Investigation of a role for noradrenaline in the neurochemical and behavioural effects of an established and putative target for antianxiety / antidepressant drugs: a microdialysis and behavioural study in rats and NK1 receptor knockout mice

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Abstract

The precise neurochemical effects of antidepressants that underlie their efficacy remain unclear. Many antidepressants modulate noradrenaline transmission *in vivo*. However, the selective serotonin reuptake inhibitors (SSRIs) and NK1 receptor antagonists target 5-HT and substance P transmission, respectively. Whether effects on noradrenaline neurones contribute to the efficacy of these drugs has been largely neglected. This study investigates modulation of central NA transmission by an SSRI (fluoxetine) in rats and by NK1 receptor ablation in mice. NK1 'knockout' mice are used as a model for lifelong inhibition of the NK1 receptor. Changes in noradrenaline transmission were determined using *in vivo* microdialysis to sample the extracellular noradrenaline concentration (efflux). Noradrenaline-related behavioural effects of these treatments were determined using a modified light/dark exploration test.

Systemic fluoxetine increased noradrenaline efflux in two brain regions (frontal cortex and hypothalamus). The effect was inconsistent, echoing the 5-HT response to SSRIs. Fluoxetine reduced noradrenaline efflux after partial lesioning of noradrenergic axon terminals (by DSP-4), suggesting an inhibitory action upstream of these terminals. Locally infused (retrodialysis) fluoxetine augmented noradrenaline efflux in the terminal field. Whereas 50μM fluoxetine elevated efflux in both regions, 5μM infusion increased efflux in the frontal cortex only. This effect was 5-HT-dependent (*i.e.* 5-HT synthesis inhibition with *p*CPA blocked the response). However, this increase was not secondary to inhibition of 5-HT reuptake (since the more potent and selective SSRI, citalopram, did not change noradrenaline efflux). Thus, fluoxetine has opposing effects on noradrenaline efflux, probably at cell bodies and terminals. The balance of processes could underlie its variable effects.

Fluoxetine reduced locomotor activity of DSP-4-pretreated rats in the novel compartment of the exploration box, suggesting a noradrenaline-related effect on behaviour. A 5-HT-related effect of fluoxetine was also found (reduced locomotor activity in the dark compartment), since this was abolished by *p*CPA.

Thus, both noradrenaline and 5-HT-related behavioural effects of fluoxetine were found.

Basal noradrenaline efflux was two-fold greater in NK1 knockout mice, indicating elevated noradrenaline transmission. Noradrenaline uptake was normal, since the uptake inhibitor DMI increased efflux equally in both strains. However, the α_2-autoreceptor antagonist, atipamezole, (which increases noradrenaline cell firing and release) had no effect on efflux in knockout mice. Also, NK1 receptor ablation produced the same behavioural changes in the light/dark box (*i.e* increased 'locomotor activity in light', reduced 'time to return') as seen with the α_2-adrenoceptor antagonists (atipamezole and yohimbine) in normal mice. These noradrenaline-related behaviours unchanged by either drug in knockout mice. This loss of sensitivity is consistent with diminished α_2-autoreceptor function in NK1 knockout mice.

Collectively, these studies demonstrate that both an established SSRI (fluoxetine) and a novel antidepressant target, (NK1 receptor) modulate central noradrenaline transmission in vivo. The results support a common role for noradrenaline in antidepressant drug therapy.

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5-HT	5-hydroxytryptamine
aCSF	artificial cerebrospinal fluid
ANCOVA	analysis of covariance
ANOVA	analysis of variance
D	Dalton
DA	Dopamine
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
ECD	electrochemical detection
ECF	extracellular fluid

g	gram
GABA	γ-aminobutyric acid
h	hour
[^{3}H]	tritium
HPLC	high performance liquid chromatography
IC_{50}	concentration causing 50% inhibition of effect
i.c.v.	intra cerebroventricular
i.d.	inner diameter
i.p.	intra peritoneal
K_d	dissociation constant
K_i	inhibitor constant
K_m	affinity constant
l	litre
m	metre
M	molar
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
min	minute
mol	moles
n	sample size
NA	noradrenaline
o.d.	outer diameter
P	probability
REDOX	reduction/oxidation
s.c.	subcutaneous
S.E.M.	standard error of the mean
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
V	volt

Prefixes

m	milli (x 10^{-3})
μ	micro (x 10^{-6})
n	nano (x 10^{-9})
p	pico (x 10^{-12})
f	femto (x 10^{-15})

Chapter 1

Introduction

1.1. Depression

Depression is a common and debilitating psychiatric illness. Its symptoms include low mood (dysthymia), loss of interest or pleasure in activities (anhedonia), sleep disturbances, reduced or increased appetite, reduced movement or agitation, fatigue, delusions, feelings of worthlessness or guilt, inability to concentrate and thoughts of death or suicide, (DSM IV, 2000). Not only do these symptoms produce a highly distressing condition for the patient, they often lead to behavioural impairment. Depressed patients are at an increased risk of premature mortality as up to 15% of individuals with major depressive disorder commit suicide.

Depression is a chronic, relapsing disorder with 17% of the population suffering from the illness, (Lepine *et al*, 1997). The World Health Organization recognizes depression as the fourth leading contributor to the global burden of disease, (Murray & Lopez, 1996). Given its debilitating nature and its prevalence, depression presents a significant economic burden. One estimate of the total cost of depression to the US economy is $83.1 billion each year (Greenberg *et al*, 2003).

The most common approach to the treatment of depression is antidepressant drug therapy. Although the precise mechanisms underlying their therapeutic effects are, as yet, unknown all antidepressant drugs share a common property in that they increase central noradrenaline (NA) and/or 5-hydroxytryptamine (5-HT) transmission on chronic administration. These neurotransmitters belong to a group known as monoamines, which also includes dopamine. The role of dopamine in the action of antidepressant drugs is less well established. In any case, the possible adverse consequences of increasing central dopamine transmission with respect to dependence liability make this an undesirable therapeutic target. Therefore, discussion of the monoamines here will be confined to NA and 5-HT.

1.2. Central NA system

Central noradrenergic neurones were first described in the CNS by Dahlström & Fuxe (1964), using the Falck-Hillarp formaldehyde vapour fluorescence technique. Noradrenergic cell bodies are located in pontine and brainstem regions (Figure 1.1). Based on cell body location, efferent target areas and axon morphology, noradrenergic nuclei are divided into two principle systems: the locus coerulus (LC; corresponding to cell groups A6 and A4 of the classification of Dahlström and Fuxe) and lateral tegmental system (A1- A3, A5, A7; Moore & Bloom, 1979). The LC is the largest noradrenergic nucleus of the brain, located along the lateral margin of the pontine fourth ventricle. This nucleus contains approximately 1400 cells bilaterally, about 45% of noradrenergic cells in the rat brain, (Swanson, 1975). In addition to fluorescent histochemical techniques, details of noradrenergic innervation have been derived from axon tracing methods in combination with immunohistochemistry and autoradiography. The most striking feature of this system is the extent to which a relatively small number of cells distribute terminals widely through the entire CNS. Efferents from the LC project caudally to all levels of the spinal cord, dorsally to the cerebellum and rostrally to the thalamus, hypothalamus, amygdala, hippocampus, olfactory system, and entire neocortex, (Foote *et al*, 1983).

The lateral tegmental system is composed of medullary and pontine groups of noradrenergic neurones. Axons arising from these cell groups differ from coerulofugal axons, with larger varicosities of more irregular size and distribution. Efferents innervate the spinal cord, basal forebrain, septal area, amygdala and thalamus. Lateral tegmental neurones do not project to the neocortex or hippocampus, their principle target being the hypothalamus. This receives a dense noradrenergic innervation from the lateral tegmental system, in addition to a minor contribution from the LC, which sends fibres to the periventricular, paraventricular, dorsomedial and supraoptic nuclei only (Moore & Bloom, 1979).

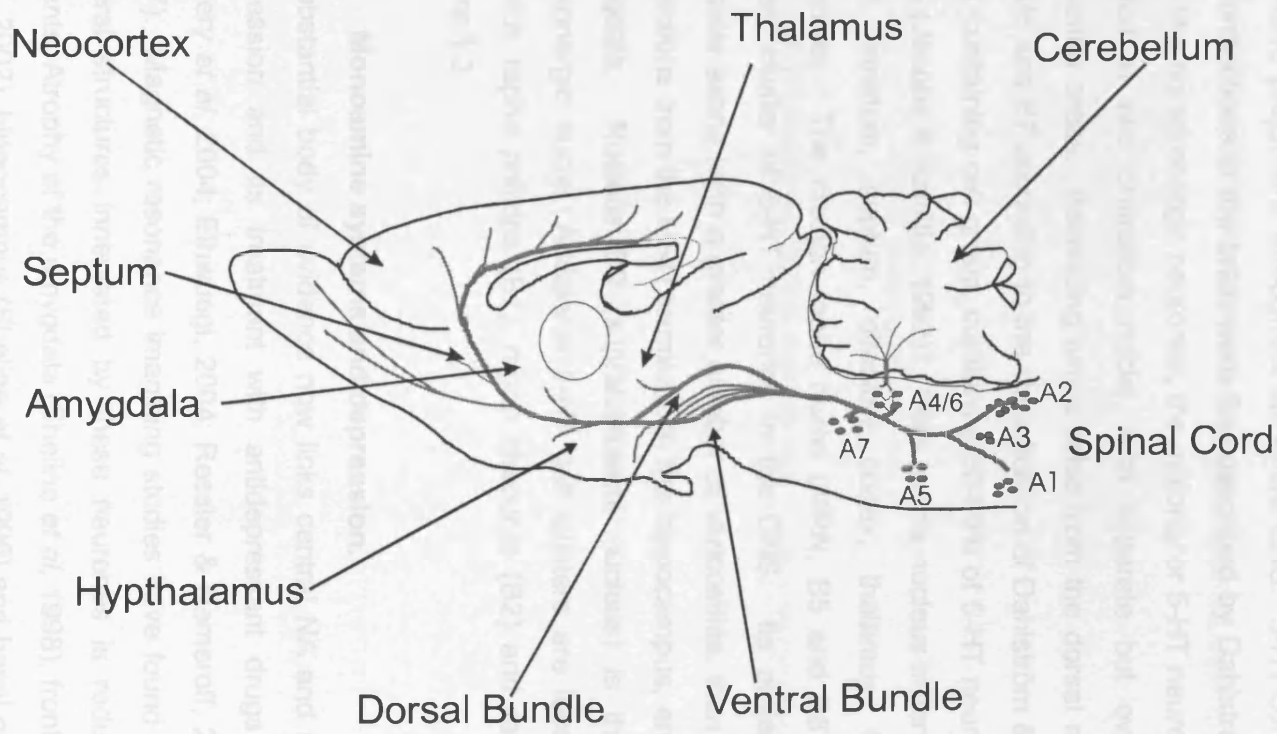


Figure 1.1. Ascending noradrenergic innervation of the rat brain. Adapted from Stanford (1999)

1.3. Central 5-HT system

The 5-HT innervation of the CNS is similar to the noradrenergic innervation in that a relatively small number of cell bodies, located in the midbrain, send extensive projections throughout the entire CNS. 5-HT-containing cell bodies and projections in the brain were first described by Dahlström & Fuxe (1964). Also like noradrenergic neurones, the majority or 5-HT neuronal cell bodies are located in two brainstem nuclei, with separate but overlapping terminal projection areas. Ascending axons arise from the dorsal raphe nuclei (DRN, B4, B6 and B7 according to the classification of Dahlström & Fuxe), the largest 5-HT-containing cell group, containing 50-60% of 5-HT neurones in the human CNS (Jacobs & Azmitia, 1992). Cells in this nucleus innervate the substantia nigra, striatum, septum, olfactory cortex, thalamus, hippocampus and neocortex. The median raphe nuclei (MRN, B5 and B8) form the second largest cluster of 5-HT neurones in the CNS. Its projections have larger diameter axons, with a greater number of varicosities, than those of the DRN. Projections from the MRN terminate in the hippocampus, entorhinal cortex and amygdala. Nucleus B9 (supralemniscal nucleus) is the most lateral of serotonergic nuclei. Additional 5-HT cell clusters are located caudally, *e.g.* nucleus raphe pallidus (B1), raphe obscurus (B2) and raphe magnus (B3). Figure 1.2.

