5±1.3**	0 / 6	12.9±2.8	7.6±2.3	58.9¥
Clonidine	0.04	7.4±3.6**	0 / 6	13.6±1.8	5.7±2.4	41.9Ω
RX801077	0.5	28.6±8.7*	2 / 6	14.6±3.1	14.5±1.7	99.3
RX801077	3.0	41.2±12.6*	3 / 6	14.2±0.9	13.9±3.7	97.9
RX801077	7.0	72.0±10.3*	4 / 6	16.1±1.6	16.8±2.8	104.3
RX801077	10.0	66.5±14.4*	4 / 6	15.3±2.8	16.4±3.1	107.2

†2-phenyl-2-imidazoline All values are means of N = 6 observations. *$P<0.05$; **$P<0.01$ versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e inability to substitute for an RX811059 cue. ¥$P<0.05$, Ω$P<0.01$ versus response rate in previous 0.9% saline control training session (Student's paired t-test).[1]n / N = Number of rats selecting RX811059-associated lever / N observations.

Figure 5.1. RX811059 stimulus substitution gradient

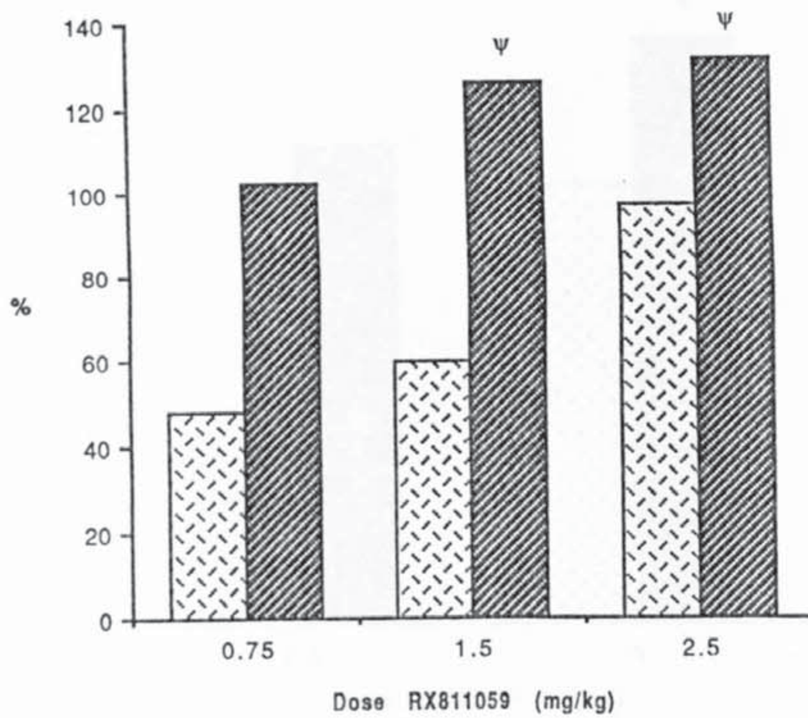
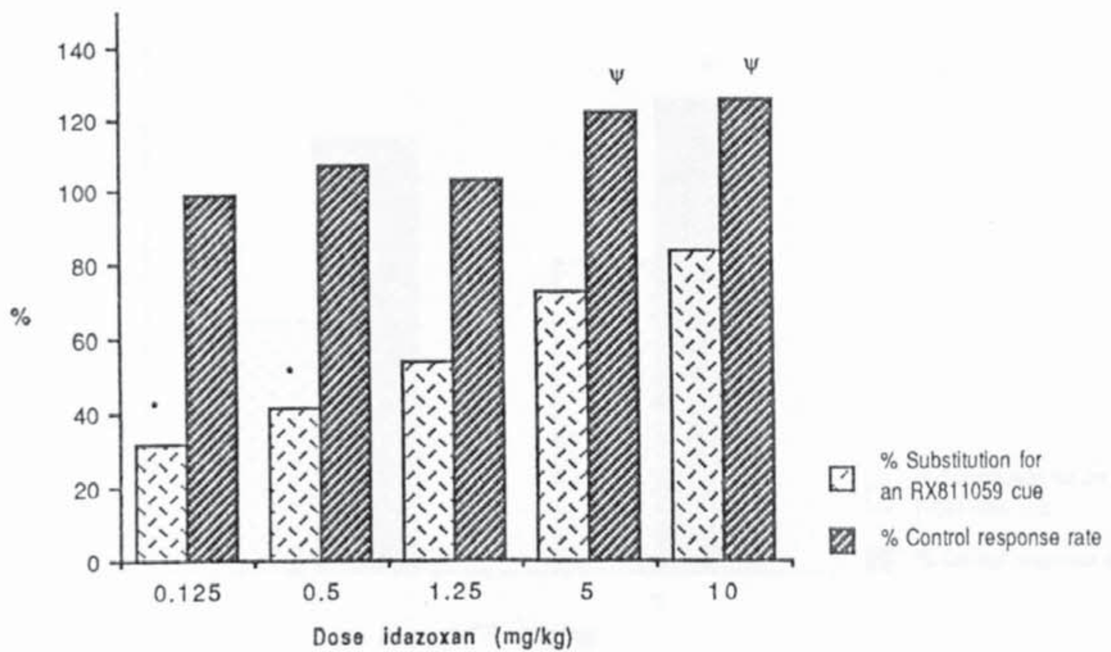


Figure 5.2. The ability of idazoxan to substitute for a 2.5 mg/kg RX811059 cue



*P<0.05 versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e. significant inability to substitute for an RX811059 cue ψP<0.05 versus response rate in previous 0.9% saline control session (Student's paired t-test).

Figure 5.3. The ability of fluparoxan to substitute for a 2.5 mg/kg RX811059 cue

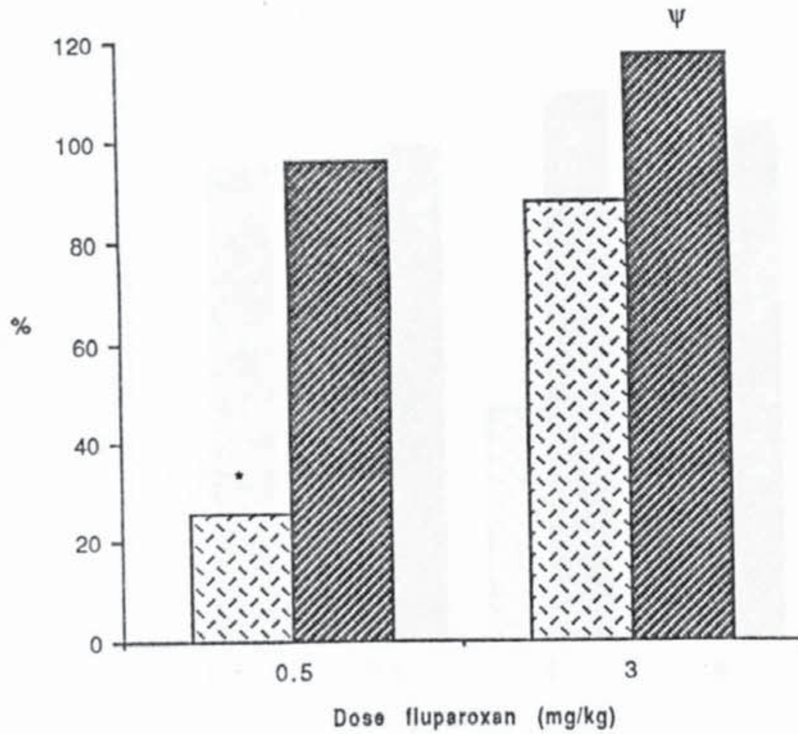
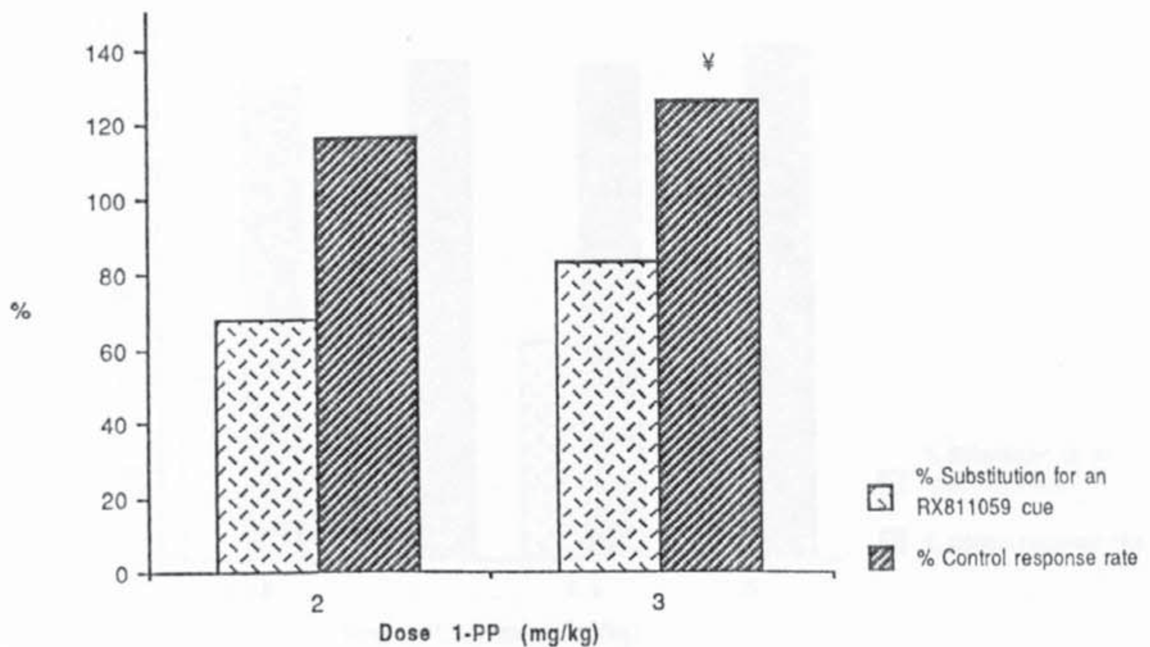


Figure 5.4. The ability of 1-(2-pyrimidinyl)-piperazine to substitute for a 2.5 mg/kg RX811059 cue



*P<0.05 versus previous 2.5 mg/kg RX811059 training session (student's paired t-test), i.e. significant inability to substitute for an RX811059 cue. ¥P<0.05 versus response rate in previous 0.9% saline training session (Student's paired t-test).

Figure 5.5. The ability of yohimbine to substitute for a 2.5 mg/kg RX811059 cue

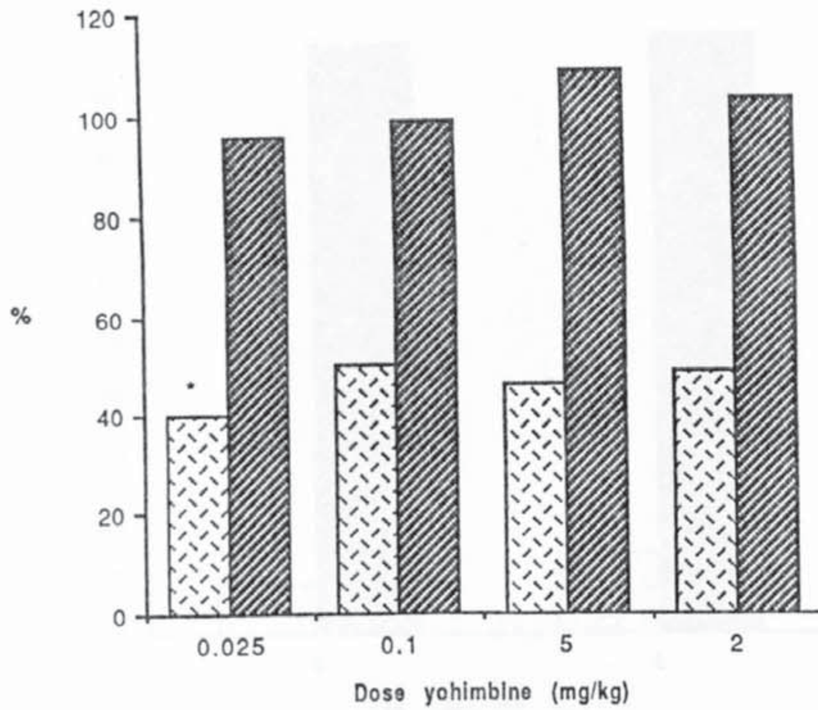
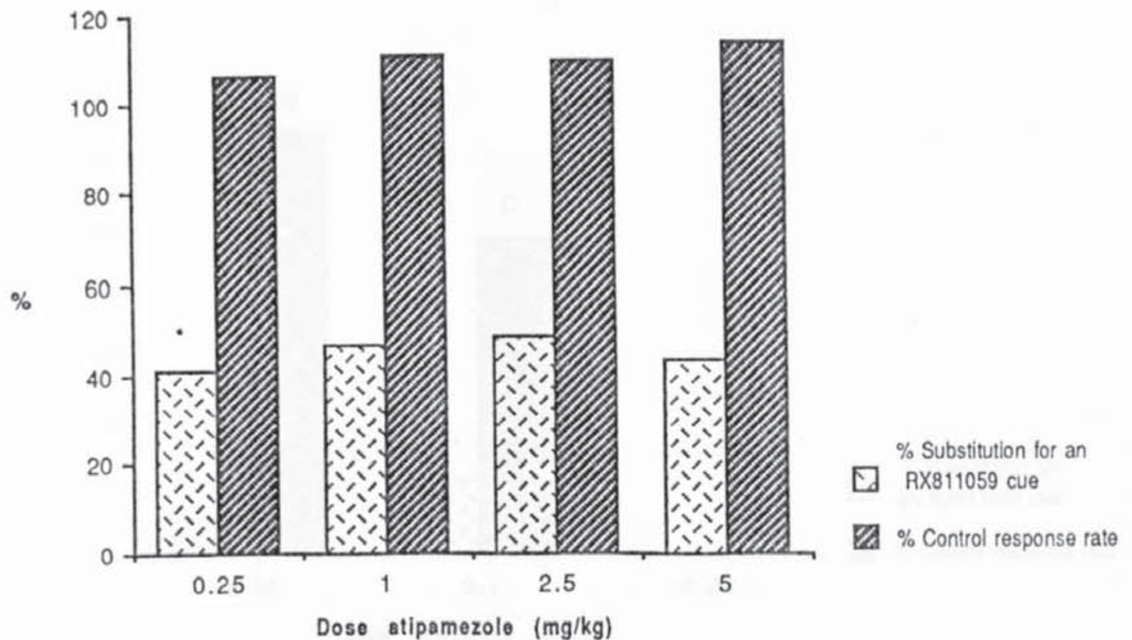


Figure 5.6. The ability of atipamezole to substitute for a 2.5 mg/kg RX811059 cue



*$P<0.05$ versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e. significant inability to substitute for an RX811059 cue.

Figure 5.7. The ability of L659,066 to substitute for a 2.5 mg/kg RX811059 cue

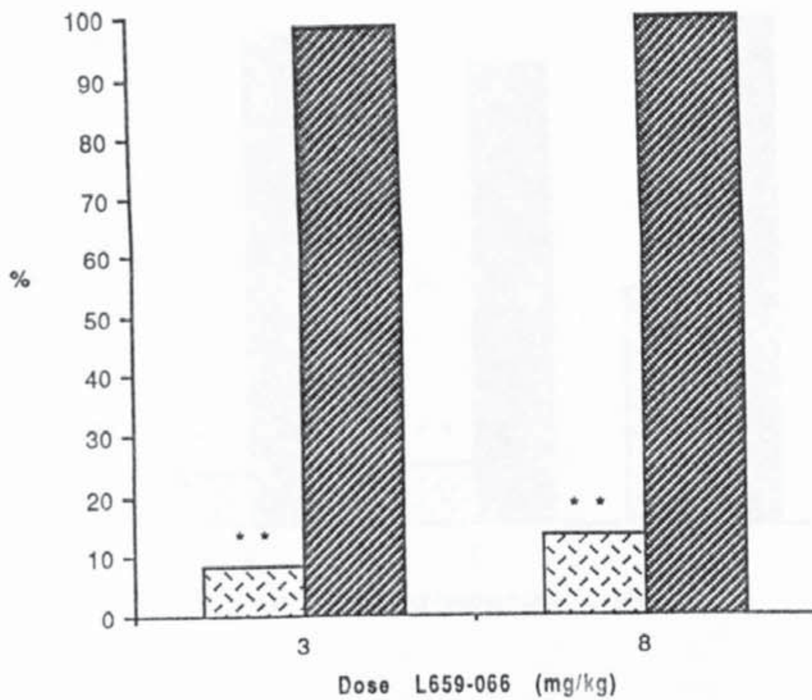
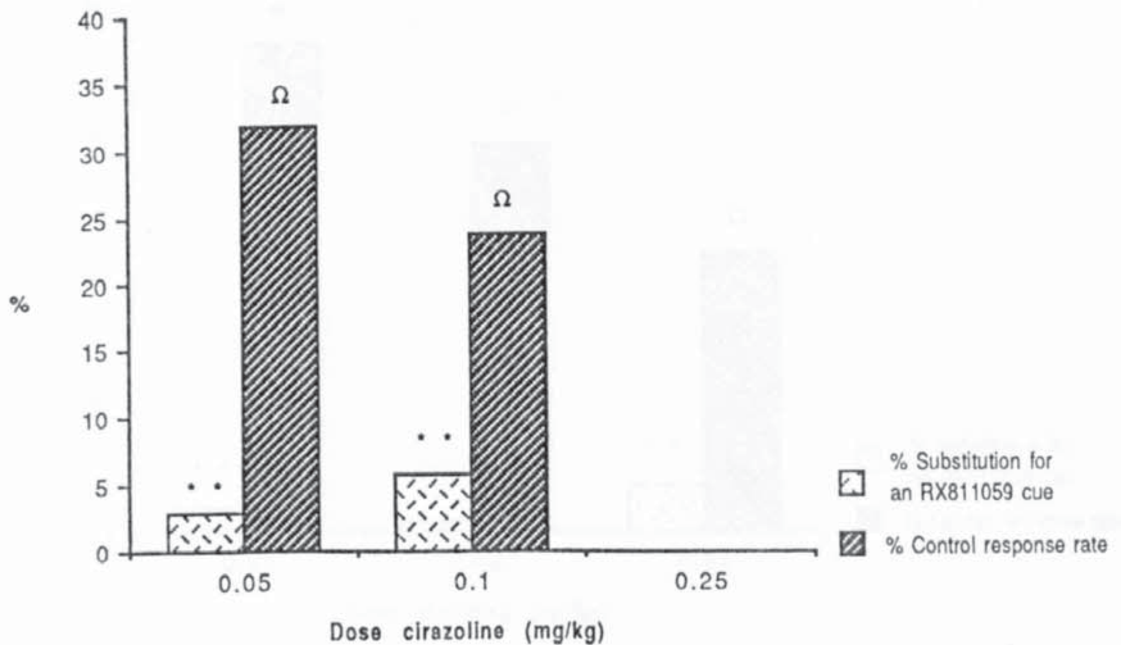


Figure 5.8. The ability of cirazoline to substitute for a 2.5 mg/kg RX811059 cue



**P<0.01 versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e. significant inability to substitute for an RX811059 cue. ΩP<0.005 versus response rate in previous 0.9% saline control training session (Student's paired t-test).

Figure 5.9. The ability of 2-phenyl-2-imidazoline to substitute for a 2.5 mg/kg RX811059 cue

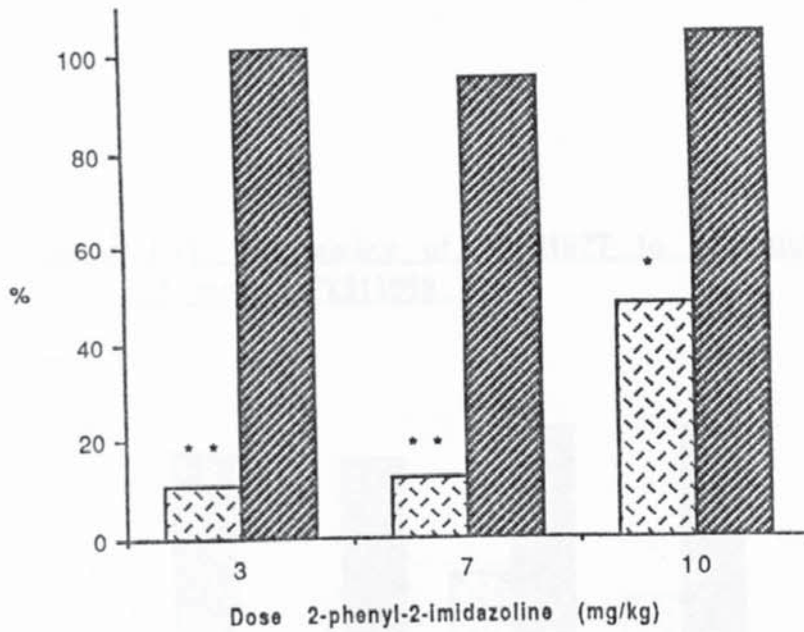
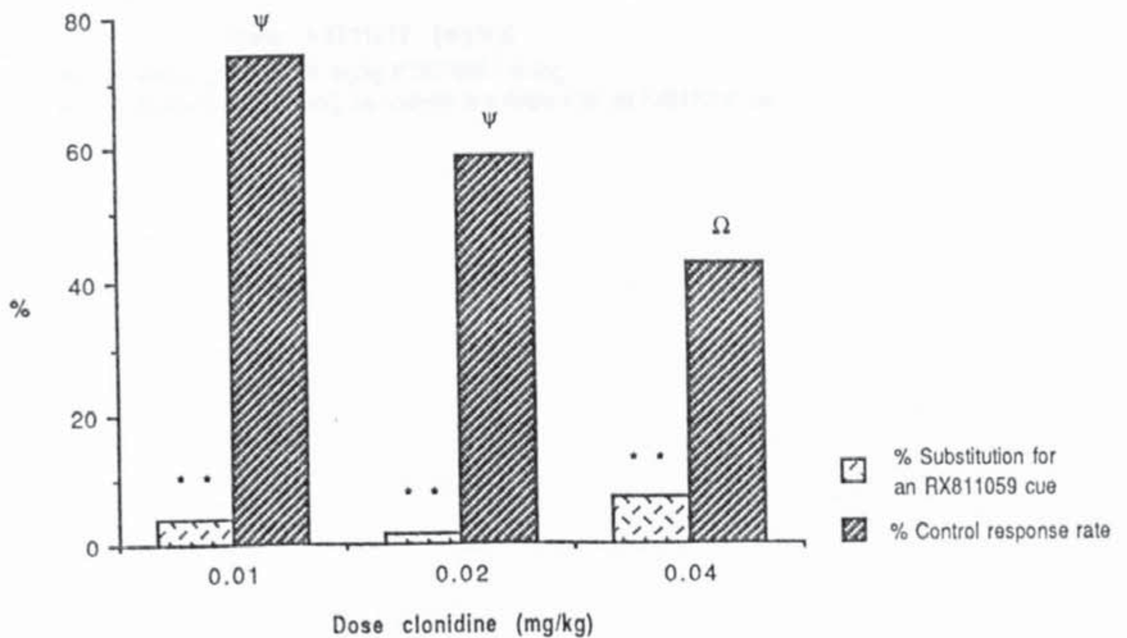
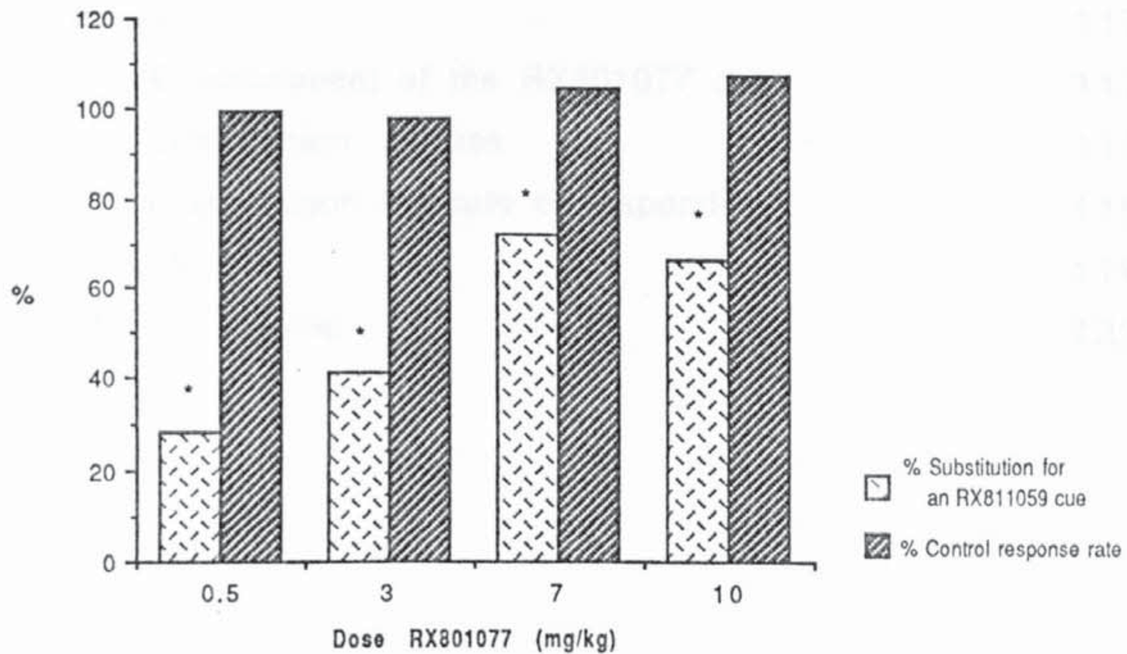


Figure 5.10. The ability of clonidine to substitute for a 2.5 mg/kg RX811059 cue



*P<0.05, **P<0.01 versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e. significant inability to substitute for a clonidine cue. ¥P<0.05, ΩP<0.005 versus previous 0.9% saline control training session (Student's paired t-test).

Figure 5.11. The ability of RX801077 to substitute for a 2.5 mg/kg RX811059 cue



*P<0.05 versus previous 2.5 mg/kg RX811059 training session (Student's paired t-test), i.e. inability to substitute for an RX811059 cue.

Chapter 6. The ability of the highly selective NAIBS compound RX801077 to produce a cue in a rat drug discrimination paradigm.

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Chapter 6

1. Introduction.

The aim of the present study was to examine the ability of the NAIBS selective agent RX801077 to produce a discriminable cue in rats. B910101 is equally selective for this site (A. Hudson - personal communication), and as such it would be expected to completely substitute (i.e. over 80% on the RX801077-associated lever) for an RX801077-induced cue.

Despite its negligible affinity for α2-adrenoceptors (A. Hudson - personal communication), several lines of evidence support a link between RX801077 and central noradrenergic systems. Firstly, by the time these experiments were performed increases in brain extracellular NA levels have been detected in *in vivo* microdialysis studies following injections of both RX801077 (see Chapter 8) and idazoxan (Thomas & Holman, 1991). Secondly, RX801077 partially substituted for (i.e. 20 to 80 % of responding occured on the drug-associated lever) cues produced by the α2-adrenoceptor agents RX811059 and clonidine (see Chapters 4 & 5 respectively), which themselves produce changes in brain extracellular NA levels (see Chapter 8). To examine this relationship further, a variety of α2-adrenoceptor agonists (i.e. clonidine and guanabenz) and antagonists (i.e. RX811059, fluparoxan and idazoxan) were to be tested for their ability to substitute for an RX801077-induced cue. Single doses of these compounds were chosen based on their activity in previous drug discrimination studies (see Chapters 4 & 5).