1.4. Monoamine systems and depression.

A substantial body of evidence now links central NA and 5-HT systems with depression and its treatment with antidepressant drugs (for reviews see Slattery *et al*, 2004; Elhwuegi, 2004; Ressler & Nemeroff, 2000; Montgomery, 1997). Magnetic resonance imagining studies have found that the volume of several structures innervated by these neurones is reduced in depressed patients. Atrophy of the amygdala (Sheline *et al*, 1998), frontal cortex (Bremner *et al*, 2002), hippocampus (Sheline *et al*, 1996) and basal ganglia (Krishnan *et al,* 1992) have been reported. Abnormalities of NA metabolites excretion in depressed patients (Maas *et al*, 1968), NA and 5-HT-depleting treatment-induction of relapse in depressed patients (Delgado *et al*, 1991, 1993) suggest that dysregulation of monoamine transmission are a feature of depression. However, most evidence for involvement of monoamines in depression comes from the apparent mode of action of antidepressant drugs.

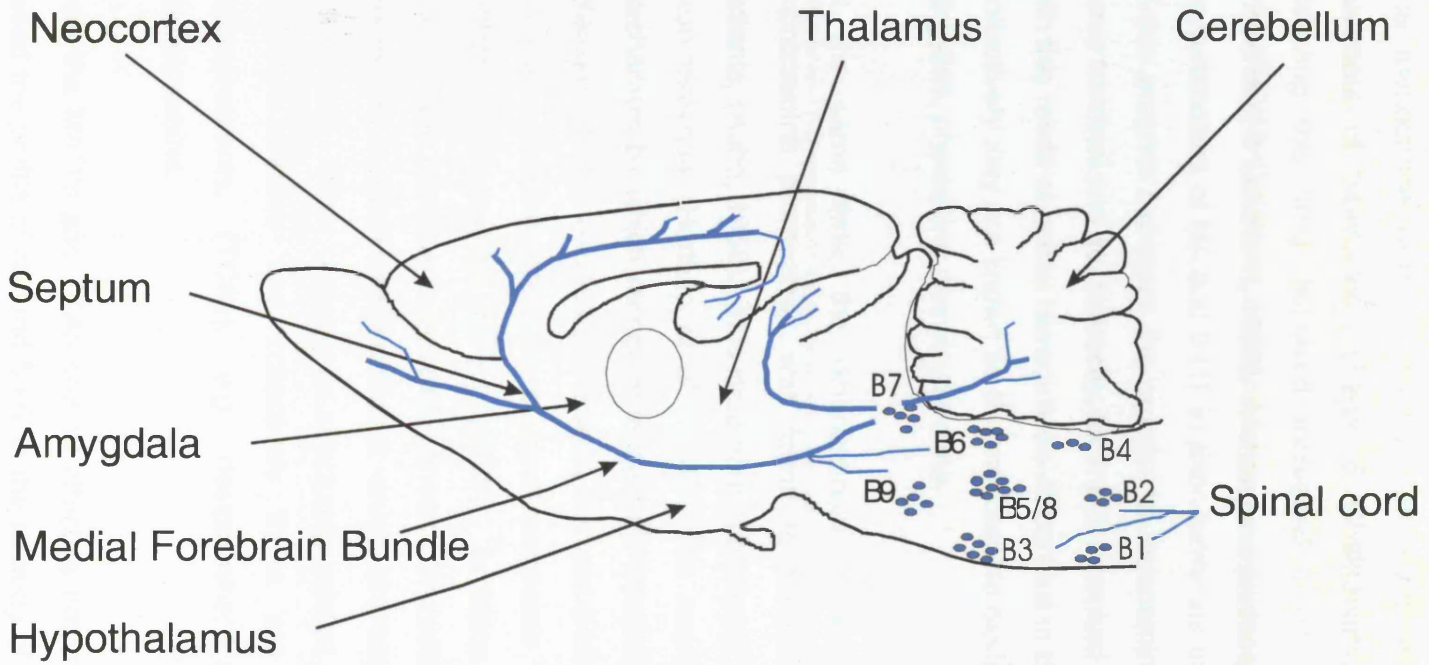


Figure 1.2. Ascending 5-HT innervation of the rat brain. Adapted from Stanford, (1999)

1.5. Monoamine systems and antidepressant drugs

The monoamine oxidase inhibitor, iproniazid, was originally used in the treatment of tuberculosis (Segal & Chakravarty, 1954). Some patients receiving this drug displayed increased locomotor activity and euphoria (Deverteuil & Lehmann, 1958). Monoamine oxidase is the enzyme responsible for metabolism of NA and 5-HT in axon terminals and other tissues. Inhibition of this enzyme increases the amount of monoamine stored in vesicles in the nerve terminal and so, indirectly, its impulse-evoked release. Many other drugs with this mode of action have proven efficacious in the treatment of depression. Collectively they are known as the monoamine oxidase inhibitors (MAOIs), *e.g.* pargyline, phenelzine, tranylcypromine.

At the same time, the dibenzazepine, imipramine, (derived from the antihistamine promazine), was found to be useful in treating depressed patients, (Kuhn, 1958). This drug is an inhibitor of NA and 5-HT reuptake into axon terminals (Hertting *et al*, 1961). The reuptake process is a principle mechanism by which monoamine synaptic transmission is inactivated. Once released into the extracellular space, NA and 5-HT are taken back into axon terminals *via* their respective trans-membrane Na^+/Cl^--dependent carrier proteins ('transporters', Nelson, 1998). Inhibition of this transport process prolongs the increase in synaptic concentration after release of the respective monoamine. Most antidepressant drugs are believed to operate *via* this mechanism. Other compounds structurally related to imipramine have proven clinically effective and collectively these are known at the tricyclic antidepressants, (TCAs), *e.g.* desipramine, amitriptyline, nortriptyline, chlomipramine.

Both the MAOIs and TCAs proved effective treatments for depression. This raised the profile of NA and 5-HT in the etiology of the disorder and led to the 'monoamine theory' of depression, (Schildkraut, 1965). This proposed that the disorder itself is due to reduced central monoamine transmission which, when corrected for by antidepressant drugs, leads to clinical improvement. The theory was supported by the mood-altering properties of the antihypertensive drug reserpine, which depletes the brain of monoamines and can induce a depressed state in humans (Freis, 1954). However, some findings are

inconsistent with the original theory: most notably the time course of action of antidepressant therapy. Despite inhibiting monoamine reuptake or metabolism on acute application, several weeks of antidepressant treatment are necessary for clinical improvement. A landmark finding was the down-regulation of β-adrenoceptors after long-term treatment with antidepressants (Vetulani & Sulser, 1975; Banerjee *et al*, 1977). This occurs after chronic treatment with a variety of antidepressant strategies. Thus, MAO inhibition (Sellinger-Barnette *et al*, 1980) tricyclic antidepressants (Bergström & Kellar, 1979) and repeated electroconvulsive shock (ECS, Stanford & Nutt, 1982) lead to down-regulation of β-adrenoceptors. Since this effect takes place over a time-scale equivalent to the 'therapeutic lag' of antidepressant therapy, it was hypothesized to be central to the therapeutic efficacy of these drugs. However, subsequent studied demonstrated that some SSRIs do not cause β-adrenoceptor down-regulation. Since this is not a common feature of all antidepressant drugs, it cannot be the mechanism by which they exert their effects.

The original monoamine hypothesis has been revised to account for the delayed onset of therapeutic efficacy of antidepressant drugs. Neurochemical changes observed after chronic administration of antidepressant demonstrate adaptive changes in a wide range of receptor populations that normally regulate the firing and release rate of 5-HT and NA. Down-regulation of presynaptic α_2-adrenoceptors, (Charney *et al*, 1981; Thomas *et al*, 1998; Guo *et al*, 1998; Mateo *et al*, 2001) 5-$HT_{1B/1D}$ receptors, (Pineyro & Blier, 1996) and somatodendritic 5-HT_{1A} autoreceptors (Blier & de Montigny, 1980; Invernizzi et al, 1994) occurs after several weeks of antidepressant therapy. These adaptations lead to greater efflux of NA and 5-HT and these long-term adaptations are thought to account for the delayed onset of antidepressant efficacy.

Despite their efficacy as antidepressants, both MAOIs and TCAs are by no means a 'cure' for depression. Both classes of drug produce side-effects that can limit their use. MAOIs, particularly irreversible inhibitors, can cause fatal hypertensive reactions when co-administered with dietary tyramine. TCAs are ligands at histamine (H_1), NA (α_1) and muscarinic (M_1) acetylcholine receptors, as well as fast-conducting Na^+ channels in cardiac tissue. In the periphery,

TCAs produce antimuscarinic effects at postganglionic parasympathetic nerve terminals and elevate noradrenergic transmission at sympathetic nerve terminals. In combination, these actions can lead to a fatal hypertension. Sympathomimetic effects, as well as nonspecific effects at α_1 (dizziness, urinary retention), H_1 (sedation) and M_1 receptors (blurred vision, dry mouth), have an adverse impact on patient compliance. Both MAOIs and TCAs are toxic in overdose, a serious problem when treating a group of patients at increased risk of suicide. These adverse effects led to a search for antidepressants with an improved side effect profile and development of the selective serotonin reuptake inhibitors (SSRIs).

1.6. SSRIs

The SSRIs are a structurally unrelated group of compounds, which all inhibit reuptake of 5-HT, with varying degrees of selectivity and potency. The SSRIs currently used in the treatment of depression are fluoxetine, paroxetine, citalopram, sertraline and fluvoxamine. They were developed specifically not to have marked affinity for NA, histamine or acetylcholine receptors in an attempt to improve on the side effect profile of their predecessors. Although of comparable therapeutic efficacy with MAOIs and TCAs, SSRIs are relatively non-toxic in overdose (Barbey & Roose, 1998). This, together with their improved side effect profile means that the SSRIs are now the 'first line' of antidepressant therapy. SSRIs are now one of the most commonly prescribed classes of antidepressant drugs.

Despite the improvement in side effects and toxicity with SSRIs, these compounds also produce unwanted side effects. These include sexual dysfunction, nausea, headache, restlessness and increased anxiety during the early stages of treatment. Increased risk of self-harm during initial treatment may also be a problem, although this remains controversial (Gunnell *et al*, 2005). These adverse effects, together with the large population of depressed patients who show no improvement after treatment with any class of antidepressant (approximately 30%), means there is still an unmet clinical need in the treatment of depression. Therefore, clinical and preclinical research into the mechanism of antidepressant drugs is still necessary.

Since the use of SSRIs has proliferated several classes of antidepressants have emerged and found use in the treatment of depression. For example, the mixed "serotonin-noradrenaline reuptake inhibitors" (SNRIs) duloxetine, venlafaxine and milnacipran. These compounds inhibit reuptake of both noradrenaline and 5-HT, and so share a similar mechanism to that of the TCAs (see above). SNRIs have similar efficacy to that of TCAs (Schweizer et al, 1994) with possibly even greater efficacy than that of SSRIs (Goldstein et al, 2004; Clerc et al, 1994). SNRIs lack affinity for adrenergic, histaminergic or muscarinic receptors, and this is reflected by their improved tolerability when compared with TCAs (Schweizer et al, 1994; Van Amergongen et al, 2002; Kasper et al, 1996).