In addition to this, RX801077 has recently been suggested to act as an MAOI (D.J. Nutt - personal communication), which might

underlie its ability to increase extracellular NA levels in brain (Chapter 8). Indeed, *in vitro* incubation studies have demonstrated this compound to selectively inhibit the MAO-A subtype of this enzyme. However, the location of central NAIBS appears to be more related to the distribution of the MAOI-B subtype (D.J. Nutt - personal communication). A further link has been suggested by the recent demonstration that chronic MAOI treatments are able to down regulate central NAIBS (Olmos et al, 1993). Moclobemide is a reversible short acting MAOI with high specificity for the A-subtype (Burkard et al, 1989), whereas pargyline is an irreversible non-specific (A+B) MAOI. These compounds were used in the present study to investigate a possible contribution of MAOI to any cue produced by RX801077. Doses of these drugs were selected on the basis of their activity in other behavioural experiments (Burkard et al, 1989). 7 mg/kg of RX801077 was chosen as the training dose on the basis of its activity in previous unpublished studies (H.C. Jackson - personal communication).

2. Results.

2.1. Establishment of the RX801077 cue. All 8 rats subjected to training learned to discriminate 7 mg/kg of RX801077 from 0.9% saline in an average of 44 (S.E.M±4) training sessions. Following training, the RX801077-associated lever was 'selected' (i.e. the first lever to recieve 10 presses to complete the session) by all animals receiving 7 mg/kg of RX801077, and no responses occured on the 0.9% saline-associated lever, i.e. 100% substitution for the RX801077-induced cue occured (Table 6.1; Figure 6.1).

2.2. Substitution studies. The RX801077-induced cue was fully substituted for (i.e. over 80% RX801077-associated responding) by RX801077 (7 mg/kg) itself, and by the other NAIBS selective ligand B910101 (7 mg/kg) under test conditions (Table 6.1; Figure 6.1). The RX801077 cue was partially substituted (i.e. between 20 and 80% RX801077-associated responding) for by 3 mg/kg doses of these compounds, although it was unable to be substituted for (i.e. less than 20% RX801077-associated responding) at by 1mg/kg doses (Table 6.1; Figure 6.1). Furthermore, the α2-adrenoceptor antagonists fluparoxan (3 mg/kg), RX811059 (3 mg/kg) and idazoxan (10 mg/kg) completely substituted for the RX801077 cue (Table 6.1; Figure 6.3), as did the MAOI drugs moclobemide (30 mg/kg) and pargyline (30 mg/kg) (Table 6.2; Figure 6.4). In contrast, the α2-adrenoceptor agonists clonidine (0.02 mg/kg) and guanabenz (0.32 mg/kg) failed to substitute for the RX801077 cue, as did the benzodiazepine diazepam (3 mg/kg) (Tables 6.1 & 6.2; Figures 6.3 & 6.4).

2.3. Effects upon the rate of responding. RX801077 and B910101 both significantly increased the rate of responding above that for saline at doses up to 7 mg/kg in each case (Table 6.1; Figure 6.2). Whereas fluparoxan (3 mg/kg) significantly increased the rate of responding, non-significant inhibitory effects were produced by RX811059 (3 mg/kg) and idazoxan (10 mg/kg) (Table 6.1; Figure 6.3). However, significant reductions in the rate of responding were produced by clonidine (0.02 mg/kg), guanabenz (0.32 mg/kg), and diazepam (3 mg/kg) (Table 6.1; Figure 6.3). An inhibitory effect was also produced by both pargyline (30 mg/kg) and moclobemide (30 mg/kg), although only the former drug produced a significant effect.

3. Discussion.

The main finding of the present study is that the NAIBS selective compound RX801077 produces a discriminable cue in rats after about 44 training sessions. Moreover, this cue may be due to an interaction with the NAIBS, as it was completely substituted for by the equally selective ligand B910101. The ability of idazoxan to substitute for the RX801077 cue may also be explicable by its affinity for the NAIBS. However, RX811059 has little affinity for the NAIBS and fluparoxan lacks an imidazoline moiety: yet both compounds completely substituted for the RX801077-induced cue. This raises the possibility that RX801077 could, despite its low affinity for the α2-adrenoceptor, have blocked a sufficient proportion of these receptors at 7 mg/kg to produce its cue by an α2-adrenoceptor related mechanism. This possibility is reinforced by the partial substitution of RX801077 for the RX811059-induced cue (see Chapter 5).

In the light of these results a further study was performed to examine the ability of 7 mg/kg RX801077 to block α2-adrenoceptors. This study tested the ability of RX801077 to block the inhibitory effect of the α2-adrenoceptor agonist clonidine upon the pinna reflex in mice (Brown & Handley, 1980). RX801077 pretreatments were adminstered 5 minutes prior to clonidine and testing began 15 minutes later (see Chapter 2 for methods). The pinna reflex was abolished in 8 out of 9 mice treated with clonidine (0.02 mg/kg, i.p) preceeded by 0.9% saline vehicle. In a group of mice pretreated with RX801077 (7 mg/kg, i.p), clonidine blocked the pinna reflex in all nine mice. Therefore, RX801077 (7 mg/kg) does not appear to act as an α2-adrenoceptor antagonist in the mouse. Further work will be necessary to fully establish that it

does not have any α2-adrenoceptor blocking activities in rats.

The effects which underlie the RX801077-induced cue are as yet uncertain, although the present results suggest a link with NA release. For instance, RX801077 has been demonstrated to produce increases in brain extracellular NA levels by the technique of *in vivo* microdialysis (see Chapter 8). Moreover, this would explain why the RX801077-induced cue was substituted for by the highly selective α2-adrenoceptor antagonists RX811059 and fluparoxan. This result is consistent with the ability of α2-adrenoceptor antagonists to stimulate NA output via an action at presynaptic α2-adrenoceptors (Dennis et al, 1987; Abercrombie et al, 1988). In contrast, the α2-adrenoceptor agonists clonidine and guanabenz, and the benzodiazepine diazepam failed to substitute for the RX801077 cue, which may be due to their inability to increase NA output (Pelayo et al, 1980; Abercrombie & Finlay, 1991). Diazepam was tested as an unrelated compound to ensure that the rats were not responding on the drug lever simply because they detected a 'non-saline' drug-induced stimulus. Conversely, the MAOI compounds moclobemide and pargyline substituted for the RX801077 cue, which could be a consequence of their ability to increase intraneuronal NA levels via an impairment of NA metabolism (Burkard et al, 1989).

Despite its minimal affinity for α2-adrenoceptors (see Chapter 1), RX801077 partially substituted for an RX811059-induced cue (Chapter 5). Therefore, the RX801077-induced cue may be due to an increase in NA output due to an action at the NAIBS and/or the α2-adrenoceptor. However, RX801077 also partially substituted for the clonidine cue and clonidine reduces NA release (Pelayo et al, 1980). This discrepancy might possibly be accounted for if RX801077 increases NA release onto postsynaptic α2-

adrenoceptors. In the present experiment, however, clonidine did not substitute for RX801077. Further work, for instance on the ability of agents such as moclobemide or DMI to substitute for clonidine would be needed to investigate this aspect more fully.

Increases in NA output might also underlie the increased response rates produced by the NAIBS selective ligands RX801077 and B910101. This effect in turn may contribute to the discriminable cue. However, pargyline inhibited the rate of responding despite its ability to increase intraneuronal NA levels. The rate of responding was also increased by fluparoxan, although the other α2-adrenoceptor antagonists RX811059 and idazoxan non-significantly reduced the rate of responding. RX811059, idazoxan and fluparoxan significantly increased the rate of responding (see Chapter 5), however, in the present work this effect was only produced by the latter compound. The reason for this discrepancy remains to be determined.

The sedative effects of clonidine (Drew et al, 1979) and guanabenz (Heal et al, 1989) may be responsible for their inhibitory effect upon the rate of responding. Similarly, the response rate inhibition produced by diazepam supports previous findings for this drug which attributed this effect to sedation (Yang et al, 1988). In fact, the sedative effect of benzodiazepines may be related to reduced NA turnover (Wise et al, 1972; Yang et al, 1988).

In conclusion, RX801077 produces a discriminable cue in rats, which may be due to its ability to increase brain NA levels via an interaction with the NAIBS. The presence of a discriminable cue suggests a functional role for the NAIBS, although the product of this interaction which serves as its discriminable cue remains uncertain.

Table 6.1. The ability of a variety of α2-adrenoceptor and NAIBS compounds to substitute for an RX801077 (7 mg/kg, i.p) induced cue.

Test drug	Dose (mg/kg)	Mean % substitution for an RX801077 cue ±SEM	[1]n / N	Mean response rate (responses/minute ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	0	0 / 8	3.5±0.8	3.4±1.2	97.1
RX801077	1	4.9±1.2**	0 / 8	3.2±0.3	3.5±1.7	109.4
RX801077	3	52.1±18.2*	4 / 8	3.5±0.2	3.6±0.4	102.9
RX801077	7	100	8 / 8	5.3±1.0	6.5±1.8	122.6¥
B910101	1	6.2±2.2**	0 / 8	5.2±0.4	4.9±0.7	94.2
B910101	3	56.4±14.9	4 / 8	5.7±0.5	5.0±0.9	87.7
B910101	7	95.8±2.7	8 / 8	5.3±0.6	7.2±1.3	135.8¥
Clonidine	0.02	6.4±2.6**	0 / 8	4.2±0.9	2.7±0.5	64.3¥
Guanabenz	0.32	5.8±3.6**	0 / 8	5.2±1.1	2.9±0.5	55.8¥
RX811059	3.0	97.8±1.5	8 / 8	4.1±1.3	3.7±0.7	90.2
Fluparoxan	3.0	91.6±3.2	8 / 8	4.1±1.0	6.8±1.6	165.9†
Idazoxan	10.0	100	8 / 8	4.0±0.9	3.4±0.4	85.0

All values are means of N = 6 observations. *P<0.05; **P<0.01 versus previous 7 mg/kg RX801077 training session (Student's paired t-test), i.e inability to substitute for an RX801077 cue. ¥P<0.05, †P<0.01 versus response rate in previous 0.9% saline control training session (Student's paired t-test).[1]n / N = Number of rats selecting RX801077-associated lever / N observations.

Table 6.2. The ability of moclobemide, pargyline and diazepam to substitute for an RX801077 (7 mg/kg, i.p) induced cue.

Test drug	Dose (mg/kg)	Mean % substitution for an RX801077 cue ±SEM	[1]n / N	Mean response rate (responses/minute ± SEM) Control training session	Test session	% Control response rate
0.9% Saline	-	0	0 / 8	4.5±0.8	3.4±1.2	97.1
Moclobemide	5	21.2±6.9	1 / 8	5.3±0.4	4.4±0.6	83.0
Moclobemide	30	92.6±5.0	8 / 8	4.9±0.7	4.2±0.5	85.7
Pargyline	30	81.2±8.9	7 / 8	5.3±0.3	2.6±0.5	49.1†
Diazepam	3.0	6.4±2.6**	0 / 8	5.5±0.9	1.2±0.1	21.8†

All values are means of N = 6 observations. *P<0.05; **P<0.01 versus previous 7 mg/kg RX801077 training session (Student's paired t-test), i.e inability to substitute for an RX801077 cue. ¥P<0.05, †P<0.01 versus response rate in previous 0.9% saline control training session (Student's paired t-test). [1]n / N = Number of rats selecting RX801077-associated lever / N observations.

Figure 6.1. Ability of the NAIBS selective ligands RX801077 and B910101 to substitute for an RX801077 (7mg/kg, i.p)-induced cue.

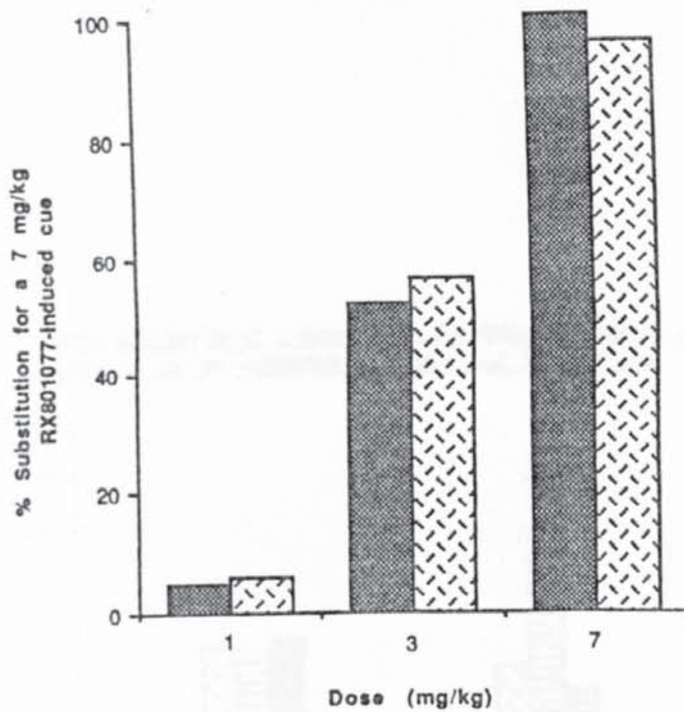
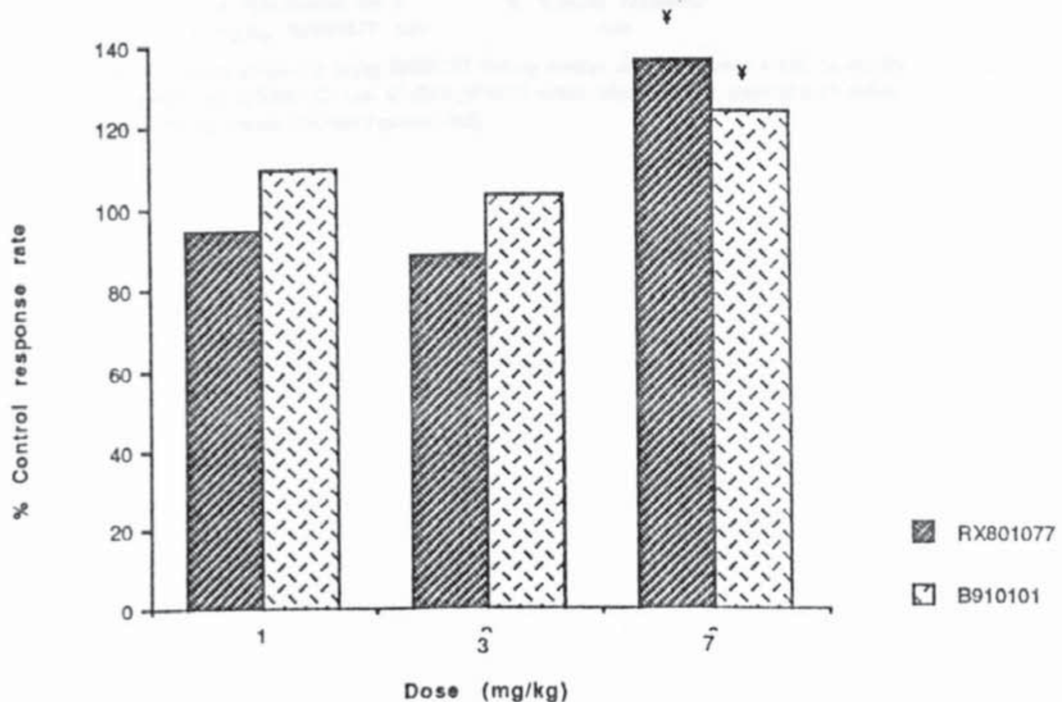
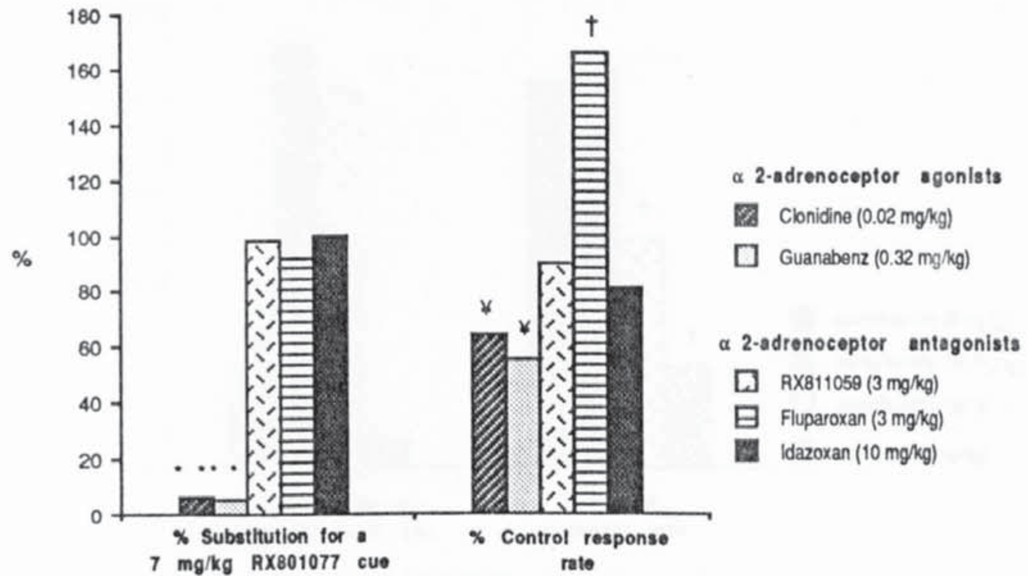


Figure 6.2. Effect of RX801077 and B910101 upon response rate (responses/minute) when compared with a 0.9% saline control.



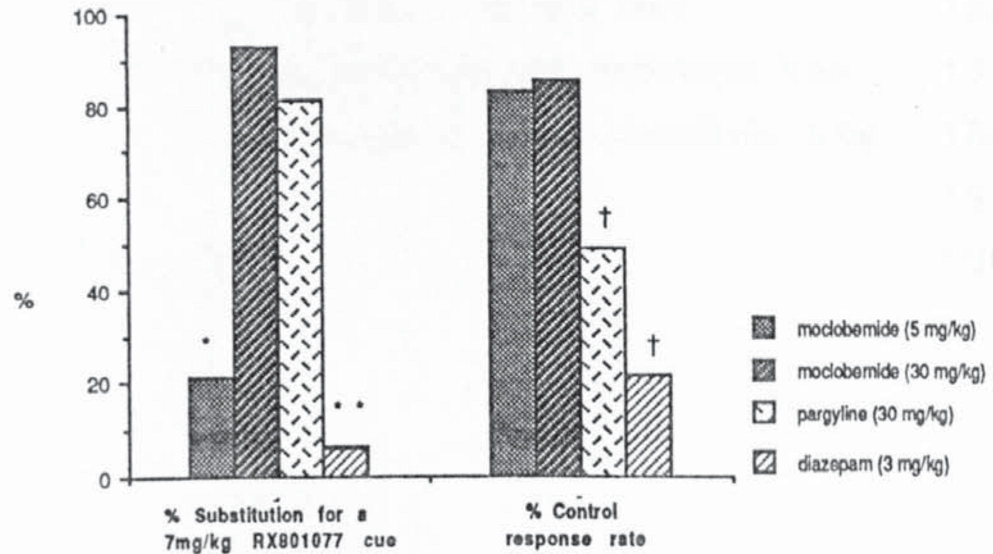
*P<0.05, **P<0.01 versus previous 7 mg/kg RX801077 training session, i.e. significant inability to substitute for the RX801077 cue (Student's paired t-test). ¥ P<0.05 versus previous 0.9% saline control training session (Student's paired t-test).

Figure 6.3. Ability of a variety of α 2-adrenoceptor agents to substitute for an RX801077 (7 mg/kg, i.p) induced cue.



**P<0.01 versus previous 7 mg/kg RX801077 training session (Student's paired t-test), i.e inability to substitute for an RX801077 cue. ¥P<0.05, †P<0.01 versus response rate in previous 0.9% saline control training session (Student's paired t-test).

Figure 6.4. Ability of moclobemide, pargyline, and diazepam to substitute for an RX801077 (7 mg/kg, i.p) induced cue.



*$P<0.05$, **$P<0.01$ versus previous 7 mg/kg RX801077 training session, i.e. significant inability to substitute for the RX801077 cue (Student's paired t-test). †$P<0.01$ versus previous 0.9% saline control training session (Student's paired t-test).

Chapter 7. The effect of RX801077, RX811059, idazoxan and desipramine in a rat forced swimming model of depression.

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Chapter 7

1. Introduction.

A wide range of compounds have proved to be useful in the treatment of human depression (see Nutt & Glue, 1989). However, a common mechanism responsible for the therapeutic efficacy of these functionally dissimilar agents does not appear to exist. The monoamine theory of depression (Schildkraut, 1965) associates this disorder with low levels of brain NA and/or 5-HT. Therefore increases in noradrenergic and/or serotonergic activity may reverse the disturbance in monoamine levels responsible for depressive states. Chapter 1 (section 1.5.2) describes the symptoms, and explains the mechanisms believed to underlie depressive states.

The forced swimming test has been proposed to induce a depressive state in animals which is characterised by an immobile posture (Porsolt et al, 1978). Animals forced to swim make an initial frenzied attempt to escape followed by an immobile posture. This state has been named "behavioural despair"; it is assumed that the animal has "given up hope of escaping" (Porsolt et al, 1977a, 1978). This "behavioural despair" seems to represent an inability or reluctance to maintain the effort of attempting to escape (Willner, 1984). Furthermore, depressed human subjects have been found to show their most pronounced psychomotor impairments in tests which require the sustained expenditure of effort (Weingartner & Silberman, 1982).

The total period of immobility is greater in a subsequent swim 24 hours after swim 1 (Porsolt et al, 1977b). Furthermore, total immobility time in swim 2 can be reduced by pretreatment with a variety of antidepressant therapies including tricyclics (eg.

imipramine and DMI), MAOI's (eg. clorgyline and deprenyl), atypical antidepressants (eg. iprindole and mianserin), ECS and REM sleep deprivation (Borsini et al, 1981; Porsolt et al, 1977b; Porsolt et al, 1978; Finnegan et al, 1987; Porsolt, 1981; Kitada et al, 1981). The ability of the forced swimming test to discriminate between antidepressant and non-antidepressant compounds is discussed in Chapter 1 (section 1.8.2.1).

The therapeutic efficacy of antidepressants becomes evident after 2-3 weeks of treatment. In the forced swimming test sub-chronic (2 or 3 injections over 24 hours) dosing schedules are consistently more effective than single or even chronic (3 intermittent injections over several weeks) antidepressant drug schedules (eg. Borsini et al, 1981; Porsolt et al, 1978; Kitada et al, 1981; review - Borsini & Meli, 1988).

Immobility appears to be under the control of noradrenergic neurons as a variety of drugs able to modulate NA output reduce immobility time. For instance, the tricyclic antidepressant DMI (10-20 mg/kg sub-chronic doses) has been demonstrated to reduce immobility by up to 70% (Borsini et al, 1981; Kitada et al, 1981; Porsolt et al, 1977b). The mechanism responsible for this action is currently uncertain, although DMI increases synaptic NA levels (Langer et al, 1981) as a consequence of its preferential blockade of NA reuptake mechanisms (Schubert et al, 1970). Alternatively, the anti-immobility effect of DMI may be due to its ability to down regulate cortical β-adrenoceptors (see Stanford, 1990). However, this effect is only apparent after seven daily 10 mg/kg treatments (Molina et al, 1989).

Highly selective α2-adrenoceptor antagonists may be useful in the treatment of clinical depression with respect to their ability to increase NA output (Dickinson, 1991) via presynaptic α2-

adrenoceptor inhibition (Langer, 1981). In a preliminary clinical study Montgomery (1988) reported an antidepressant effect following chronic idazoxan treatment (3 daily administrations over 4 weeks). Moreover, an anti-immobility effect has been reported for the α2-adrenoceptor antagonist yohimbine (Porsolt et al, 1979), which stimulates the firing of noradrenergic neurons (Marwaha & Aghajanian, 1982). Nevertheless, this compound also interacts with α1-adrenoceptors, DA and 5-HT receptors which may mediate its anti-immobility effect (Scatton et al, 1980; Goldberg & Robertson, 1983). The more selective α2-adrenoceptor antagonist idazoxan is inactive under the same conditions (Cervo et al, 1990), although this compound also binds with high affinity to the NAIBS (Michel & Insel, 1989). It is therefore possible that the interaction of idazoxan with the NAIBS could mask an anti-immobility effect due to α2-adrenoceptor antagonism. The highly selective α2-adrenoceptor antagonist RX811059, which has minimal NAIBS affinity (Mallard et al, 1991), may be used to examine this possibility. Moreover, idazoxan attenuates DMI's anti-immobility effect (Cervo & Samanin, 1991), this attenuation may also involve an action at the NAIBS or the α2-adrenoceptor.

In the present study RX801077 and B910101 were investigated for their effects upon immobility time in a second forced swimming test, using DMI as a reference compound. The α2-adrenoceptor antagonists idazoxan and RX811059 (minimal NAIBS affinity; Mallard et al, 1991) were also tested in an attempt to distinguish between the effects of the NAIBS and the α2-adrenoceptor upon immobility. A sub-chronic dosing schedule (i.e. drugs adminstered 0.5, 19 and 23 hours after swim 1, second swim at 24 hours) was used throughout the present studies as repeated administrations appear to be necessary for DMI to exert its anti-

immobility effect (Borsini et al, 1984; Kitada et al, 1986). Moreover, drugs were administered over a 24 hour period to avoid any down-regulation of β-adrenoceptors (Molina et al, 1989). Doses of each test compound were selected on the basis of their ability to increase brain extracellular NA levels (see Chapter 8). Observations of immobility during three periods (0-5, 3-8 and 0-8 minutes) of each 8 minute swimming session enabled the time course of each drug effect to be determined.

2. Results.

2.1. The effect of DMI upon immobility time. DMI (15 mg/kg) significantly reduced the total time rats remained immobile during each observation period (i.e. 0-5, 3-8 and 0-8 minutes) of a second 8 minute forced swim (Table 7.1; Figure 7.1).

2.2. NAIBS selective compounds and immobility time. RX801077 (3 & 10 mg/kg) significantly reduced total immobility time in each observation period (Table 7.1; Figure 7.1). However, this effect was only significant with a 10 mg/kg dose of B910101 during the first 5 minutes of observation. Lower doses of B910101 (3 & 7 mg/kg) non-significantly reduced immobility time (mg/kg; Table 7.1; Figure 7.2).

2.3. α2-adrenoceptor antagonists and immobility time. The α2-adrenoceptor antagonist idazoxan (3 mg/kg) produced a significant reduction in total immobility time during the final 5 minutes of swimming (Table 7.1; Figure 7.4). However, during the other time bins (i.e. 0-5 and 0-8 minutes) the same dose produced trends towards anti-immobility effects which were non-significant. The highly selective α2-adrenoceptor antagonist RX811059 (0.3, 1 & 3 mg/kg) also produced only non-significant trends towards reductions in immobility (Table 7.1; Figure 7.3), as did idazoxan at 1 & 10 mg/kg (Table 7.1; Figures 7.3 & 7.4).

3. Discussion.

The highly selective NAIBS agent RX801077 and the tricyclic antidepressant DMI both significantly reduced immobility time using the same sub-chronic dosing schedule. The anti-immobility effect produced by the tricyclic antidepressant DMI in the present study confirms previous findings with similar doses of this drug (Borsini et al, 1981; Porsolt et al, 1977b). However, in contrast to RX801077, a significant effect was observed only with the highest dose of the NAIBS selective compound B910101 during the first 5 minutes of swimming.