The "Noradrenaline and Specific Serotonin Receptor Antagonists" (NaSSAs, *e.g.* mianserin, mirtazapine) increase transmission of noradrenaline by inhibiting presynaptic α_2-adrenoceptor-mediated inhibition of NA cell firing and release rate, which may explain the antidepressant efficacy of these drugs. Simultaneous antagonism of 5-HT2C and 5-HT3 receptors could underlie the reduced rate of 5-HT related adverse effects that has been reported for mirtazapine compared with TCAs (Smith et al, 1990).

Another novel antidepressant is the "Noradrenaline Reuptake Inhibitor" (NARI) reboxetine. This compound selectively inhibits reuptake of noradrenaline, free from effects on 5-HT reuptake. It has comparable efficacy to TCAs and SSRIs. In particular, reboxetine may have even greater efficacy that other drugs in the treatment of severe from of the illness, and has been shown to have a beneficial effect on social functioning of depressed patients. The emergence of a NA-selective antidepressant demonstrates the central role this transmitter plays in the action of most, if not all antidepressant drugs.

1.7. NK1 receptor antagonists as putative, novel antidepressants

Neurokinin 1 (NK1) receptors are the primary target for the peptide neurotransmitter substance P. Along with neurokinin A and neurokinin B, substance P belongs to a family of peptides known as tachykinins (*i.e,* peptides that share the sequence Phe-X-Gly-Leu-Met-NH2). In addition to the NK1 receptor two other tachykinin receptors have been identified that mediate the postsynaptic effects of these peptides (NK2 and NK3 receptors). NK2 receptors show the greatest efficacy for neurokinin A and NK3 receptors neurokinin B. However, the selectivity of the receptors for each neurokinin is low since each is able to illicit full-agonist responses at each receptor type (see Maggi, 1995). Tachykinin-mediated transmission is complicated further by the biological activity of metabolite fragments that may differ in their rate of clearance from the extracellular space (Michael-Titus et al, 2002).

Substance P is released from primary afferent fibres in superficial layers of the spinal cord during noxious thermal, chemical or mechanical stimulation (Duggan *et al*, 1988). Binding sites for substance P are also localized in limbic areas, such as the hippocampus, striatum, olfactory bulb, central nucleus of the amygdala, thalamus and superior colliculus, (Dam & Quirion, 1986). Non-peptide NK1 receptor antagonist drugs were developed in the hope that they would be useful analgesics. However, drugs that act at the NK1 receptor have effects other than analgesia, (see below). In particular, a double blind, placebo-controlled study demonstrated that the NK1 receptor antagonist MK-869 was of similar efficacy in reducing symptoms in depressed patients as the SSRI, paroxetine, (Kramer *et al*, 1998). Moreover, MK-869 produced fewer adverse effects than paroxetine. Taken alone, this study demonstrated that NK1 receptor antagonists might be effective and well-tolerated antidepressants. This was particularly interesting since they represent the first class of putative antidepressants that apparently do not target monoamine metabolism or reuptake as their primary mode of action. However, development of this compound in the treatment of depression was abandoned since subsequent data failed to demonstrate of efficacy.

Other NK1 receptor antagonists are in the development pipeline. GlaxoSmithKline (GSK) are conducting Phase I trials of the NK1 antagonist

GW823296 for depression and anxiety. Combination treatment of the NK1 antagonist Vestipitant with the SSRI paroxetine is in Phase II development. Also, Pfizer have an NK1 antagonist (CP-122,721) undergoing Phase II trials.

NK1 receptor antagonists may have therapeutic potential for additional indications. Currently, GSK are testing the NK1 antagonist Casopitant, (679769) for symptoms of depression and anxiety, urinary incontinence and chemotherapy-induced nausea and emesis.

In addition to NK1 receptor ligands, NK2 and NK3 receptor antagonists may prove useful in the treatment of depression and anxiety states and some additional indications. The NK2 antagonist Saredutant, (SR48968, Sanofi Aventis) is currently in Phase III testing for depression/anxiety. Their compound SAR-102279 is at the preclinical stage of testing for the same indication. The mixed NK2/NK3 antagonist, SSR-241586 is at the preclinical stage of development for depression and anxiety. This compound may also have therapeutic potential for irritable bowel syndrome (IBS) and chronic obstructive pulmonary disorder. NK2 receptor antagonist have been suggested as possible treatments for IBS, also (see Lecci *et al*, 2004). The NK3 receptor antagonist, Talnetant (SB223412) is at the Phase III stage of development for IBS and urinary incontinence.

There is evidence that NK3 antagonists may prove useful in the treatment of positive symptoms of psychosis (see Spooren *et al*, 2005). Two compounds are currently in development for this indication (GSK's Talnetant and Sanofi's SSR-146977).

The fact that neurokinin receptors antagonists are being developed for a broad range of seemingly unrelated clinical states is reflective of their widespread distribution and principle role as chemical mediators in a range of tissues. Also, the overlap regarding clinical indications that each selective antagonists may treat reflects coexpression of tachykinins (and the ability of neurokinins to act at multiple tachykinin receptors (Maggi et al, 1995; Drew et al, 2005)

1.7.1 Antidepressant-like properties of NK1 receptor antagonist-preclinical evidence.

Preclinical experiments using NK1 receptor antagonists have used a variety of tests and several rodent species. This is because antagonists with high affinity for the rat and mouse NK1 receptor have low affinity for the human NK1 receptor (Gitter *et al*, 1991). Species variations in the receptor amino acid sequence produce differences in antagonist binding affinities (Fong *et al*, 1992). Also, drugs that have high affinity for rat and mouse NK1 receptors have poor brain penetration and short half-lives (Rupniak *et al*, 1997). These problems may be overcome in two ways: by using species that have NK1 receptor pharmacology similar to humans, (*e.g.* guinea pig, gerbil, hamster) or by using NK1 receptor knockout mice (see below).

NK1 receptor antagonist drugs

Several studies demonstrate antidepressant-like effects of NK1 receptor antagonist. For example, acute treatment with either fluoxetine or imipramine blocks the vocalization of gerbils induced by i.c.v. infusion of the NK1 receptor agonist, GR73632. This response is also prevented by the NK1 receptor antagonist, L760735, (Kramer *et al*, 1998). Fluoxetine, phenelzine and imipramine all prevent ultrasonic vocalization of guinea pig pups in response to transient maternal separation. This behaviour is also blocked by the NK1 receptor antagonists L760735 (Kramer *et al*, 1998) or RP67580 (Santarelli *et al*, 2001). Thus, blockade of the NK1 receptor inhibits behavioural responses to aversive stimuli in the same way as conventional antidepressants. Moreover, NK1 receptor antagonists and conventional antidepressants have the same effect on behaviours such as the mouse forced swim test (Rupniak *et al*, 2001; Zocchi *et al*, 2003), hamster resident intruder (Rupniak *et al*, 2001) and mouse marble burying (Millan *et al*, 2002). The modulation of behaviours sensitive to antidepressant drugs by NK1 receptor antagonist indicates their potential for use as antidepressant therapy.

NK1 receptor knockout mice

Although studies are limited, NK1 ablation produces the same pattern of results as NK1 receptor antagonist drugs. Thus, NK1 receptor knockout mice behave like normal mice that have been treated with an established antidepressant.

For example, NK1 receptor knockout mice struggle for longer in the forced swim and tail suspension tests (Rupniak *et al*, 2001), exhibit less ultrasonic vocalization on transient maternal separation (Santarelli *et al*, 2001) as well as increased latency to initiate aggression in the resident intruder test, (Rupniak *et al*, 2001). These studies demonstrated that the NK1 receptor knockout mouse is a valid preclinical model of treatment with NK1 receptor antagonist drugs.

1.8. SSRIs and NK1 antagonists- free from effects on NA transmission?

The increased use of SSRIs over older classes of antidepressants led to a more prominent role for 5-HT in theories of the etiology and treatment of depression. The clinical efficacy of SSRIs was not believed to involve an action at NA transmission, since they were thought to be free from effect on this transmitter *in vitro* (see Wong *et al*, 1995). Likewise, despite evidence for substance P activating noradrenergic cell firing (Guyenet & Aghajanian, 1977; Cheeseman *et al*, 1983) the NK1 receptor antagonists had no established effects on noradrenergic transmission, (Kramer *et al*, 1998). However, evidence is emerging that, like other established antidepressants, both SSRIs and NK1 antagonist modulate NA transmission *in vivo*.

1.9. Augmented NA transmission by SSRIs

Evidence has accumulated over the last decade for augmentation of NA transmission *in vivo* by SSRIs. The majority of studies have employed *in vivo* microdialysis in freely-moving rats. Table 1.1 summarizes the results from microdialysis studies showing the effect of acute SSRI administration on NA `efflux'. Efflux is defined as the fraction of extracellular transmitter that collects in the perfusate of a microdialysis probe. An increase in the extracellular concentration of NA will be followed by an increase in its probe concentration ('efflux'). These studies examined the effects of systemic and locally applied SSRIs in several brain regions receiving a noradrenergic innervation. Overall, they can be divided into studies where increased efflux has been seen and those where it has not (Table 1.1 A and B, respectively). In general, increased NA efflux after acute SSRI treatment has been reported most commonly in the frontal cortex, with one report in the hypothalamus but not, so far, in the hippocampus or nucleus accumbens.

A) Increased NA efflux reported.

SSRI	REGION	ROUTE	DOSE	REFERENCE
FLUOX	FC	IP	10,20	Jordan *et al* , 1994
	FC	SC	10	Bymaster *et al*, 2002
	FC	SC	10	Gobert *et al* ,1997
	FC	IP	30	Hatanaka *et al*, 2000
	FC	IP	40	Zocchi *et al*, 2003
	FC	SC	1,3,10	Koch *et al*, 2004
	FC	SC	10	Zhang *et al* 2000
	FC	LI	5,50μM	Hughes & Stanford, 1996,1998
	FC	LI	100	Jordan *et al* , 1994
	HYP	IP	10	Perry & Fuller, 1997
	VTA	LI	10-1000μM	Chen & Reith, 1994
FLUVOX	FC	IP	12	Shachar *et al*, 1997
	FC	LI	10μM	Jordan *et al* 1994
CITAL	FC	IP	4,8	David *et al*, 2003
	FC	LI	50μM	Hughes & Stanford, 1998
	LC	LI	100μM	Mateo *et al*, 2000
PAROX	FC	IP	1,4,8	David *et al*, 2003
	FC	SC	30	Beyer *et al*, 2002
SERT	FC	SC	10	Zhang *et al*, 2000

Table 1.1(A). Summary of microdialysis studies of the effect of acute SSRI administration on NA efflux. Doses are in mg/kg, except local infusion probe concentrations, which are given in μM. FC= frontal cortex, HIPP= hippocampus, HYP= hypothalamus, LC= locus coerulus, NAC= nucleus accumbens, VTA= ventral tegmental area. IP= intraperitoneal, SC= subcutaneous, LI= local infusion (*via* microdialysis probe).

B) No change in NA efflux reported

DRUG	REGION	ROUTE	DOSE	REFERENCE
FLUOX	FC	SC	5	Li *et al*, 2002
	FC	SC	30	Beyer *et al*, 2002
	HIPP	IP	10	Page & Abercrombie, 1997
	HYP	SC	10	Li *et al*, 2002
	NAC	SC	10	Li *et al* 2002
FLUVOX	FC	IP	1-20	Jordan *et al*, 1994
	FC	SC	10,20	Bymaster *et al*, 2002
CITAL	FC	IP	1	David *et al*, 2003
	FC	SC	10	Bymaster *et al*, 2002
	FC	IP	30	Hatanaka *et al*, 2000
	VTA	LI	100μM	Chen & Reith, 1994
PAROX	FC	SC	3	Bymaster *et al*, 2002
	FC	SC	1,3,10	Beyer *et al* 2002
	HIPP	SC	5	Hajos-Korcsok *et al*, 2000
SERT	FC	SC	10	Bymaster *et al*, 2002
	FC	IP	10	Thomas *et al*, 1998
	HIPP	IP	10	Thomas *et al*, 1998

Table 1.1(B). Summary of microdialysis studies of the effect of acute SSRI administration on NA efflux. Doses are in mg/kg, except local infusion probe concentrations, which are given in μM. FC= frontal cortex, HIPP= hippocampus, HYP= hypothalamus, LC= locus coerulus, NAC= nucleus accumbens, VTA= ventral tegmental area. I.P.= intraperitoneal, S.C.= subcutaneous, L.I.= local infusion (*via* microdialysis probe).

There could be several different mechanisms by which SSRIs modulate NA transmission *in vivo* (see below). Precisely which or how many of these factors contribute to the action of SSRIs on NA transmission remains undetermined.

1.9.1. Effects of SSRIs on NA reuptake

SSRIs inhibit NA uptake *in vitro*, albeit at higher concentrations than those required for inhibition of 5-HT uptake (for review see Stanford, 1996). Most studies of SSRI inhibition of the 5-HT and NA transporters measure inhibition of uptake of tritiated monoamines in synaptosomal or brain slice preparations *in vitro*. Richelson & Pfenning, (1984) reported K_is for inhibition of [^{3}H]-NA uptake into synaptosomes from rat occipital cortex. Fluoxetine inhibits NA uptake with a K_i value of 0.28μM. K_is for fluvoxamine and citalopram induced inhibition of [^{3}H]-NA uptake were 0.5μM and 4.0μM respectively. Using a different measure of potency (IC_{50} which is valid only for 'within' study comparisons), Koe *et al*, (1983) reported inhibition of NA uptake to hypothalamus synaptosomes for sertraline (1.2μM) fluvoxamine, (1.9μM) and fluoxetine (0.74μM).

Most studies have compared uptake of individual monoamines in different brain regions, (*e.g.* NA uptake in the hypothalamus with 5-HT uptake in the striatum). However, uptake of monoamines varies between different brain regions, (Snyder & Coyle, 1969). For example, Wong *et al*, (1975) determined inhibition of 5-HT uptake by fluoxetine in synaptosomes prepared from different regions of the rat brain. The cerebral cortex and brain stem were most affected, (70 and 50% reduction respectively), with only 23% inhibition in the diencephalon, 19% in the corpus striatum and no effect in the cerebellum. Therefore, the selectivity ratios of SSRIs for inhibition of uptake of 5-HT and NA in different brain regions is misleading.

The effects of fluoxetine on NA/5-HT uptake have been compared in the same brain region. Its selectivity for inhibition of 5-HT uptake over that of NA is only 2-fold in slices of frontal cortical tissue (Harms, 1983) and 20-fold in the hypothalamus, (Thomas *et al*, 1987). Hughes & Stanford (1996) reported marked inhibition of [^{3}H]-NA uptake into cortical synaptosomes by 5.0μM fluoxetine. Comparison of the effects of fluoxetine with desipramine, (regarded as a selective NA reuptake inhibitor) in brain slices (Harms, 1983) or

synaptosomes (Hughes & Stanford, 1996) reported roughly similar potencies of both these drugs for inhibition of NA transport.

Most relevant to effects on NA transmission is the *potency* of a particular SSRI. In other words, regardless of effects on 5-HT uptake, to what extent do these drugs modulate NA transmission by a direct inhibition of its transport? Estimates of potency of inhibition of [^{3}H]-NA uptake by fluoxetine vary from 0.1μM - 9.5μM (Bolden-Watson & Richelson, 1993; Richelson & Pfenning, 1984; Thomas *et al*, 1987; Wong *et al*, 1975). Estimates of plasma concentration of fluoxetine after chronic dosing in humans are 0.5-1.5μM (Amsterdam *et al*, 1997; Baumann P, 1996). Therefore, *in vitro* studies demonstrate direct inhibition of the NA transporter at clinically relevant doses of fluoxetine.

In vivo studies suggest that SSRIs do inhibit NA transport. Gobert, *et al* (2000) reported increased NA efflux in the frontal cortex of freely moving rats given systemic fluoxetine. The authors suggested this effect was due to direct inhibition of NA uptake by fluoxetine, since it was not modified by a range of 5-HT receptor antagonists and unlikely, therefore, to be due to 5-HT-mediated effects (see below).

However, several studies have found no evidence for inhibition of NA reuptake by fluoxetine. Acute and 5-day administration of fluoxetine did not decrease [^{3}H]-NA uptake into rat heart tissue, *ex vivo*, (Wong *et al*, 1975). Also, systemically administered fluoxetine did not decrease *ex vivo* binding of the NA reuptake inhibitor [^{3}H]-nisoxetine in rat cerebral cortex (Bymaster *et al*, 2002). A relatively low concentration of fluoxetine (0.5μM) did not reduce [^{3}H]-NA uptake into synaptosomes from rat cortical tissue (Hughes & Stanford, 1996). Fuller *et al*, (1975) used the neurotoxins α-ethyl-3-hydroxy-4-methylphenethylamine (H75/12) and 6-hydroxydopamine (6-OHDA) to deplete intracellular stores of NA and 5-HT. These agents enter monoamine neurones *via* their respective transporters. Fluoxetine inhibited uptake of the neurotoxin via the 5-HT transporter and prevented depletion of 5-HT. However, fluoxetine treatment did not prevent the depletion of NA by either of these agents. This

inability to prevent the effects of these drugs on NA concentration was taken to indicate that fluoxetine does not block uptake *via* the NA transporter *in vivo*.

1.9.2. SSRI receptor actions

SSRIs have relatively high affinity for several neurotransmitter receptor subtypes, (see Stanford, 1999 for review). SSRIs could modulate NA transmission by directly activating these receptors. Cusack *et al* (1994) reported equilibrium dissociation constants (K_ds) for a variety of antidepressants at a number of different receptor classes. Fluoxetine binds to 5-HT_{2A} receptors with a K_d value of 280nM. Fluoxetine also binds to 5-HT_{2C} receptors (Jenck *et al*, 1993). These authors reported 5-HT_{2C} receptor binding in the pig myenteric plexus for a variety of antidepressants. The rank order of affinities of the SSRIs that were studied was fluoxetine> citalopram> sertraline> fluvoxamine. Palvimaki, *et al*, (1999) reported 5-HT_{2C} receptor occupancy with fluoxetine, with 10-fold greater selectivity over 5-HT_{2A} receptors. Using a phosphoinositide assay, they demonstrated 5-HT_{2C} receptor antagonist actions of fluoxetine. The stereoisomer R-fluoxetine binds to the 5-HT_{2A} and 5-HT_{2C} receptor (Koch *et al*, 2002). Functional GTP-γ-S binding studies indicate that R-fluoxetine acts as an antagonist at both these receptor subtypes. Given that 5-HT has a tonic, inhibitory action on NA neurones (see section 1.12.), antagonism at 5-HT_{2C} receptors would increase noradrenergic cell firing and release. Indeed, systemic administration of 5-HT_{2C} antagonists increases NA efflux in the frontal cortex (Millan *et al*, 1998). This effect of fluoxetine has been proposed to explain its effects on NA transmission, (Millan *et al*, 1998; Bymaster *et al*, 2002) and antidepressant its action (Palvimaki *et al*, 1999, Koch *et al* 2002).

1.9.3. Secondary effects of SSRIs on NA transmission *via* effects on 5-HT transmission.

Interactions between central 5-HT and NA systems are well documented. One way SSRIs could augment NA efflux is by the secondary effects of elevated extracellular. 5-HT can modulate noradrenaline transmission by effects both at the somatodendritic and terminal level. SSRI will affect both these processes by increasing the extracellular concentration of 5-HT.

5-HT effects at noradrenergic cell bodies

Immunocytochemistry and autoradiography studies demonstrate a dense 5-HT innervation of noradrenergic neurones in the LC of the rat (Pickel *et al*, 1977, Leger & Decarries, 1978). Also, 5-HT synthesis inhibition with *p*CPA increases synthesis of NA (McRae-Degueurce *et al*, 1985) and LC firing rate (Ferron *et al*, 1988). These studies demonstrate an inhibitory effect of 5-HT on NA transmission.

Electrophysiological and microdialysis studies in anaesthetized rats confirm that 5-HT has an inhibitory effect on LC neurones (for review see Millan *et al*, 2000). For example, systemic administration of 5-HT agonists, such as quipazine, the 5-HT_2 receptor agonists, 1-(4-bromo-2, 5-dimethoxyphenyl)-2-aminopropane (DOB; Gorea & Adrien, 1988) and 2,5-dimethoxy-4-methylamphetamine (DOM; Rasmussen & Aghajanian, 1986) decrease the discharge frequency of LC neurones in the rat. The effect of these drugs is prevented by the 5-HT_{2A} antagonist, ritanserin. Quipazine and the 5-HT_2 agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) also decrease NA efflux in the hippocampus (Done & Sharp, 1992). Again, these effects are prevented by ritanserin, which also increases LC neuronal activity (Clement *et al*, 1992).

These studies show that 5-HT inhibits noradrenergic neurones in the LC *via* 5-HT_{2A} receptor activation. However, this is not a direct effect on noradrenergic cells since direct application of 5-HT_2 receptor agonists to the LC does not alter the firing-rate of noradrenergic neurones, (Gorea *et al*, 1991). An indirect effect *via* excitation of an inhibitory GABAergic input is more likely, since the inhibitory effects of systemic 5-HT_2 receptor agonists are blocked by the GABA

antagonist, bicuculine (Chiang & Aston-Jones, 1993) or by lesions of the prepositus hypoglossal nucleus, (Gorea *et al*, 1991). This nucleus provides GABAergic afferents to the LC ((Ennis & Aston-Jones, 1989). Therefore, increased extracellular 5-HT after SSRI administration could inhibit noradrenergic neurones *via* this multi-synaptic pathway (Figure 1.3).

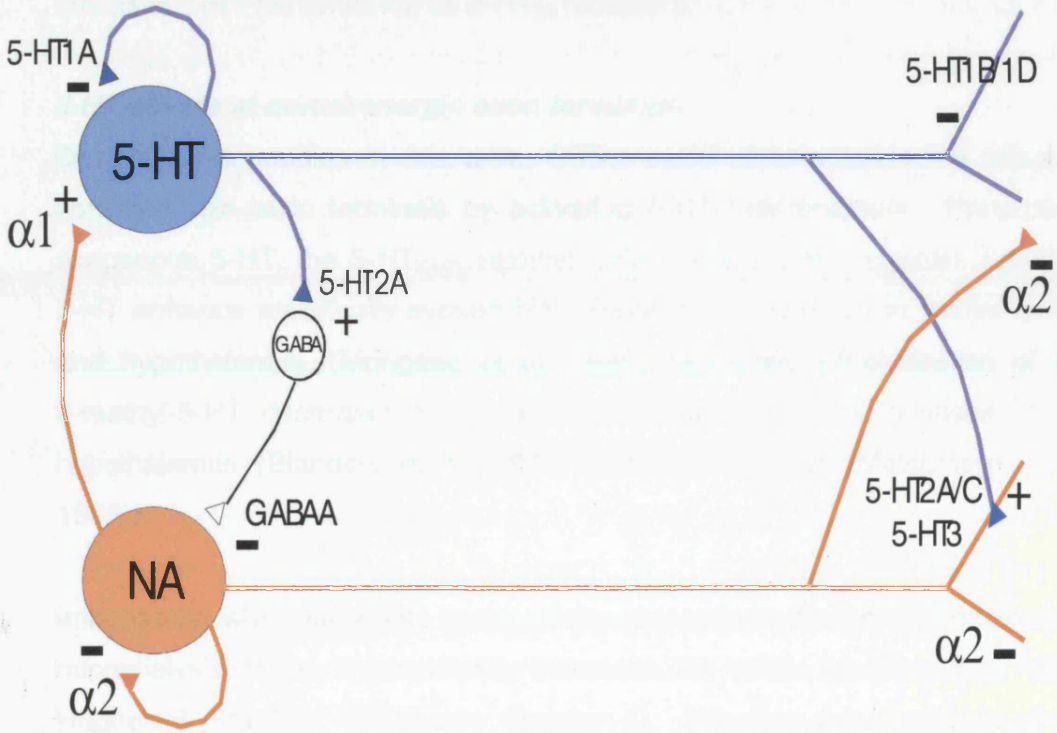


Figure 1.3. Schematic diagram describing possible interaction between noradrenaline and 5-HT neurones. 5-HT= 5-HT-releasing neurones of the DRN; NA=noradrenaline-releasing neurones of the LC; GABA= GABAeric neurones of the prepositus hypoglossal nucleus '+' = excitatory effect; '-' = inhibitory effect. See section 1.12.