Idazoxan significantly reduced immobility at a single dose during the latter 5 minutes, whereas RX811059 produced non-significant anti-immobility effects at all doses. Since the action of idazoxan resembled that of B910101 its anti-immobility effect might be due to its high affinity for the NAIBS (Michel & Insel, 1989). The lack of significant effects of RX811059 introduce the possibility that the anti-immobility effect of idazoxan is due to its NAIBS affinity and not due to its ability to block α2-adrenoceptors. On the other hand, idazoxan's anti-immobility effect may be due to its ability to increase locomotor activity in this laboratory (Dickinson et al, 1990).

Drugs which increase synaptic NA content appear to reduce immobility time (Porsolt et el, 1979), although in the present study the highly selective α2-adrenoceptor antagonist RX811059 failed to significantly reduce immobility time at doses which increase extracellular NA content in brain (see Chapter 8). However, an increase in the number of α2-adrenoceptors present in the brain have been reported after a period of acute stress (i.e. electrofootshock or restraint stress; see Stanford, 1990). Such

changes may have occurred in the present study, as a consequence of the initial forced swimming exposure, which could have attenuated an effect of RX811059 upon NA output and thus immobility time.

The α2-adrenoceptor agonist clonidine has also been reported to decrease immobility time (Cervo & Samanin, 1991). Therefore, it could be argued that α2-adrenoceptor antagonists ought to increase immobility time. Indeed, such increases may be masked by the ability of RX811059 to increase locomotor activity, as previously demonstrated in this laboratory (Dickinson et al, 1990). The relationship between immobility time and motor activity therefore needs further investigation. Borsini & Meli (1988) suggested a possible role for different brain regions in mediating motor responses in a cage or in water. For instance, amphetamine-induced hypermotility is prevented by electrolytic lesions of rat amygdala (Allikmets et al, 1967), although imipramine-induced activity is unaffected (Gorka et al, 1979).

· The relatively smaller saline control scores in the idazoxan and RX811059 studies might underlie their apparent inability to both significantly reduce immobility. In retrospect, the inclusion of a positive control group (eg. DMI) in each experiment would have assisted interpretation. Lower immobility scores appeared to occur during the initial 5 minutes of each test. This observation is in agreement with the proposed existence of a period of struggling prior to the onset of immobility (Porsolt et al, 1977b).

In conclusion, sub-chronic treatments with the tricyclic antidepressant DMI and the NAIBS selective agent RX801077 both reduced immobility time during a second forced swimming period. The mechanism responsible for the antidepressant-like effect of RX801077 is uncertain, although increases in neuronal NA levels

may be involved. Further studies with RX801077 are therefore required to examine a possible role for the NAIBS in the treatment of depression.

Table 7.1. Effects of various compounds, with differing selectivity profiles for the α2 adrenoceptor, IPR and the NAIBS, upon total immobility time during 0-5, 3-8 and 0-8 minutes of a second 8 minute forced swimming test (n=8 per treatment). A sub-chronic dosing schedule (0.5, 19 & 23 hours after the first swimming period) was used for all drugs.

DRUG	DOSE (mg/kg)	MEAN DURATION OF IMMOBILITY (seconds ± S.E.M) 0-5 mins	3-8 mins	0-8 mins
0.9% saline	-	62.0±5.8	150.9±18.6	178.3±19.0
DMI	15	20.0±7.0* (32%)	58±11.5* (38%)	71.4±15.0* (40%)
RX801077	3	35.8±4.6* (58%)	88.7±12.7* (59%)	113±16.4 (64%)
	10	34.8±7.3* (56%)	92.4±18.7* (61%)	111.6±21.9* (63%)
		$F_{3,27}$=7.664, P=0.0004	$F_{3,26}$=5.788, P=0.0036	$F_{3,26}$=5.68, P=0.004
0.9% saline	-	103.4±21.3	91.0±18.5	119.3±21.0
B910101	3	69.4±10 (67%)	69.4±9.4 (76%)	86.9±12.4 (73%)
	7	64.6±11.3 (62%)	74±10.4 (81%)	89.6±11.7 (75%)
	10	51.6±8.5* (50%)	64.9±17.7 (71%)	83.7±18.8 (70%)
		$F_{3,25}$=2.566, P=0.0772	$F_{3,25}$=0.61, P=0.6149	$F_{3,25}$=1.002, P0.4082
0.9% saline	-	36.5±10.5	62.0±16.5	73.0±18.6
RX811059	0.3	25.4±8.2 (70%)	27.9±10.4 (45%)	40.8±13.7 (56%)
	1	34±5.8 (93%)	33.4±8.9 (54%)	50.6±10.3 (69%)
	3	36.4±7.7 (99.7%)	50.1±5.8 (91%)	67.6±8.8 (93%)
		$F_{3,28}$=0.408, P=0.748	$F_{3,28}$=1.98, P=0.141	$F_{3,28}$=1.243, P=0.3129
0.9% saline	-	43.0±8.1	66.17±14.9	78.67±18.1
Idazoxan	1	31.3±6.5 (73%)	34.3±8.0 (52%)	52.8±10.5 (67%)
	3	25.6±10.9 (60%)	25.4±6.6* (38%)	40.1±11.7 (51%)
	10	39.9±8.3 (93%)	43.4±8.6 (66%)	69.4±12.8 (88%)
		$F_{3,27}$=0.835, P=0.0004	$F_{3,26}$=5.788, P=0.0036	$F_{3,26}$=5.68, P=0.004

Values in parentheses represent immobility time as a % of saline control. *P<0.05, versus 0.9% saline control (one-way analysis of variance (ANOVA) and Dunnett's t-test).

Figure 7.1. Effect of DMI (15 mg/kg, ip) and RX801077 (3 & 10 mg/kg, ip) upon total immobility time during 0-5, 3-8 and 0-8 minutes of a second 8 minute forced swimming test (values are means where n=8 per treatment).

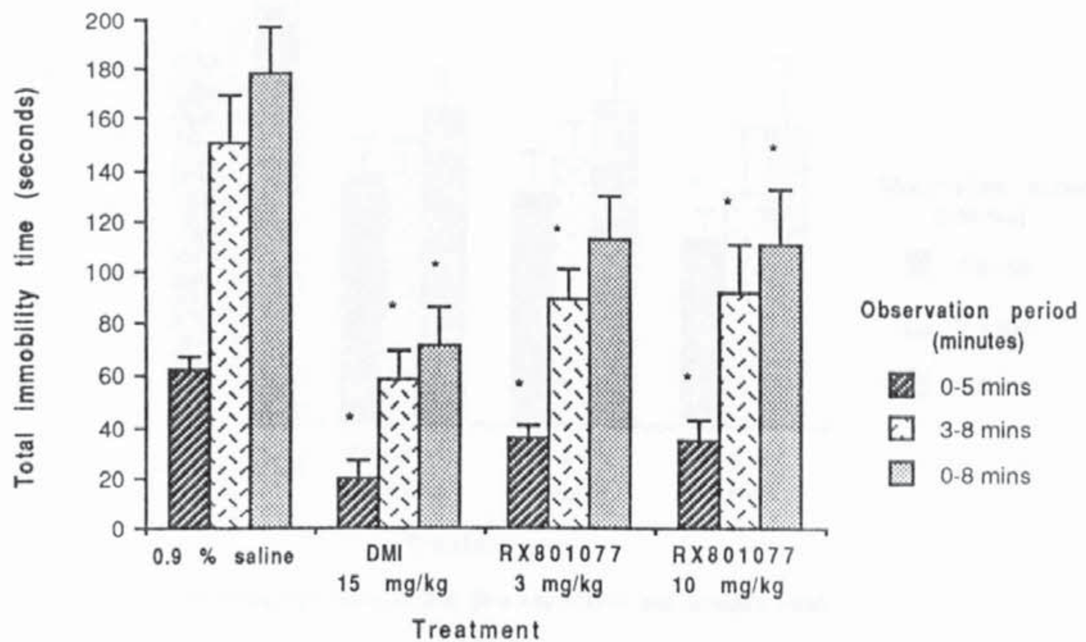
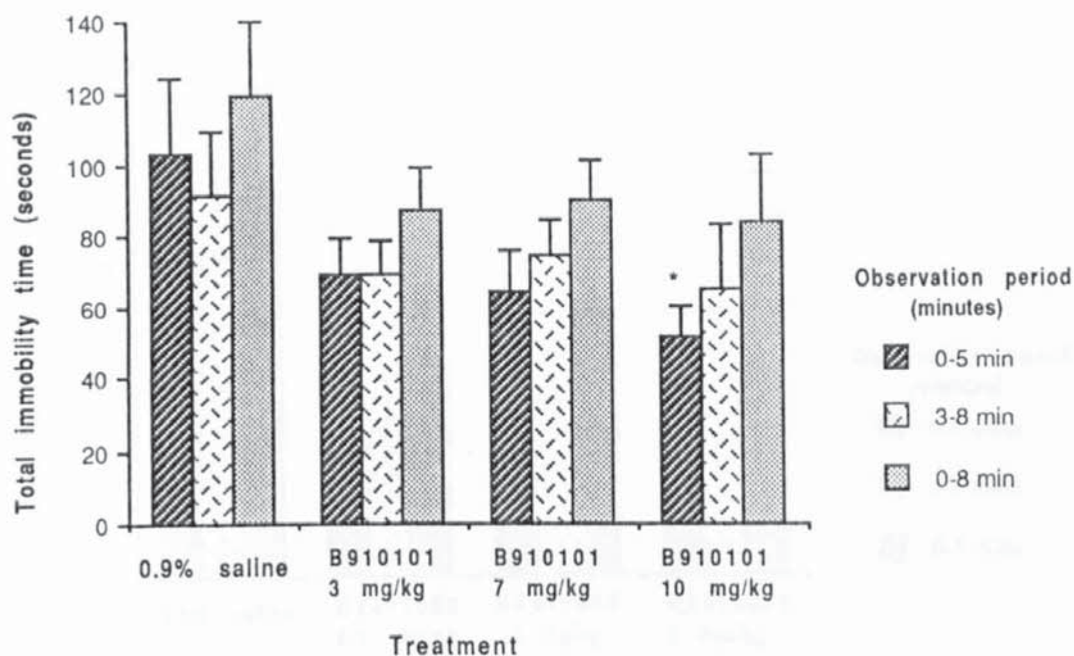


Figure 7.2. Effect of B910101 (3, 7 & 10 mg/kg, ip) upon total immobility time during 0-5, 3-8 and 0-8 minutes of a second 8 minute forced swimming test, values are means where n=8 per treatment.



*P<0.05 versus 0.9% saline control (one-way ANOVA and Dunnett's t-test).

Figure 7.3. Effect of RX811059 (0.3, 1 and 3 mg/kg, ip) upon total immobility time during 0-5, 3-8 and 0-8 minutes of a second 8 minute forced swimming test. values are means where n=8 per treatment.

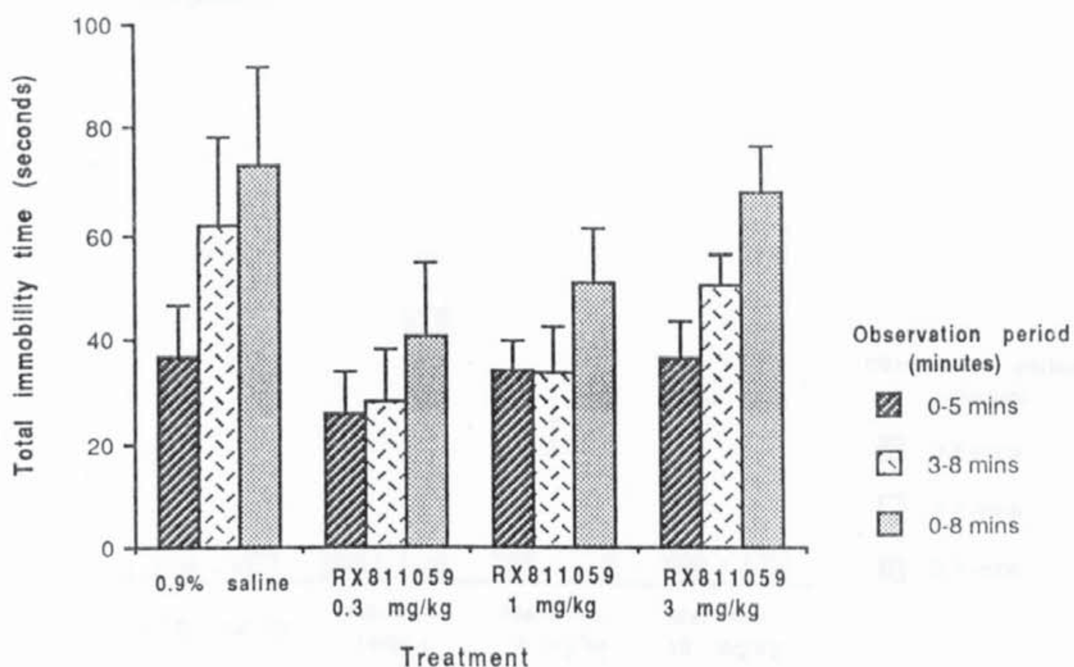
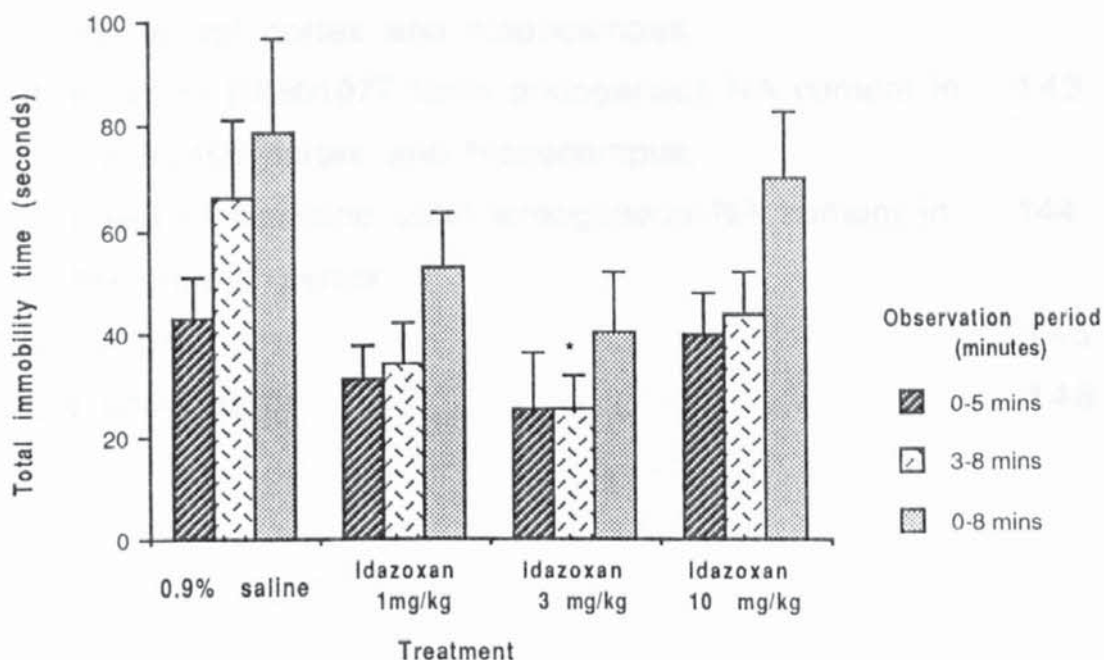


Figure 7.4. Effect of idazoxan (1, 3 & 10 mg/kg, ip) upon total immobility time during 0-5, 3-8 and 0-8 minutes of a second 8 minute forced swimming test, values are means where n=8 per treatment.