Systemic administration of SSRIs preferentially activates presynaptic 5-HT_{1A} autoreceptors in the DRN that reduce 5-HT cell firing and release (Hervas & Artigas, 1998). This effect could increase NA efflux by removing the 5-HT_{2A}-mediated inhibition of noradrenergic cell firing and release (see above). Using the push-pull cannula technique, Kaehler *et al*, (1999) demonstrated a long-lasting reduction of 5-HT overflow in the LC after microinjection of the 5-HT_{1A} receptor agonist 8-OHDPAT to the DRN. Systemic administration of 5-HT_{1A} agonists, (MKC-242, 8-OHDPAT, buspirone, MDL73005EF, S15535) increases

NA efflux in the frontal cortex, hippocampus and hypothalamus of rats (Suzuki *et al*, 1995; Millan *et al*, 1997; Hajos-Korcsok & Sharp, 1996; Hajos-Korcsok *et al*, 1999). 8-0HDPAT and buspirone also increase Fos-like immunoreactivity in the LC (Hajos-Korcsok & Sharp, 1999), while 8-OHDPAT increases the firing of LC noradrenergic neurones (Lejeune & Millan, 2000). Since SSRI administration preferentially activates 5-HT_{1A} receptors on the cell bodies on 5-HT neurones, they could increase NA efflux by diminishing the inhibitory effects of 5-HT transmission as 5-HT_{2A} receptors.

5-HT effects at noradrenergic axon terminals

Despite fewer studies in this area, SSRIs could also increase NA efflux at noradrenergic axon terminals by activating 5-HT heteroceptors. Paroxetine, exogenous 5-HT, the 5-$HT_{2A/2C}$ agonist, DOI and the 5-HT_3 agonist 2-methyl-5-HT enhance electrically-evoked NA release from slices of rat frontal cortex and hypothalamus, (Mongeau *et al*, 1994). However, administration of the 2-methyl-5-HT *decreases* K^+-evoked noradrenaline release in slices of the hypothalamus (Blandina *et al*, 1991) and hippocampus (Matsumoto *et al*, 1995).

Importantly, when fluoxetine is administered locally to the frontal cortex *via* a microdialysis probe, it consistently increases NA efflux, (Jordan *et al*, 1994; Hughes & Stanford, 1998; see Chapter 4). Whether this is due to direct inhibition of NA reuptake (see above) or secondary to its effect on 5-HT remains unresolved. Experiments described in Chapter 4 address this issue.

If the effects of SSRIs on NA efflux are secondary to their effects on 5-HT, they will be attenuated or blocked by a reduction in 5-HT transmission. This can be achieved using the 5-HT synthesis inhibitor, *para*-chlorophenylalanine, (*p*CPA, Koe & Weissman, 1966). This amino acid is a potent and selective inhibitor of tryptophan hydroxylase (Jequier *et al*, 1967), the rate-limiting enzyme in 5-HT biosynthesis. However, if *p*CPA does not abolish fluoxetine's effect on NA efflux, this would support a direct effect of fluoxetine on noradrenergic neurones, possibly due to inhibition of the noradrenaline transporter (see above).

1.9.4. Selectivity of monoamine transporters

The effects of SSRIs *in vivo* will depend on the substrate selectivity of the 5-HT transporter. For example, if NA is also taken into neurones *via* the 5-HT transporter, inhibition of this would elevate the extracellular the concentration of both 5-HT and NA. However, the 5-HT transporter appears to be highly selective. Thus, uptake of [^{3}H]-5-HT in the rat lung was unchanged by NA (10μM) administration (Paczkowski *et al*, 1996). However, a higher concentration of 5-HT (50μM) caused a 56% inhibition of NA uptake, suggesting that 5-HT competes with NA transport at high concentrations. If this is also the case in the brain, the increase in extracellular 5-HT produced by SSRIs could cause a competitive reduction in NA uptake, which could explain the increase in its extracellular concentration.

1.10. Effect of NK1 antagonists on NA and 5-HT

Since all established antidepressants target the noradrenergic and/or 5-HT systems, it is vital to determine whether NK1 antagonists act independently of these systems or if, in fact, they also modulate monoamine function. When originally proposed as antidepressants (Kramer *et al*, 1998) NK1 antagonists were suggested to function independently of monoamines and hence, represent an entirely new approach to the drug treatment of depression. However, substance P affects monoamine function (see below) and preliminary evidence suggests that NK1 antagonists may have similar effects on monoamine transmission as conventional antidepressants. There are several mechanisms that could explain these interactions.

Millan *et al*, (2001) examined the effects of NK1 receptor antagonist administration on NA and 5-HT efflux in the frontal cortex and hypothalamus of freely moving rats. The NK1 receptor antagonist GR205,171 increased NA efflux in both brain regions, but had no apparent effect on 5-HT efflux. A lack of effect of this drug on 5-HT efflux was also reported in the frontal cortex of freely moving mice (Guiard *et al*, 2004). DRN cell firing in anaesthetized rats is similarly unchanged by the NK1 antagonists WIN 51,708 or CP-96,345 (Haddjeri & Blier, 2000).

Despite this lack of effect of NK1 receptor antagonists on 5-HT efflux and cell firing, several studies report changes in 5-HT regulation after inhibition of the NK1 receptor, either using antagonist drugs, or receptor knockout mice (see below). The NK1 receptor antagonist, RP67580, increased the firing-rate of 5-HT neurones in the DRN of anaesthetized mice (Santarelli *et al*, 2001). Spontaneous DRN cell firing was also greater in NK1 receptor knockout mice. This study also demonstrated reduced 5-HT_{1A} receptor function in NK1 knockout mice, since the decrease in 5-HT cell firing and body temperature after administration of the 5-HT_{1A} receptor agonist, 8-OHDPAT, were diminished in these animals.

Down-regulation of 5-HT_{1A} autoreceptors is a common effect of chronic treatment with many antidepressants and is thought to be central to their long-term effects on 5-HT transmission and therapeutic efficacy (Blier & DeMontigny, 1983; Artigas *et al*, 1996). The above study demonstrates that loss of NK1 receptor function leads to changes in 5-HT regulation in a manner common to that of conventional antidepressants. Confirmation of decreased 5-HT_{1A} receptor function was provided by a study by Froger *et al*, (2001): they reported reduced DRN 5-HT_{1A} receptor labeling by [^{3}H]-WAY100,635, decreased DRN 5-HT_{1A} receptor mRNA, a reduced hypothermic response to the 5-HT_{1A} agonist 8-OHDPAT and reduced efficacy of the 5-HT_{1A} agonist, ipsapirone, to diminish 5-HT cell firing in NK1 receptor knockout mice. Nevertheless, NK1 knockout mice had no difference in spontaneous DRN cell firing or basal efflux of 5-HT in the frontal cortex.

Despite evidence for modulation of 5-HT transmission by NK1 receptor antagonists, or in NK1 receptor knockout mice, a direct effect on 5-HT neurones is unlikely. NK1 receptors are located on few if any 5-HT cell bodies of the DRN. Thus, Froger *et al*, (2001), using double labeling with antibodies raised against 5-HT and the NK1 receptor, found no co-expression of these by neurones of the DRN. Likewise, Santarelli *et al*, (2001), found little overlap between 5-HT neurones and NK1 receptors in the DRN. Therefore, the effects of NK1 receptors antagonists on 5-HT transmission, and those reported in NK1 receptor knockout mice, may be due to indirect effects.

In contrast to its relationship with 5-HT, there is anatomical evidence of a direct substance P- NA interaction. Substance P-containing fibres form axodendritic synapses with noradrenergic neurones of the LC, (Pickel *et al*, 1979). Using double immunolabelling in the rat, Chen *et al* (2000) found that 100% of tyrosine hydroxylase labeled neurones in the LC complex co-express NK1 receptors. Localization of NK1 receptors on noradrenergic neurones in the LC has also been reported in mice, (Santarelli *et al*, 2001). Functional evidence also suggests substance P-mediated effects on NA transmission. Thus, application of substance P *in vivo*, (Guyenet & Aghajanian, 1977), or *in vitro* slice preparations, (Cheeseman *et al*, 1983) increases LC cell firing-rate. Paradoxically, systemic administration of an NK1 antagonist also increases NA efflux in the frontal cortex of freely moving rats (Millan *et al*, 2001) and increases the number of LC c-fos-immunoreactive cells in response to restraint stress (Hahn & Bannon, 1998). Since NK1 receptor activation depolarizes neuronal membranes, the fact that both agonists and antagonists at this receptor increase the firing-rate of LC neurones seems problematic. This could reflect populations of NK1 receptors, both on LC neurones themselves, and on inhibtory afferents to the LC. Thus, inhibition of NK1 receptors located on GABAergic afferents to the LC from the prepositus hypoglossal nucleus would produce a disinhibition of noradrenergic neurones.

Down-regulation of α_2-adrenoceptors is a common feature of long-term treatment with antidepressant drugs of several different classes (see Invernizzi & Garattini, 2004). NK1 antagonists attenuate the suppressant effect of α_2-adrenoceptor activation on LC firing-rate, suggesting a common mechanism of action of NK1 antagonist and conventional antidepressants, (Haddjeri & Blier, 2000). However, despite increased burst firing of guinea pig LC neurones following chronic treatment with the NK1 antagonist, L760,735, no down-regulation of α_2-adrenoceptors was found, (Maubach *et al*, 2002). The impact of NK1 receptor ablation on NA transmission has not been studied so far (see below).

1.11. Approach

NA is central to our understanding of depression and its treatment, since the original monoamine theory of Schildkraut to the development of new treatments that selectively target this transmitter, (*e.g.* the selective NA reuptake inhibitor, reboxetine). Given the large proportion of depressed patients who show no improvement after antidepressant treatment, an increased understanding of the neurochemical effects of these drugs is necessary. Unlike all other antidepressants, effects on the noradrenergic system are not considered central to the effects of SSRIs. Likewise, a role for NA has yet to be established in the action of the putative, novel NK1 receptor antagonists. However, there is growing evidence that these drugs modulate NA function *in vivo*. The aim of experiments in this thesis was to determine the effects of acute SSRI treatment (fluoxetine) and NK1 receptor blockade (using NK1 receptor knockout mice) on NA transmission *in vivo*. A dual approach is used, recruiting both *in vivo* neurochemical measurement of NA efflux using microdialysis and scoring of behaviours sensitive to changes in NA transmission in a modified light/dark exploration test.

The effects of fluoxetine were studied in rats. Previously microdialysis studies, and experiments presented here, suggest that fluoxetine augments NA efflux (see above). However, often this effect is not replicated. In addition, fluoxetine's effects on NA efflux vary across brain regions. As yet, it is unknown whether this variation represents differences in NA regulation between brain regions, or simply the greater number of studies performed in the frontal cortex. The aim here was to examine the effect of fluoxetine on NA efflux by comparing its effects in two brain regions receiving noradrenergic innervation from different brainstem sources (frontal cortex and hypothalamus). Since 5-HT can augment NA transmission, the ability of fluoxetine to increase NA efflux could be secondary to its effects on 5-HT. This was determined by administering fluoxetine to rats pretreated with the 5-HT synthesis inhibitor *para*-chlorophenylalanine (*p*CPA).

Because of the lack of availability of compounds with high affinity for the rat and mouse receptor NK1 receptor, a strain of NK1 receptor knockout mice was used. Receptor knockout models represent 'lifelong' inhibition of that receptor,

since it is absent throughout life. This may be advantageous when studying a receptor related to antidepressant therapy, since long-term treatment with antidepressant is required for clinical efficacy.

Knockout mice are generated by genetic modification of embryonic stem cells. These cells are transfected with a DNA vector- a portion of DNA that is identical to the allele of interest (*e.g.* NK1 receptor), but altered to render it non-functional. When these cells divide, a proportion of them have the parent allele replaced by that containing the non-functional vector. When these transfected cells are introduced to a blastocyst and incubated in a foster mother, the resultant offspring contain the targeted allele in cells of their germline. These offspring are backcrossed with normal mice to generate heterozygotes for the non-functional, 'null' allele. Inbreeding of heterozygotes generates offspring that are homozygous for the null allele, with no functional copy of the targeted gene, *i.e.* 'knockouts'.

NK1 receptor knockout mice offer a model of lifelong inhibition of the receptor. By genetically ablating the NK1 receptor, the lack of high affinity, poor brain penetrance and short half-life of antagonist drugs are obviated. However, loss of function of a receptor could produce compensatory adaptations during development, which could lead to functional changes not seen after long-term administration of an antagonist. Ultimately, both approaches offer advantages and both have limitations.

In summary, experiments here use the same line of NK1 receptor knockout mice as that used in the study by Froger *et al*, (2001, see above), which demonstrated diminished 5-HT_{1A} receptor function in the knockout mice. It is unknown how NK1 receptor ablation modulates 5-HT transmission since these receptors are not found on 5-HT neurones (see above). One possibility is *via* effects on NA transmission. So far, the impact of NK1 receptor ablation on this transmitter has not been explored.

1.12. Aims

The aim of experiments in this thesis was to determine the effects of fluoxetine and NK1 receptor ablation on NA transmission *in vivo*. Since drugs that belong to these classes (*i.e.* SSRIs, NK1 receptor antagonists) are not considered to target NA as their primary mode of action, demonstration that they modulate this transmitter *in vivo* will add to the growing body of evidence that NA is implicated in the mechanism of all antidepressant drugs.

Chapter 2

Materials and methods

2.1. Introduction

The techniques used in this study were chosen to measure changes in NA transmission elicited by drug administration or genetic ablation. Neurochemical changes in NA efflux were monitored using *in vivo* microdialysis, a now widespread *in vivo* sampling technique that is used to study many endogenous substances. The effects of these treatments on NA function were also measured using a behavioural assay: a modified version of the light/dark exploration box. Both techniques are discussed here.

2.1.1. *In* vivo sampling techniques

Technologies that enable measurement of neurotransmitters *in vivo* have been developed over the past 40 years. This became possible when assay procedures of suitable sensitivity were developed. Several techniques are employed, both in freely-moving and anaesthetized animals. A brief description of each of these follows.

The Cortical cup

The cortical cup (MacIntosh & Oborin, 1953) enables recovery of substances from the surface of the cerebral cortex for subsequent *ex vivo* analysis. A small cylinder (0.2 – 1 cm^2) is placed in tight contact with the surface of the cerebral cortex. The cylinder is filled with artificial CSF, into which neurotransmitters diffuse from the surface of the brain. The fluid is exchanged periodically and analyzed for neurotransmitter content. The cortical cup can be applied in anaesthetized or conscious animals. The technique has been used to study the relationship between behavioural changes and neurotransmitter release (Rasmusson & Szerb, 1976). However, applications of the cortical cup are limited as it only allows measurement of neurotransmitter release from the neurones at the upper layers of the cortex.

The Push-pull cannula

The push-pull cannula (Gaddum, 1961; Stadler *et al*, 1975) is composed of two concentric hollow fibres or steel cannula, the inner for delivery of perfusion medium, the outer for collection of the fluid (outer diameter >2 mm). Neurotransmitters and other substances are taken up by the flow of fluid from the cannula for subsequent analysis. The push-pull cannula was a significant advancement over the cortical cup, since neurotransmitters could be measured from discrete locations within the brain. Drugs can be included in the perfusion medium and applied directly to the site of interest (Dietl *et al* 1981). The response of local neurones to direct electrical stimulation can also be studied by using the outer tube of the cannula as a monopolar electrode (Philippu *et al*, 1973). However, the push-pull cannula causes extensive tissue damage due to the flow of artificial CSF in direct contact with nervous tissue. Since the ends of the inlet and outlet tubing are exposed, they can become blocked by tissue debris.

In vivo voltammetry

In vivo voltammetry (for reviews see: Adams, 1978; Stamford, 1989) enables measurement of monoamine concentration *in vivo,* by exploiting the electrochemical properties of these neurotransmitters. The voltammetry probe is a fine diameter (8 – 300 μm) carbon fibre microelectrode that causes minimal tissue damage when inserted into small structures of the brain. A positive potential is applied to the electrode, which oxidizes monoamines at its surface. The current produced by the release of electrons is amplified and detected. This current is directly proportional to the number of neurotransmitter molecules oxidized, in accordance to Faraday's Law. 'Fast-cyclic' voltammetry allows almost real time measurement of changes in the extracellular concentration of neurotransmitters and, therefore, has the greatest temporal resolution of all *in vivo* sample techniques. However, since there is no separation of the ECF solutes prior to detection of transmitters, problems of selectivity can arise. The oxidation potentials of monoamines and their metabolites (and certain drugs) are very similar. This is the main drawback of the technique.

2.2. *In vivo* microdialysis

Dialysis is defined as any process by which solutes of different kinds are selectively removed from a liquid as a consequence of their capacity to pass through a semi-permeable membrane. The first use of *in vivo* dialysis was by Bito *et al*, (1966). They implanted semi-permeable membrane sacs filled with 6% dextran in saline to the cerebral cortex of dogs. 10 weeks later, the sacs were removed and their contents analyzed for amino acid content and other electrolytes. Using this method, the composition of the brain extracellular fluid was compared with that of blood plasma and subcutaneous tissue. This group later attempted to take repeated samples from *in vivo* dialysis fluid by using dialysis sacs fitted with polythene tubes.

Delgado *et al* (1972) developed the 'dialytrode'. This was a push-pull cannula with a small membrane sac fitted over the exposed ends of an inlet and outlet tubing. This created a closed environment through which Ringer's solution was passed. Amino acids and other molecules passed into the membrane sac. As well as causing less damage to the surrounding tissue, the addition of a membrane protects recovered neurotransmitters from enzymatic degradation.

Modern microdialysis involves insertion of a probe (250-300 μM diameter) into the brain region or tissue of interest. The end of the probe is a small cylinder of semi-permeable membrane (Ungerstedt & Pycock, 1974). The membrane allows diffusion of small molecules and water. It is fitted with an inlet and outlet and continuously perfused with a medium devoid of the substance of interest. This creates a concentration gradient between the ECF and the 'perfusate' that results in movement of small molecules across the membrane and into the probe, where they are extracted for subsequent analysis. In this way, a microdialysis probe acts as an artificial capillary: the amount of transmitter recovered is proportional to its concentration in the ECF.

2.2.1. Probe design

Several different probe designs have been developed. The 'transcranial' probe (Imperato & DiChiara, 1984) is composed of a length of dialysis membrane passing through the brain transversely. This design has the advantage of

offering a large surface area for exchange of molecules to occur, but is obviously limited in terms of spatial resolution.

The 'loop' probe (Hernandez, *et al*, 1983; Abercrombie *et al,* 1988) is made from a length of membrane, bent into a vertical loop at the end of the probe. Again this increases the surface area over which dialysis occurs, but can also increase damage to the area being studied.

The 'concentric' probe design has the inlet tubing placed inside the outlet, with a small diameter, hollow fibre of dialysis membrane at the end (Tossman & Ungerstedt, 1986).

All experiments described in this thesis use the 'side by side' probe (Sandberg *et al*, 1986). This is similar to the concentric probe design, but has the inlet and outlet tubing next to one another with a small diameter hollow membrane fibre at the tip.

2.2.2. Neurotransmitter 'efflux'

Brain tissue has three fluid compartments: the intracellular fluid, the extracellular fluid and the vascular fluid. When neurotransmitters are released, they diffuse into the extracellular space. At the same time, neurotransmitters are cleared from the ECF by reuptake and enzymatic degradation. What dialysis is sampling, therefore, is the net result of these processes. It is not measuring release *per se*, but rather the concentration of substances that pass into the extracellular fluid, which is determined by the balance of release and reuptake/degradation. The term 'efflux' is used to describe the amount of transmitter that reaches the probe.

2.2.3. Probe 'recovery'

Since the perfusion medium in the probe is not static, the concentration of substances within the probe will not equal that in the surrounding tissue. The 'recovery' of a microdialysis probe is defined as the ratio between the concentration of a substance in the perfusate and the concentration of the same substance in the solution outside the probe: this is normally expressed as a percentage (Zetterstrom *et al*, 1982). Recovery can be calculated *in vitro* by

perfusing the probe at a constant flow rate in a solution of known concentration of the substance of interest. The recovery is calculated from the concentration of substance in the perfusate relative to that in the external solution. However, extrapolation from an *in vitro* model to the situation *in vivo* is not straightforward. Passage of substances in brain tissue in not the same as in aqueous solution, since mass transport is confined to the extracellular space, which comprises only 20% of the total brain volume (Levin *et al* 1970).

For experiments described in this thesis, the concentration of NA in the perfusate was not corrected for probe recovery, since the objective was not to quantify the absolute concentration of NA in the ECF. The aim was to monitor changes in NA efflux relative to spontaneous, 'basal' efflux. However, both approaches require a constant probe recovery. Several factors are known to influence the recovery of transmitters by microdialysis. Therefore, these must be carefully controlled.

The main factors that determine probe recovery are:

- membrane surface area
- perfusion ('flow') rate
- composition of the perfusion medium
- membrane material

Membrane surface area

The larger the surface area of the dialysis membrane, the greater area available for exchange of molecules to occur. Therefore, the greater the recovery (Sandberg & Lindstom, 1983; Tossman & Ungerstedt, 1986). Increasing the membrane area is normally achieved by increasing the length of membrane fitted to the end of the probe. However, the greater the membrane length, the lower the spatial resolution and greater the tissue damage.

Perfusion ('flow') rate

The flow rate of the perfusion medium will determine the concentration gradient between inside and outside the probe, *i.e.* lower flow rates will allow greater recovery and a lower concentration gradient. Increasing the flow rate will

increase the concentration gradient, but also reduce the relative recovery, since the volume of perfusate flowing per unit time will be increased in relation to the amount of substance recovered. High flow rates should be avoided since they can produce hydrostatic pressure within the membrane, expelling fluid to the ECF and causing tissue damage. A flow rate in the region of 2 μl/min is typical.

Composition of perfusion medium ('perfusate')

The perfusion medium is a critical consideration for microdialysis experiments. Differences in composition between the medium used and the ECF will lead to additional concentration gradients between the two compartments that could affect the recovery of the substance of interest. Also, removal of ions from the ECF, or addition of ions from the perfusate to the ECF can disrupt the electrical activity of neuronal cell membranes and the *in vivo* release of neurotransmitters. K^+ and Ca^{2+} ions are particularly important in this respect (Abercrombie *et al*, 1988; Westerink *et al*, 1988). A perfusion medium with a K^+ concentration greater than that of the ECF will raise the ECF K^+ concentration as it crosses the membrane down its concentration gradient. Elevated ECF K^+ concentration leads to depolarization of neurones, increasing neurotransmitter release. Conversely, if Ca^{2+} concentration is lower in the perfusion medium that ECF, these ions will be removed from the area surrounding the probe. Since neurotransmitter release is a Ca^{2+} dependent process, this will reduce neurotransmitter release (Dalley & Stanford, 1995).

The perfusion medium used for microdialysis in this thesis was a modified Ringer's solution containing 4 mM KCl, 145 mM NaCl and 1.3 mM $CaCl_2$, pH 6.8. Continuous perfusion with this medium produces a stable spontaneous efflux of NA, which is within the limits of detection of the assay (see below).