*P<0.05 versus 0.9% saline control (one-way ANOVA and Dunnett's t-test)

Chapter 8. *In vivo* brain microdialysis: the effect of α2-adrenoceptor and NAIBS selective compounds upon endogenous noradrenaline release in rat hippocampus and frontal cortex

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CHAPTER 8

1. Introduction.

The technique of *in vivo* microdialysis has been used to determine the effect of selective α2-adrenoceptor antagonists such as idazoxan (Doxey et al, 1984) upon extracellular NA levels in rat frontal cortex and hippocampus (Dennis et al, 1987; Holman & Thomas, 1991). In these studies systemic idazoxan increased NA output in samples from rat frontal cortex but not hippocampus. In addition, NA levels were elevated in both regions when the NA reuptake inhibitor DMI was administered into either region via a microdialysis probe. This latter effect was potentiated by systemic idazoxan in both the frontal cortex and hippocampus. These studies suggest that the regulation of extracellular NA in the frontal cortex appears to be mainly due to activation of presynaptic α2-adrenoceptors; whereas, in the hippocampus reuptake seems to be the primary effector mechanism (Dennis et al, 1987; Holman & Thomas, 1991). This hypothesis was tested further in this study by using the more selective α2-adrenoceptor antagonist RX811059 (Mallard et al, 1991) alone and in combination with DMI. The DMI was delivered via the dialysis probe to restrict its delivery to the brain region of interest. In addition, the ability of clonidine to mimic the autoinhibitory effect of the endogenous α2-adrenoceptor agonist NA was examined. The highly selective NAIBS compound RX801077 was also tested alone and in combination with DMI for its effects upon basal NA content in the frontal cortex. A comparison between basal NA content in conscious and anaesthetised rats was carried out in an attempt to determine the effect of an anaesthetic upon endogenous release.

2. Results.

2.1. Basal NA content in frontal cortex and hippocampus.

Similar basal NA concentrations were observed in samples obtained from the frontal cortex and hippocampus of anaesthetised rats (Table 8.1), which remained stable following 0.9% saline control inections (Tables 8.1). However, in the presence of DMI (5μM) continuously infused via the dialysis probe, significantly higher basal concentrations occured in the frontal cortex and hippocampus (Table 8.1). In addition, basal NA content in both regions was unaffected by 0.9% saline control injections (Table 8.1). Furthermore, no statistical difference existed between basal NA output in conscious and anaesthetised rats (Student's unpaired t-test; Table 8.1 versus Table 8.2).

2.2. Effect of RX811059 upon endogenous NA content in the frontal cortex and hippocampus. The highly selective α2-adrenoceptor antagonist RX811059 (3 mg/kg, ip) significantly increased the NA content of samples from the frontal cortex for 45 minutes after injection, although basal levels in the hippocampus remained stable (Figures 8.1 &8.3). However, in the presence of DMI (5μM) hippocampal NA was more than doubled during the first 30 minutes following systemic RX811059 (3 mg/kg, ip; Figure 8.7).

2.3. Effect of RX801077 upon endogenous NA content in the frontal cortex and hippocampus. The highly selective NAIBS compound RX801077 (10 mg/kg) did not significantly increase basal NA content in either the frontal cortex or hippocampus (Figures 8.2 & 8.4), although a strong trend towards such an effect was observed in frontal cortex. However, in the

presence of DMI (5μM) NA levels in the frontal cortex and hippocampus were doubled during the 90 minutes following systemic RX801077 (10 mg/kg; Figures 8.5 & 8.8).

2.4. Effect of clonidine upon endogenous NA content in the frontal cortex. In the presence of DMI the α2-adrenoceptor agonist clonidine (0.02 mg/kg, ip) significantly decreased NA content in frontal cortex samples during the second and third 15 minute periods following its injection (Figure 8.6).

3. Discussion.

In the present studies similar basal NA concentrations were recorded in samples from the frontal cortex of anaesthetised and conscious rats. However, previous studies report basal NA levels to be higher in the conscious rat, which may suggest anaesthetics to inhibit noradrenergic activity (Dennis et al, 1987; Thomas & Holman, 1991). Basal NA content was increased in the frontal cortex and hippocampus in the presence of DMI. Furthermore, RX811059 and RX801077 significantly elevated NA concentrations in the frontal cortex and hippocampus, although these effects were only observed in the presence of DMI. In contrast, clonidine significantly inhibited basal NA content in the frontal cortex, which has been reported elsewhere (Abercrombie & Finlay, 1991).

The ability of DMI to maintain elevated NA baselines in the frontal cortex and hippocampus confirms its inhibitory action upon NA reuptake mechanisms (Langer et al, 1981), which leads to an increase in extracellular NA. In the hippocampus an effect of presynaptic $\alpha 2$-adrenoceptor blockade with RX811059 was only evident when NA reuptake mechanisms were blocked with DMI. These results are in agreement with the proposal that the effect of an $\alpha 2$-adrenoceptor antagonist will depend on the tonic input to the $\alpha 2$-adrenoceptors, i.e the concentration of the endogenous NA already available in the synaptic cleft (Dennis et al, 1987). Furthermore, the present findings support the suggestion that in the frontal cortex presynaptic $\alpha 2$-adrenoceptor activation appears to be the primary NA regulatory mechanism; whereas NA reuptake seems to be more important in the hippocampus (Dennis et al, 1987; Thomas & Holman, 1991). This difference may be due to biochemical differences between the hippocampus and frontal

cortex. For instance, a low NA turnover rate or the existence of highly efficient NA reuptake mechanisms may restrict synaptic NA levels in the hippocampus. Differences between the density or threshold of cortical and hippocampal α2-adrenoceptors might also underlie the lower potency of RX811059 in the latter of these brain regions.

RX811059 increased basal NA output in the frontal cortex, but not the hippocampus. The ability of idazoxan to produce this effect (Thomas & Holman, 1991) may therefore be due to its blockade of cortical α2-adrenoceptors rather than its affinity for the NAIBS (Michel & Insel, 1989). Conversely, in the presence of a DMI-elevated baseline the α2-adrenoceptor agonist clonidine reduced basal NA output in the frontal cortex. These results are consistent with the ability of α2-adrenoceptor agonists and antagonists respectively to inhibit and stimulate the firing rate of NA neurons in the LC (Svensson et al, 1975; Marwaha & Aghajanian, 1982; Freedman & Aghajanian, 1984). Indeed, sytemic RX811059 may act upon somatodendritic α2-adrenoceptors in the LC to increase cell body firing in addition to an action upon presynaptic sites on NA terminals in the frontal cortex and hippocampus.

The highly selective NAIBS compound RX801077 did not significantly increase basal NA content in the frontal cortex and hippocampus. However, RX801077 did increase extracellular NA in both areas when given in combination with DMI. This effect was unlikely to be mediated by presynaptic α2-adrenoceptors because it has a low affinity for this site (A. Hudson - personal communication). Also, RX801077 differed from RX811059 in that RX811059 did increase NA in the frontal cortex while RX801077 produced a non-significant trend in this direction. However, RX801077 has been suggested to act as an MAOI (D.J. Nutt -

personal communication). A link between the NAIBS and MAOI activity is suggested by the recent demonstration of chronic MAOI treatments being able to down regulate central NAIBS (Olmos et al, 1993).

The present findings support the suggestion that in the frontal cortex presynaptic α2-adrenoceptor activation appears to be the primary NA regulatory mechanism; whereas NA reuptake seems to be more important in the hippocampus (Dennis et al, 1987; Thomas & Holman, 1991). Moreover, the highly selective NAIBS agent RX80107 increases extracellular NA levels by an as yet unidentified mechanism. Future studies with a range of drug doses may confirm the single dose effects observed in the present studies.

Table 8.1. Basal NA content in samples from rat frontal cortex and hippocampus with and without 5µM DMI in the dialysis medium continuously infused via the dialysis probe. All values are means where n=3 except -45-0 minutes which represents the mean of three consecutive 15 minute samples in three rats prior to 0.9% saline injection.

	Time interval after 0.9% saline injection (minutes)								
Treatment	-45 - 0	0 - 15	15 - 30	30 - 45	45 - 60	60 - 75	75 - 90	90 - 105	105 - 120
Frontal cortex									
0.9% saline	4.21±0.98	3.98±1.17	4.07±0.28	4.16±0.84	4.31±0.98	4.02±0.43	3.97±0.37	3.67±1.13	4.05±0.53
0.9% saline + DMI*	20.32±3.21†	19.64±3.87†	21.92±1.53†	20.42±2.25†	20.38±2.31†	19.63±2.04†	20.78±1.29†	20.04±0.94†	19.45±1.01†
Hippocampus									
0.9% saline	4.01±0.37	4.21±0.22	3.89±0.48	4.16±0.56	3.95±0.42	4.03±0.11	4.15±0.31	3.78±0.78	3.91±0.61
0.9% saline + DMI*	37.65±4.12†	36.89±3.63†	36.27±2.98†	36.04±1.02†	35.67±2.76†	34.54±2.01†	35.20±2.77†	36.90±4.16†	34.29±2.82†

* 5µM DMI delivered in dialysis medium continuously via the dialysis probe. *Significantly higher ($P<0.001$) than 0.9% saline in same brain region (Student's unpaired t-test).

Table 8.2. Basal concentration of NA (pg/15 mins ±SEM) in samples from frontal cortex of conscious rats 24 and 48 hours after probe implantation. All samples are means where n = 3.

Time after probe	Time (minutes)							
Implantation (hours)	0 - 15	16 - 30	31 - 45	46 - 60	61 - 75	76 - 90	91 - 105	106 -120
+24	6.46±0.38	5.32±1.52	5.70±1.14	5.45±0.22	5.82±0.88	5.06±1.16	6.08±0.44	6.33±1.22
+48	8.11±1.79	7.09±1.51	7.37±1.61	6.83±0.98	6.50±1.39	7.80±0.85	7.00±0.85	7.20±1.53

Figure 8.1. Effect of RX811059 (3 mg/kg, i.p.) upon NA concentration in samples from rat frontal cortex. values are means where n=4.

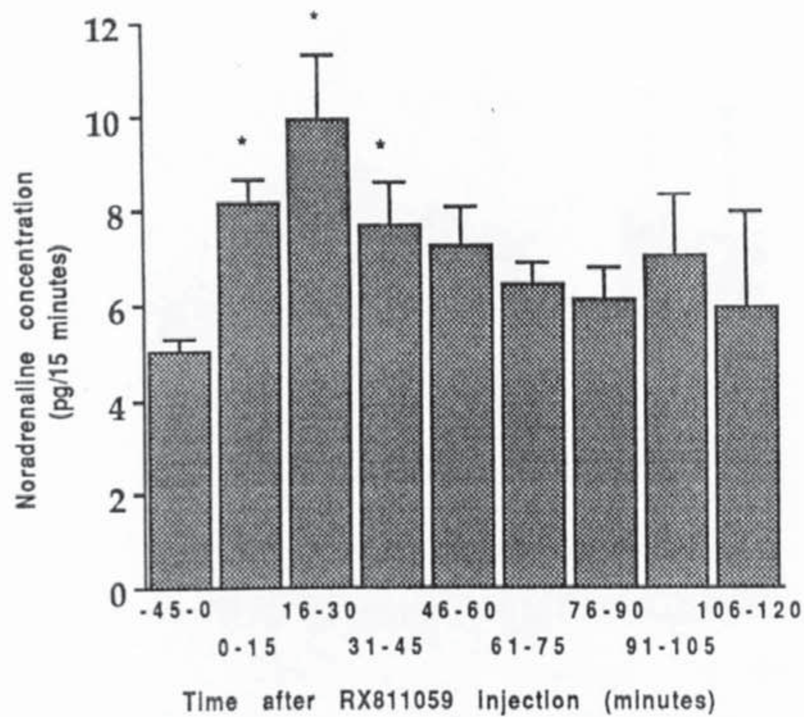
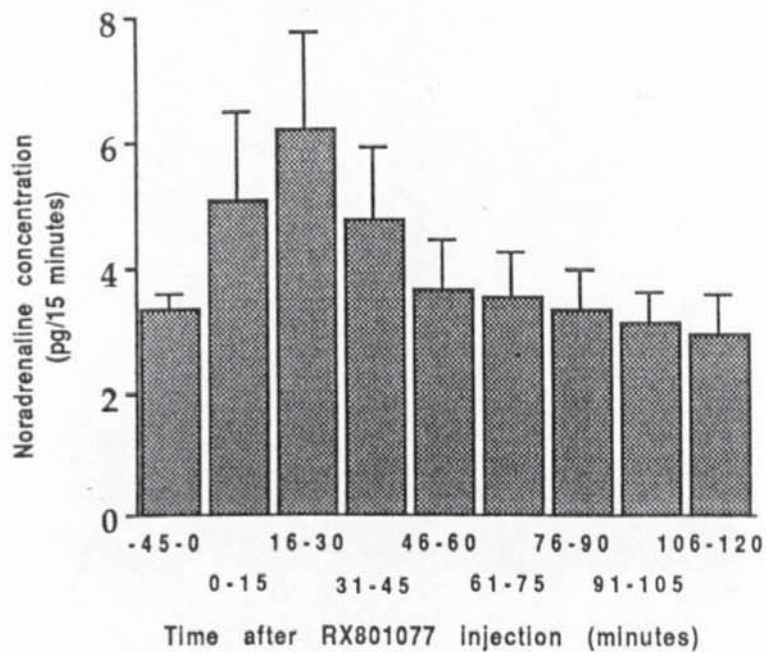


Figure 8.2. Effect of RX801077 (10 mg/kg, i.p.) upon NA concentration in samples from rat frontal cortex. Values are means where n=6.