Membrane material

Most non-commercial microdialysis probes are made with renal dialysis membrane. A variety of materials is available. Each must be porous enough to allow passage of water and substances of interest, but impermeable to larger molecules to prevent passage of unwanted components of the ECF, which could potentially contaminate the detection assay. Typically, the molecular

weight cut-off varies between 10-50 KDa. A vital property of dialysis membrane is biocompatability, *i.e.* the membrane should not interact physiologically with living tissue, cause immune responses or have toxic effects. In this thesis, two types of membrane fibre were used. For experiments using rats, a synthetic AN69, acrylonitrile and sodium methallyl sulfonate copolymer (Hospal industries) was used. For experiments using mice, a smaller diameter, regenerated cellulose membrane (cuprophan) was used, to minimize tissue damage. Since no comparisons were made between data from microdialysis experiments using rats and mice, the use of different membrane materials is of no consequence. What is critical is that all properties of the microdialysis probe are consistent within studies where comparisons are made.

2.3 *In vivo* Microdialysis procedure

2.3.1 Construction of probes

The microdialysis probes used in this study were custom-made in the laboratory.

Probes for use in rats

Two 4cm lengths of silica tubing (i.d. 75μm, o.d. 150μm, SGE) are inserted to a 2cm length of stainless steel cannula (i.d. 380μm, o.d. 500μm, Goodfellow). These are glued in place with epoxy resin. Two 0.5cm lengths of stainless steel tubing are placed over the fixed ends of the silica, which act as the inlet and outlet channels. The lengths of stainless steel are fixed in position with epoxy resin. Two 5cm lengths of Portex fine bore tubing (i.d. 280μm, o.d. 610μm) are fitted over the silica and glued in place. A length of dialysis membrane (i.d. 240 μm, o.d. 300 μM; molecular weight cut-off 40KDa; Filtral 12, AN69; Hospal Industries) is placed over the end of the silica and glued inside the steel cannula to leave a length of exposed membrane. This is trimmed to 2.5 cm and the end sealed with epoxy resin. The length of membrane exposed is 2 cm. Figure 2.1 shows the main stages of probe construction.

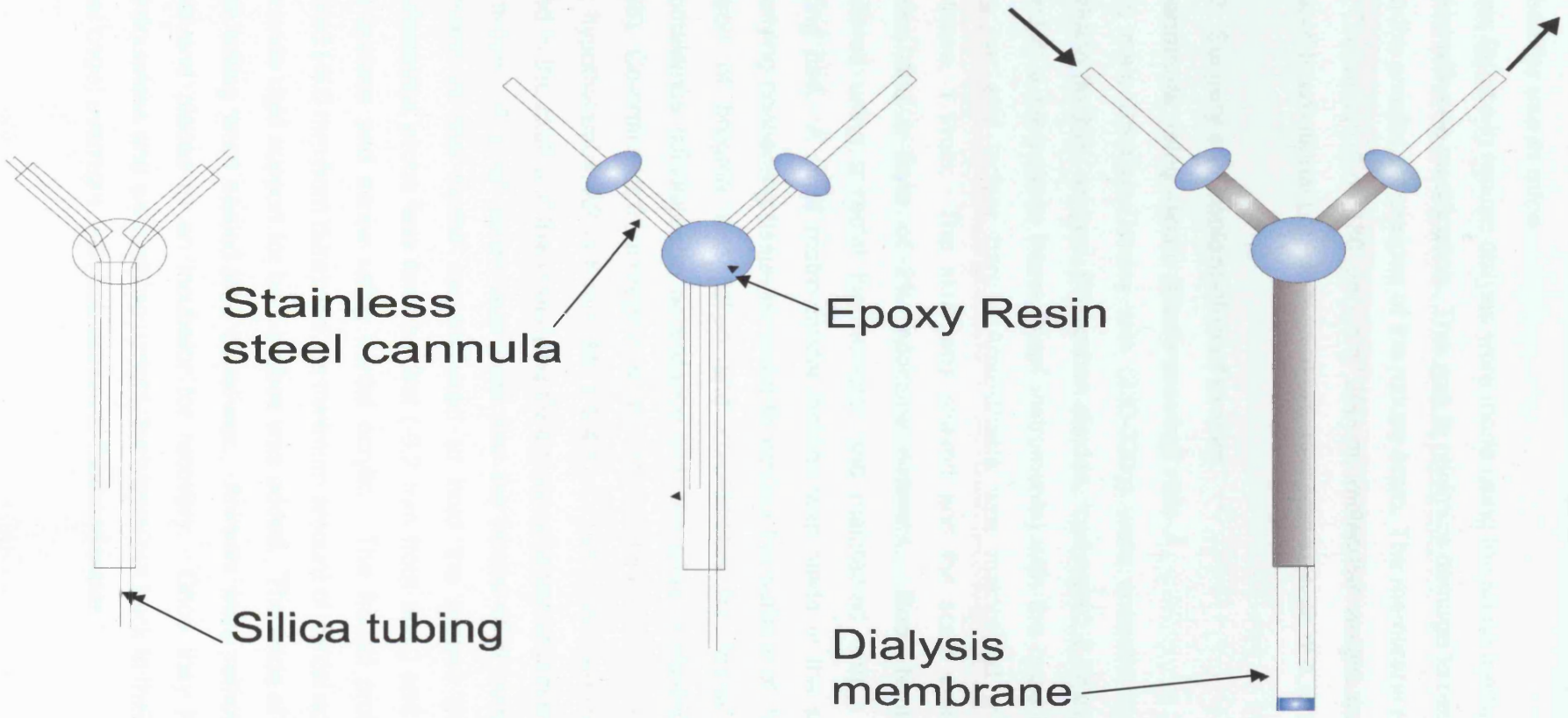


Figure 2.1. Diagram showing the main stages of microdialysis probe construction. Arrows depict direction of perfusion medium flow. Details of construction are given in section 2.3.1. Drawings are not to scale.

Probes for use in mice

Probes for use in mouse dialysis were made using the same method as used in rats but with one modification. This was to minimize damage to neuronal tissue, given the smaller dimensions of the mouse brain. The membrane material used was Cuprophan (i.d. 180 μm, o.d. 250μm; molecular weight cut-off 10KDa; Medicell International Ltd), with an active membrane length of 1.5mm.

2.3.2. Surgery and implantation of probes

Experiments using awake (freely-moving) rats

Adult, male Sprague-Dawley rats (230-320g) were anaesthetized using 5% halothane (in 95% oxygen, 5% carbon dioxide, 'carbogen'; 2 l/min). They were placed in a stereotaxic frame (Kopf instruments) with the head held in place using ear and incisor bars. Anaesthesia was maintained at 2.0 - 2.5% halothane, 1 l/min. The skull was shaved and the scalp coated in iodine solution and a layer of 2% xylocaine ointment. Body temperature was monitored using a rectal thermometer and maintained with a thermostatic heating pad. A small rostro-caudal incision was made in the scalp and the underlying connecting tissue removed to expose the surface of the skull. The position of bregma was taken and co-ordinates for frontal cortex and hypothalamus calculated in accordance with the atlas of Paxinos & Watson (1998). Co-ordinates from bregma were (mm): frontal cortex; AP +3.5, ML ± 1.5 mm; hypothalamus; AP -1.8 mm, ML ± 0.4 mm. Two small trephine holes were drilled in the skull and the underlying dura mater pierced to prevent damage to the probe. A small screw was fixed into the frontal skull surface for extra purchase of the dental cement used to hold the probes in place. The hypothalamus probe was lowered first (-9.2 mm from dura) and glued to the skull surface and screw using dental acrylic. The frontal probe was then lowered (-4.0 mm from dura) and the minimum amount of dental acrylic required to provide rigid support for both probes was added. The ends of the inlet and outlet tubing were sealed with bone wax. Animals were removed from the frame and placed in an incubator for recovery. Once they had regained consciousness and were sitting upright, they were put back in their homecages (1 per cage) overnight, with free access to food and water.

Experiments using anaesthetized mice

Adult, male NK1 wild type and NK1 knockout C57BL/6 x SvEv mice (25-30g) were anaesthetized with 5% halothane in 70% nitrogen, 30% oxygen mixture (1.5 l/min). They were placed in a stereotaxic frame (Kopf instruments) with the head fixed in place with a mouse adapter. Anaesthesia was maintained throughout at approximately 1.5% halothane. An incision was made in the scalp to expose the surface of the skull. The position of bregma was taken and probe placement determined relative to bregma using the atlas of Paxinos and Watson (2001). Co-ordinates for the frontal cortex were (mm): AP +2.1, ML ±1.0. A small trephine hole was drilled in the skull and the dura mater pierced to prevent damage to the dialysis membrane on entry. The probe was fitted to the holder of the stereotaxic frame and slowly lowered into position, (-2.0 mm from the surface of the dura).

2.4. The detection assay- High Performance Liquid Chromatography with Electrochemical Detection, (HPLC-ED)

2.4.1. HPLC

Given the low concentrations of neurotransmitters collected using microdialysis, a highly sensitive assay must be available for the technique to be useful. The development of liquid chromatography for separation of catecholamines was developed over 20 years ago (Kissinger *et al,* 1975; Refshauge *et al.* 1974). This is used to separate the components of the dialysate prior to their measurement with electrochemical detection. Separation is achieved by exploiting differences in the relative affinities of the constituent compounds to a solid, non-polar phase (the 'stationary phase') and a polar, liquid phase (the 'mobile phase').

In this type of 'reversed phase' chromatography, the stationary phase is a hydrocarbon-bonded surface, usually octadecyl silyl (ODS) bonded to silica. This particulate phase is densely packed within a length of stainless steel housing (the 'column'). The mobile phase is a pH buffered water/methanol mixture. Samples are introduced to the flow of mobile phase and passed through the stationary phase at a constant flow rate. The most polar components of the sample will elute first, having lowest affinity for the non-polar stationary phase. By adjusting the pH of the buffer, the polarity of compounds

within the sample can be adjusted, therefore determining their retention to the stationary phase. Individual compounds are identified by their 'retention time' *i.e.* the time taken to pass through the column and onto the electrochemical cell.

2.4.2. Electrochemical detection

After passing through the column, the mobile phase undergoes oxidation at an electrochemical cell. Electrochemical detection of NA is possible since this molecule is readily oxidized. A positive potential is applied to the working electrode of the cell in contact with the mobile phase. The release of electrons creates a current, which is amplified and detected. The amount of current produced is directly proportional to the number of molecules of NA present in the sample, in accordance with Faraday's Law.

The optimum oxidizing potential for detection of NA was determined systematically. A standard solution of NA was used to determine the peak heights produced by a range of applied potentials. Figure 2.2 shows the resulting 'voltammagram'. A potential of +180mV was chosen as, at this potential, NA oxidation is saturated. Lower potentials will result in incomplete oxidation of NA (and reduced sensitivity of the system). Higher potentials are unnecessary and will lead to oxidation of additional component of the perfusate, producing unwanted noise or peaks on the chromatogram, making separation of the NA more difficult.