*$P<0.05$ versus mean basal, i.e. mean [NA] during -45-0 mins. (One-way ANOVA and Dunnett's t-test; Figure 8.1. $F_{8,33} = 4.59$, $P = 0.0008$; Figure 8.2. $F_{8,55} = 1.72$, $P = 0.1127$).

Figure 8.3. Effect of RX811059 (3 mg/kg, i/p) upon NA concentration in samples from rat hippocampus, values are means where n=3

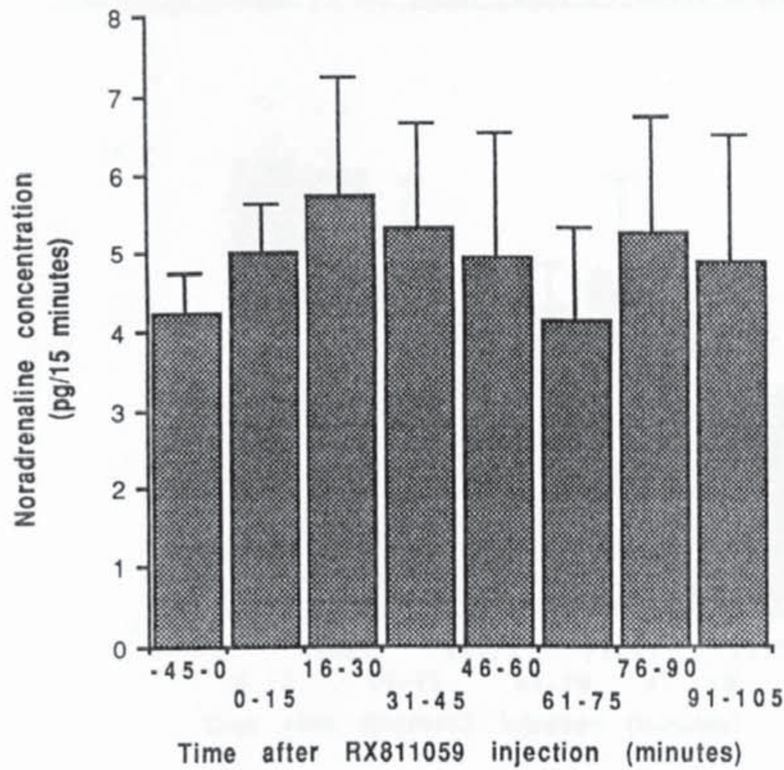
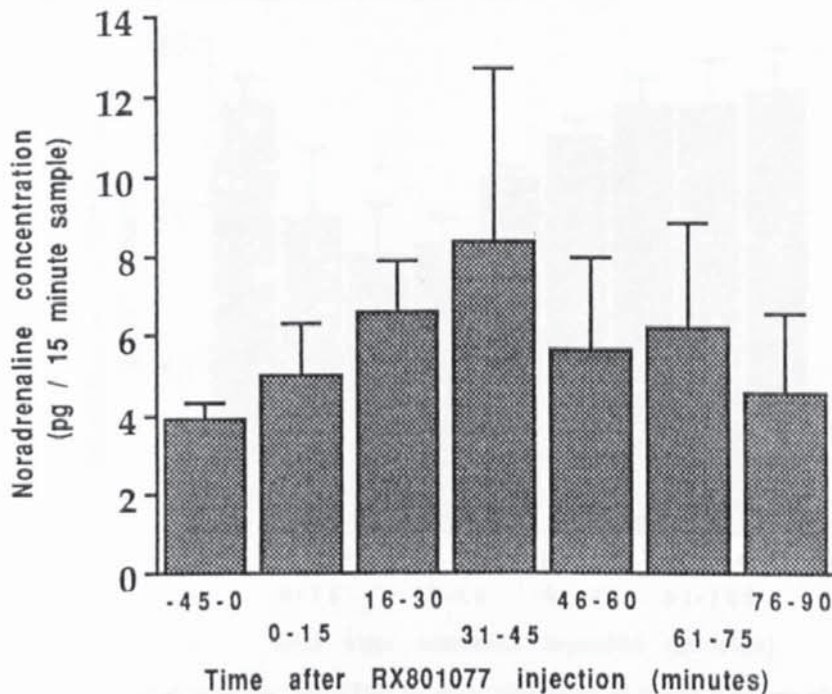


Figure 8.4. Effect of RX801077 (10 mgkg-1, i.p) upon NA concentration in samples from rat hippocampus, values are means means where n=4.



*$P<0.05$, **$P<0.01$ versus mean basal, i.e. mean [NA] during -45-0 mins. (One-way ANOVA and Dunnett's t-test; Figure 8.3. $F_{6,20}=0.359$, $P = 0.8958$; Figure 8.4. $F_{6,25} = 0.812$, $P = 0.578$).

Figure 8.5. Effect of RX801077 (10mg/kg, i.p) upon NA concentration in samples from rat frontal cortex, with 5μM DMI in the dialysis medium continuously infused via the probe. Values are means where n=5.

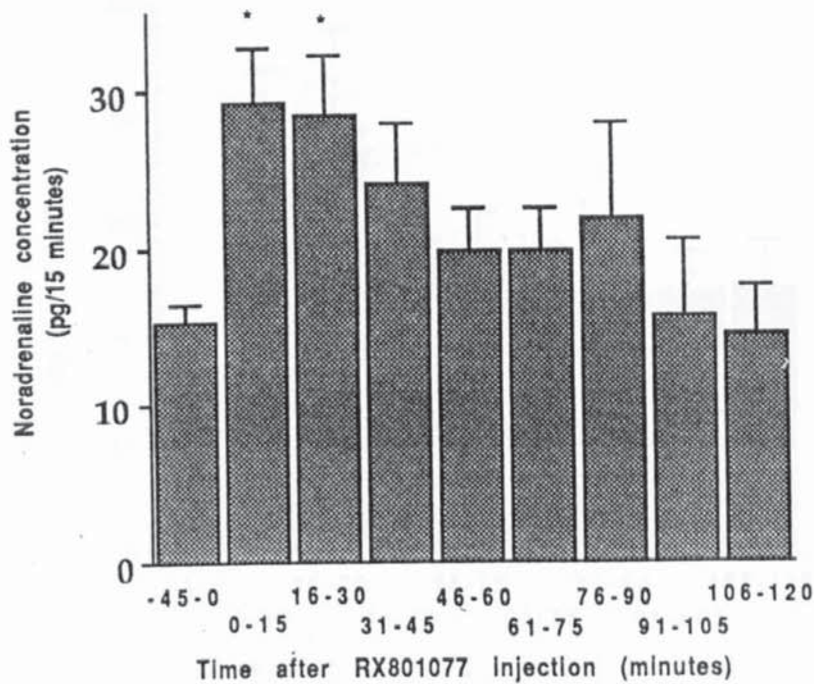
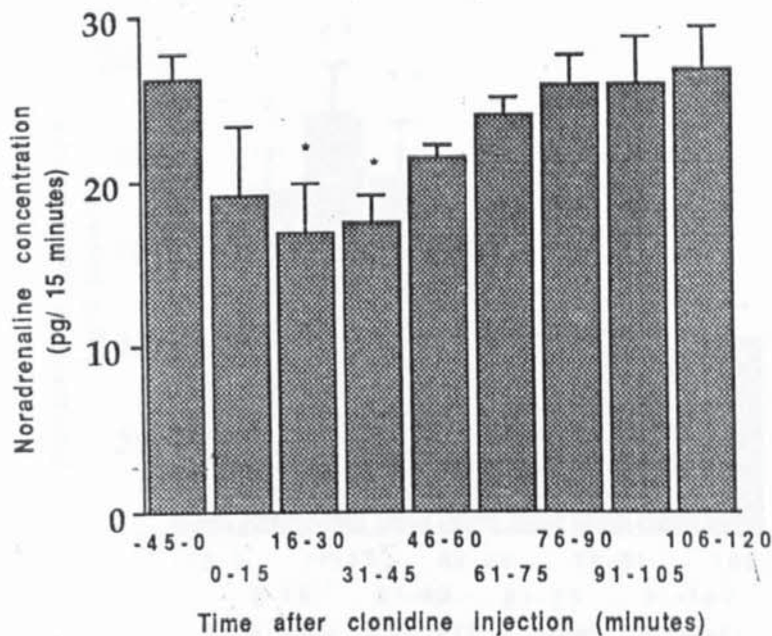


Figure 8.6. Effect of clonidine (0.02 mg/kg, i.p) upon NA concentration in samples from rat frontal cortex, with 5μM DMI in the dialysis medium continuously infused via the probe. Values are means where n=4.



*P<0.05 versus basal + DMI, i.e. mean [NA] during -45-0 mins. (One-way ANOVA and Dunnett's t-test; Figure 8.5. $F_{8,34}$ =2.75, P = 0.0186; Figure 8.6. $F_{8,42}$ =3.025, P = 0.0089).

Figure 8.7. Effect of RX811059 (3 mg/kg, i.p) upon NA concentration in samples from rat hippocampus, with 5μM DMI in the dialysis medium continuously infused via the probe, values are means where n=3.

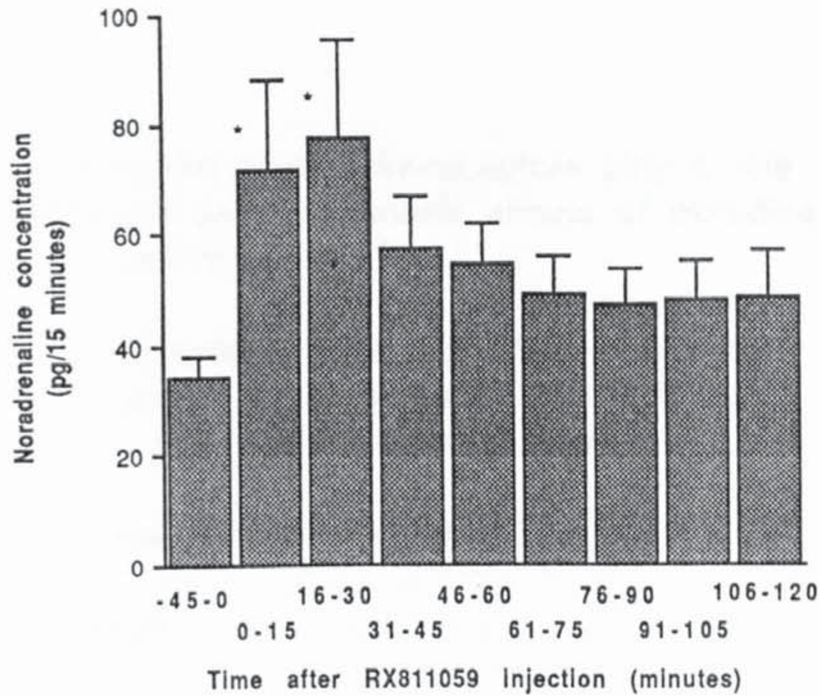
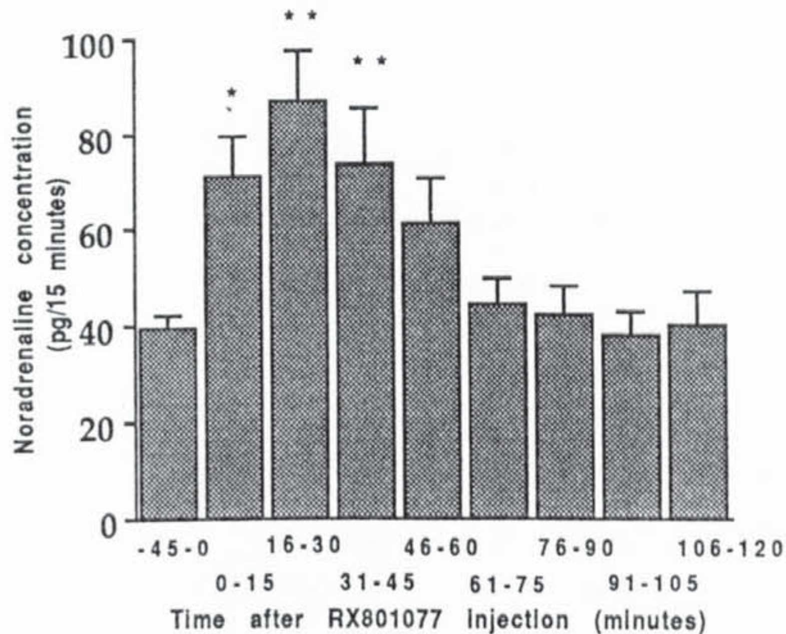


Figure 8.8. Effect of RX801077 (10 mg/kg, i.p) upon NA concentration in samples from rat hippocampus, with 5 μM DMI in the dialysis medium continuously infused via the probe, values are means where n=5.



*P<0.05, **P<0.01 versus basal + DMI, i.e. mean [NA] during -45-0 mins. (One-way ANOVA and Dunnett's t-test; Figure 8.7. $F_{8,34} = 2.705$, $P = 0.0203$; Figure 8.8. $F_{8,42} = 7.152$, $P = 0.0001$).

CHAPTER 9. General Discussion.

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GENERAL DISCUSSION

The primary aim of this project was to determine whether the recently identified NAIBS is merely a binding site or a receptor linked to a function. A variety of agents with differing selectivity profiles for these sites and the α2-adrenoceptor were employed in a number of behavioural and neurochemical situations to examine this problem.

9.1. To what extent do α2-adrenoceptors play a role in mediating the behavioural effects of clonidine, idazoxan and RX811059?

A preliminary survey was undertaken to examine the effects of clonidine, idazoxan and RX811059 upon a range of behavioural and physiological parameters in mice (see Chapter 3). In this study clonidine produced a number of behavioural effects which have been attributed to its action as an α2-adrenoceptor agonist (Fielding & Lal, 1981), for instance, it produced several signs of behavioural suppression, such as an impairment of the righting reflex and reduced locomotor activity. In addition to this, it produced signs of analgesia and sedation in the form of reduced responses to touch and the tail pinch. These findings confirm previous reports for this drug and other α2-adrenoceptor agonists (Harsing et al, 1989; Heal et al, 1989; Paalzow & Paalzow, 1976; Drew et al, 1979).

Conversely, both idazoxan and the RX811059 produced a number of signs of behavioural stimulation, such as increases in locomotor activity, which may be due to their ability to act as α2-

adrenoceptor antagonists (Doxey et al, 1983; Mallard et al, 1991). However, this behavioural survey was employed as a preliminary system to identify gross drug-induced effects, and as such certain clonidine-induced effects (eg. clonidine-induced hypothermia) were unable to be detected using these techniques (see page 68 of this thesis). The only effect of RX801077 was a decrease in grooming. This effect was not shared by RX811059 or idazoxan and might therefore be due to an interaction with the NAIBS. However, this effect might equally be due to an MAOI effect of RX801077. A further comparative investigation with MAOI compounds is therefore required to examine this problem.

Clonidine produced a discriminable cue in a rat drug discrimination study (see Chapter 4), which has been demonstrated elsewhere (Bennett & Lal, 1982; Lal & Yaden, 1985). Furthermore, RX811059 is the most selective α2-adrenoceptor antagonist to date to produce a cue under the same conditions (see Chapter 5). The inhibitory effect of α2-adrenoceptor agonists demonstrated here (see Chapter 3) may underlie the ability of such agents (viz. clonidine, UK-14,304 and guanabenz) to reduce the rate of responding in a rat drug discrimination study (Chapter 4). Similarly, the stimulant effects of α2-adrenoceptor antagonists may be responsible for the increased response rates produced by RX811059, idazoxan, fluparoxan and 1-PP in rats (Chapter 5)

Several lines of evidence suggest the clonidine and RX811059-induced cues to be due to an interaction with α2-adrenoceptors. For instance, the clonidine cue was substituted for by the more selective α2-adrenoceptor agonists UK-14,304 and guanabenz (Cambridge et al, 1981; Langin et al, 1990), and it was abolished by pretreatments with the highly selective α2-

adrenoceptor antagonists RX811059 and fluparoxan (Mallard et al, 1991; Halliday et al, 1991). Moreover, the RX811059-induced cue was substituted for by other highly selective α2-adrenoceptor antagonists such as fluparoxan and 1-PP (Halliday et al, 1989; Bianchi et al, 1988).

Furthermore, the clonidine-induced cue was unable to be blocked by the peripherally active α2-adrenoceptor antagonist L659,066 (Clineschmidt et al, 1988), which suggests a role for central as opposed to peripheral α2-adrenoceptors in producing its cue. Similarly, the inability of L659,066 to substitute for the RX811059-induced cue suggests it to be mediated by central rather than peripheral α2-adrenoceptors.

Alternatively, the clonidine and/or RX811059-induced cues may be the functional consequence of several receptor interactions acting together to form a compound cue. However, the IPR does not appear to play a role in mediating the cues produced by either of these drugs. The clonidine and RX811059-induced cues were respectively blocked and substituted for by the α2-adrenoceptor antagonist fluparoxan, which would not be expected to bind to the IPR due to the lack of an imidazoline moiety in its chemical structure. Moreover, the clonidine cue was substituted for by the α2-adrenoceptor agonist guanabenz which is unable to bind to the IPR (Ernsberger et al, 1990; Gomez et al, 1991). In addition to this, the minimal binding affinities of clonidine and RX811059 for central and peripheral NAIBS in rat (Michel & Insel, 1989; Mallard et al, 1991) excludes a role for this site in mediating either of their cues. Finally, clonidine has been suggested to act as a partial α1-adrenoceptor agonist (Bradshaw et al, 1982), although this interaction does not appear to be involved in mediating its cue, as it was substituted for by the α2-adrenoceptor agonists UK,14,304

and guanabenz which are inactive at α1-adrenoceptors (Bradshaw et al, 1982).

9.2. The neurochemical effects of α2-adrenoceptor agents, and a possible relationship with their actions in the forced swimming test.

Antidepressants known to influence synaptic NA levels produce anti-immobility effects in the forced swimming test (see Introduction Chapter 7). The NA re-uptake inhibitor DMI substantially reduced immobility time in the present studies, whereas comparitively weaker effects were produced by both RX811059 and idazoxan (Chapter 7). In accordance with these observations DMI, administered via the dialysis probe, dramatically increased extracellular NA levels in frontal cortex and hippocampus whereas systemic RX811059 produced a comparitively smaller increase in the frontal cortex alone (see Chapter 8). The weaker effect of RX811059 may be due to its delivery via the systemic route. In fact, smaller 'RX811059-like' increases in cortical NA occur with systemic DMI (Abercrombie & Finlay, 1991). Furthermore, acute stress down-regulates α2-adrenoceptors (Stanford, 1990), therefore this phenomenon may have occured due to the first forced swimming exposure which may have attenuated the effect of RX811059 upon NA output and hence immobility time.

Despite its inability to influence hippocampal NA output when given alone, RX811059 increased NA output in this region in the presence of a DMI-elevated baseline (Chapter 8). Therefore, whereas NA re-uptake appears to be an important regulator of endogenous NA in both frontal cortex and hippocampus, the α2-

adrenoceptor seems to have a detectable additional role in the frontal cortex. So it would appear that in both regions DMI raises synaptic NA to a point where presynaptic α2-adrenoceptors start to reduce its release. In the frontal cortex, α2-adrenoceptors are doing this under basal conditions so blocking them would increase NA release. However, in the hippocampus the α2-adrenoceptors are not appreciably affecting release under basal conditions, therefore the addition of DMI would bring synaptic NA levels to a point where α2-adrenoceptors do affect release. This potentiation of the effect of DMI indicates a possible antidepressant role for α2-adrenoceptor antagonists when given in combination with existing tricyclic antidepressant drugs.

9.3. A functional role for the NAIBS?

RX801077 produced a variety of behavioural and neurochemical effects in rodents. However, uncertainty exists as to the extent to which these effects are related to this compound's high NAIBS affinity and/or its ability to modify brain NA levels. For example, RX801077 produced a single inhibitory effect upon the rate of grooming in mice (Chapter 3). This effect may be due to an action of RX801077 at sites other than the NAIBS, as grooming was not affected by the equally selective NAIBS ligand B910101. In contrast, idazoxan stimulated grooming behaviour despite its high NAIBS affinity (Boyajian et al, 1987). However, both idazoxan and RX811059 (inactive at the NAIBS; Mallard et al, 1991) stimulated grooming probably due to their action as α2-adrenoceptor antagonists. Moreover, this latter effect may have masked any inhibitory effects of RX801077 mediated via an interaction with the NAIBS. However, it seems unlikely that RX801077 may act as

an $\alpha 2$-adrenoceptor antagonist in mice as it was unable to block the inhibitory effect of clonidine upon the pinna reflex in mice (see Discussion of Chapter 6). Future studies using more specific and reliable techniques in a variety of animal species may help to identify the mechanisms responsible for this inhibtory effect of RX801077. In addition to this, such studies may reveal other behavioural effects for this drug unable to be detected due to the preliminary nature of this survey.

RX801077 produced non-significant increases in extracellular NA levels in rat frontal cortex and hippocampus (Chapter 8). Highly efficient NA re-uptake mechanisms appear to have blunted this effect upon NA output as significant increases occured in both brain regions in the presence of the re-uptake inhibitor DMI. Such increases in NA output do not appear to involve a direct action of RX801077 upon presynaptic $\alpha 2$-adrenoceptors as this drug has a minimal affinity for this site (A. Hudson - personal communication). Moreover, RX811059 significantly increased NA levels in frontal cortex in the absence of DMI. The possibility exists that RX801077 may exert its effect upon NA levels via its proposed inhibition of MAO, although B910101 is inactive in this respect (D.J. Nutt - personal communication). To test this hypothesis further a study is required to examine the ability of B910101 to increase cortical and/or hippocampal NA levels.

The anti-immobility effect of RX801077 in the rat forced swimming test (Chapter 7) may be due to its ability to increase brain NA (Chapter 8) via an MAOI effect. In fact, a variety of MAOI antidepressants, such as clorgyline, selegiline and pargyline produce anti-immobility effects in this paradigm probably because of their ability to inhibit the metabolism of neuronal NA (Porsolt, 1981; Duncan et al, 1985). A further link between the NAIBS and

MAOI activity is provided by the recent demonstration of the down-regulation of central NAIBS by chronic MAOI treatments (Olmos et al, 1993). Moreover, increased NAIBS binding has been detected in the postmortem brains of depressed suicide victims (Barturen et al, 1992). Therefore, a down-regulatory effect of RX801077 upon brain NAIBS may underlie its anti-immobility effect in rats. On the other hand, NAIBS selective agents may reduce immobility via a direct or indirect stimulation of NA release from noradrenergic neurones. However, RX811059 increased brain NA levels in the same regions as for RX801077, although RX811059 failed to significantly reduce immobility (Chapter 7). Thus, the anti-immobility effect of RX801077 appears to be related to its high NAIBS affinity, although a noradrenergic involvement can not be ruled out at this stage.

The ability of RX801077 to produce a discriminable cue in rats implies a functional role exists for the NAIBS (Chapter 6). For instance, the RX801077 cue was substituted for by both B910101 and idazoxan, both of which bind with high affinity to the NAIBS (A. Hudson - personal communication; Boyajian et al, 1987). Alternatively, an α2-adrenoceptor antagonist effect of RX801077 may contribute toward the formation of its cue. For example, a number of α2-adrenoceptor antagonists, including RX811059 and fluparoxan, substituted for the RX801077-induced cue. RX811059 has a minimal affinity for the NAIBS (Mallard et al, 1991) and fluparoxan would not be expected to bind to the NAIBS due to its non-imidazoline structure, although its affinity for this site remains to be determined. Nevertheless, RX801077 has a minimal affinity for α2-adrenoceptors, and its inability to block the inhibitory effect of clonidine upon the pinna reflex in mice would appear to exclude its action as an α2-adrenoceptor antagonist (see

Discussion of Chapter 6). Alternatively, the ability of RX801077 to increase brain NA levels (Chapter 8) by a mechanism other than α2-adrenoceptor blockade (eg. MAOI activity) may contribute toward the formation of its cue. For example, the RX801077 cue was substituted for by the MAOI drugs moclobemide and pargyline (Chapter 6). However, its cue is probably not MAOI selective as it was substituted for by B910101 which lacks MAOI activity (D.J. Nutt - personal communication). Further experimentation is therefore required to determine the ability agents, which stimulate NA output by a non α2-adrenoceptor related mechanism, to substitute for an RX801077-induced cue.

Despite its minimal affinity for α2-adrenoceptors, RX801077 partially substituted for cues produced by both clonidine (Chapter 4) and RX811059 (Chapter 5). The clonidine cue may be mediated by its action upon postsynaptic α2-adrenoceptors. RX801077 may substitute for this component of the clonidine cue via its ability to increase endogenous NA output. This NA in turn may then mimic the action of clonidine upon postsynaptic α2-adrenoceptors. The ability of non-α2-adrenoceptor compounds, which stimulate extracellular NA output (eg. MAOI drugs and DMI), to substitute for the clonidine cue may help to examine this hypothesis further.

In summary, it currently seems uncertain as to what extent the behavioural and/or neurochemical effects of RX801077 are related to its high NAIBS affinity and/or its ability to increase brain NA output. Further studies may reveal the mechanisms responsible for this effect upon brain NA levels. The present studies appear to support an as yet unidentified functional role to exist for the NAIBS.

9.4. Conclusions.

The major findings of this project are that the highly selective ligand RX801077 produced a number of behavioural and neurochemical effects which suggest the NAIBS might be a 'true' functional receptor.

The functional consequences of NAIBS stimulation appears to be an increase in NA output which may be related to an MAOI effect of this site or of RX801077 independently of NAIBS. This increase in NA output may be responsible for producing the physiological and/or behavioural effects which underlie the ability of this receptor to produce a discriminable cue in rats. However, the behaviours and/or physiological mechanisms which are affected by the activity of RX801077 at the NAIBS still remains to be determined, although continued research may reveal a role for this site in the treatment of human depressive disorders. Further studies are also required to determine whether the effects produced by RX801077 were due to its activity as an agonist or antagonist at the NAIBS.

The development of highly selective NAIBS compounds may reveal an endogenous ligand for this site which may have a unique therapeutic profile or serve as a useful research tool.

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