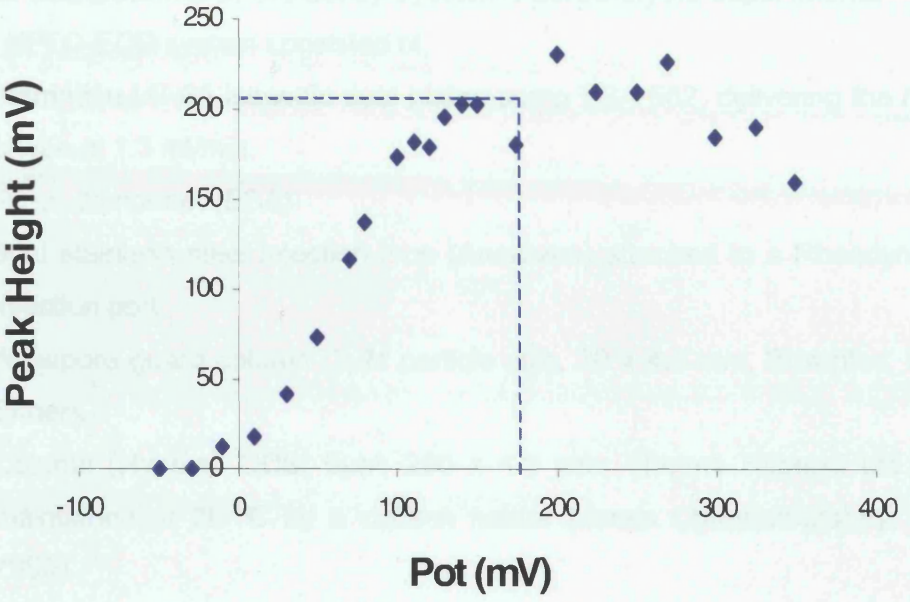


Figure 2.2. Voltammagram demonstrating optimum oxidation potential of NA. X-axis shows the oxidation potential applied to the working electrode of the electrochemical cell; Y-axis shows the resulting peak height produced on the chromatogram. A standard NA solution of 100fmol/50µl was used to generate all peaks. Dotted line shows the optimum potential (+180mV). See section 2.4.2.

In addition to measuring the content of NA in microdialysis samples, HPLC-ECD was also used to measure the content of NA, 5-HT and dopamine in brain tissue. This was to establish the extent of monoamine-depleting chemical lesions in experiments using DSP-4 and *p*CPA pretreatment in rats (Chapters 3-5). Some differences exist between the HPLC-ECD procedures used for these tasks, reflecting the approximately 1000-fold lower concentration of NA in dialysis samples, compared to homogenized brain tissue.

2.4.3. Components of the assay system- microdialysis experiments

The HPLC-ECD system consisted of:

- Shimadzu LC 6A isocratic dual piston pump ESA 582, delivering the mobile phase at 1.3 ml/min.
- Pulse dampener (ESA)
- 50µl stainless steel injection loop (Anachem) attached to a Rheodyn 7125 injection port.
- Aquapore guard column (7µM particle size, 30 x 4.6 mm, Brownlee, Perkin Elmer),
- Column (Hypersil ODS; 5µM; 250 x 4.6 mm; Thermo Hypersil Ltd., UK), maintained at 26 ^{0}C by a column heater (Jones Chromatography, model 7955).
- An analytical cell: ESA 5014 electrochemical cell, with two electrodes in series; a conditioning electrode set at –280mV and a measuring electrode set at +180mV.
- An ESA model 5100A coulometric detector.
- As Spectraphysics Chromjet integrator.

The mobile phase consisted of HPLC grade water with 12% methanol containing 2 mM octanesulphonic acid (OSA), 0.1 M sodium dihydrogen orthophosphate (NaH_2PO_4, anhydrous), 0.67mM ethylenediaminetetraacetic acid (EDTA, disodium salt). The pH of the mobile phase was adjusted to 3.75 using hydrochloric acid.

2.4.4 Measurement of brain tissue monoamine content.

To determine the effect of pretreatment with either DSP-4 or *p*CPA, the frontal cortex and hypothalamus were dissected from the rest of the brain post-mortem. These were homogenized in 10µl/mg perchloric acid ($HClO_4$) and centrifuged at 13 000 x g for 5 min. The supernatant was injected manually onto a Hypersil ODS (5µm particle size, 250 x 4.6 mm, Thermo Hypersil Ltd., UK) HPLC column using a 50 µl stainless steel injection loop (Anachem) and a Rheodyn 7125 injection port. The system was fitted with an Aquapore guard column (7µm, 30 x 4.6 mm, Brownlee, Perkin Elmer) between the sample loop and the main column. Electrochemical detection of NA, dopamine and 5-HT

was achieved using a BAS LC4B detector and an amperometric cell, with a Ag/AgCl reference electrode and a working electrode set to an oxidizing potential of +750 mV. The mobile phase consisted of water and 10.5% methanol with 0.1 M sodium dihydrogen orthophosphate buffer (Na_2HPO_4, anhydrous), 2.8 mM octanesulphonic acid (OSA, sodium salt) and 0.7 μM ethylenediaminetetraacetic acid (EDTA, disodium salt). The mobile phase was adjusted to pH 4 using orthophosphoric acid.

Chromatogram peaks were converted to monoamine concentration (ng/50μl) by reference to standard solutions of known monoamine concentration (Figure 2.3). This was expressed and ng/g of wet tissue.

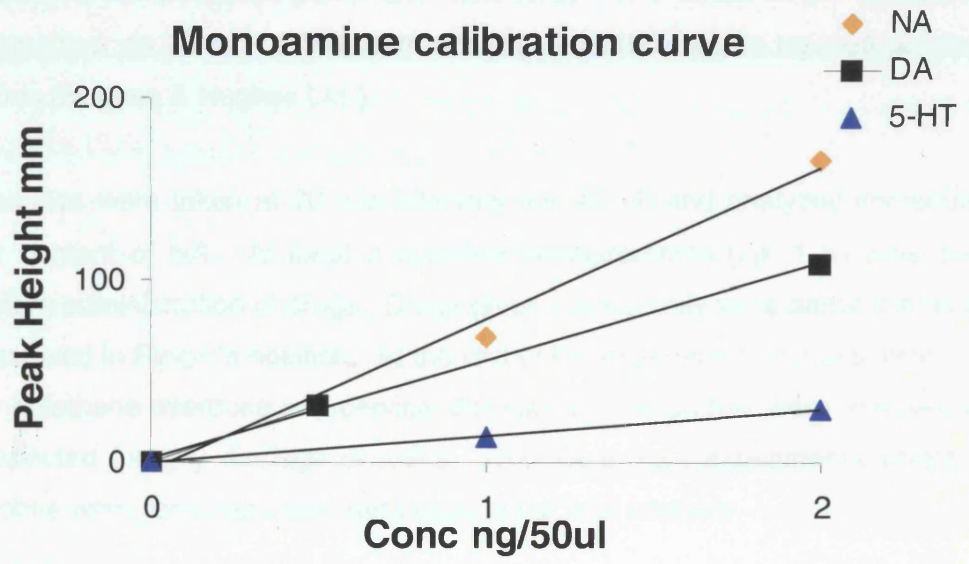


Figure 2.3. Monoamine calibration curve. Standard solutions of NA, DA and 5-HT were used to calibrate sample peak heights to fmoles. This was then converted to ng/g of wet tissue.

2.5. *In vivo* microdialysis- experimental procedure

Chapters 3 & 4 describe experiments using *in vivo* microdialysis in freely moving rats. Chapter 6 describes microdialysis experiments performed on anaesthetized mice. The methods sections of the individual chapters give details of the precise drug treatments and experiments performed. The following descriptions are of procedures common to all the experiments using microdialysis.

2.5.1. Freely-moving rats

The morning after surgery, (see 2.3.2) rats were moved (in their homecages) to the experiment room. The sealed ends of the inlet and outlet tubing were trimmed and the inlet connected *via* polythene tubing to a 2 ml syringe containing Ringer's solution. The probes were perfused at 2.0 µl/min using a multi-channel perfusion pump (KD Scientific). The outlet of the probes was connected via 20 cm of fine-bore tubing (1.2 µl/100 mm, to tapered polythene tubes (Hughes & Hughes Ltd.).

Samples were taken at 20 min intervals (*i.e.* 40 µl) and analyzed immediately for content of NA. At least 3 baseline measurements (*i.e.* 1 h) were taken before administration of drugs. Drugs given systemically were administered in a dissolved in Ringer's solution. At the end of the experiment, animals were killed by halothane overdose and cervical dislocation. The probes were removed and inspected for any damage or leaks. Only data from experiments where the probes were removed intact were used in the final analysis.

2.5.2. Anaesthetized mice

Microdialysis probes were perfused at 1.5 µl/min with fractions collected every 20 min (*i.e.* 30µl samples). The first three samples were discarded as the perfusate equilibrated with its surroundings. At least 3 basal samples were taken before administration of any drugs. Drugs (given i.p.) were dissolved in saline and given in a volume of 10ml/kg. Local infusion of drugs *via* the probe was achieved by switching the probes' perfusion medium to Ringer's solution containing the drug. At the end of the experiment mice were killed by cervical dislocation. The probes were removed and inspected for any damage or leaks.

Only data from experiments where the probes were removed intact were used in the final analysis.

2.6. Analysis of behaviour

In addition to effects of fluoxetine and NK1 receptor ablation on NA efflux, their effects on behaviour were also determined. The aim was to determine whether any of the behavioural effects of either fluoxetine or NK1 receptor ablation could be attributed to effects on NA transmission. A modified version of the light/dark exploration box was used. This procedure was chosen since its principle component is exposure to a novel, brightly-lit arena. Exposure to such environments is paralleled by increased NA transmission, (Aston-Jones & Bloom, 1981; Dalley & Stanford, 1995; McQuade *et al*, 1999; Mason *et al*, 1998; Rajkowski *et al*, 1994). Moreover, drugs that target central NA transmission (Mason *et al*, 1998; Haller *et al*, 1997; Berridge & Dunn, 1986; Stone *et al*, 2004), as well as NA -depleting lesions (Mason & Fibiger 1977; Delina-Stula *et al*, 1984; Berridge & Dunn 1990; Harro *et al*, 1995) modulate the behavioural response of rodents to novel environments (see Chapters 5 & 7)

The rationale was to identify behaviours that were modulated by manipulation of central NA transmission (using DSP-4 pretreatment in rats and α_2-adrenoceptor antagonists in mice) then to determine whether these behaviours were sensitive to fluoxetine or NK1 receptor ablations.

2.6.1. The light/dark exploration box

The light/dark exploration test apparatus consists of a box partitioned into two compartments joined by a small door (Figure 2.4). The larger compartment (normally two thirds) is a brightly-lit area, while the remaining one third is in darkness. Rodents find the larger, brighter area more aversive than the smaller, dark area. This is borne out by the finding that when placed in the box, animals spend more time in the dark area. A random behaviour predicts that they would spend only on third of the time in the dark area, (reflecting its relative size).

Box design can differ among groups with some using light/dark components of equal size (Griebel *et al*, 1994), while others use separate boxes joined by a connecting tube (Belzung *et al*, 1987) or a black box in the corner of an open

field (Merlo-Pich *et al*, 1986). Other factors which can affect behaviour in the test are strain differences (Griebel et al, 2000), prior test experience (Holmes *et al*, 2001), the time of day (Costall *et al,* 1989) and age of the animals (Hascoet *et al,* 1999)

A variety of parameters are measured in the light/dark exploration test. For example, latency to leave the light area after initial placement there, number of transitions between areas, total time spent in the lit area, locomotor activity in both areas and exploratory rearing. Also, stretch attend postures and thigmotaxis (wall hugging) have been recorded in the mouse (Holmes *et al*, 2001). Traditionally, the aim of scoring these behaviours is to identify those that are sensitive to the anxiolytic effects of drugs. This has meant that most studies measure a limited number of behaviours, such as the total time in the novel arena, or the number of transitions between arenas, both of which are increased by benzodiazepines and barbiturates (Crawley & Goodwin, 1980; Crawley, 1981).

For experiments described here, the light/dark exploration box is used in a novel way *e.g.* is it not used as a screen for 'anxiolytic' activity. Since behaviour in this test is paralleled by changes in central NA transmission (see above) it is employed as an assay to determine whether any behavioural effects of fluoxetine or NK1 receptor ablation could be due to effects on this transmitter.

For this reason, a relatively large number or behavioural parameters were recorded, in addition to those normally taken in studies using the exploration box. This approach was used to increase the likelihood of identifying any behaviours sensitive to manipulation of central NA transmission, since these will not necessarily equate with those that are sensitive to classical anxiolytic drugs.

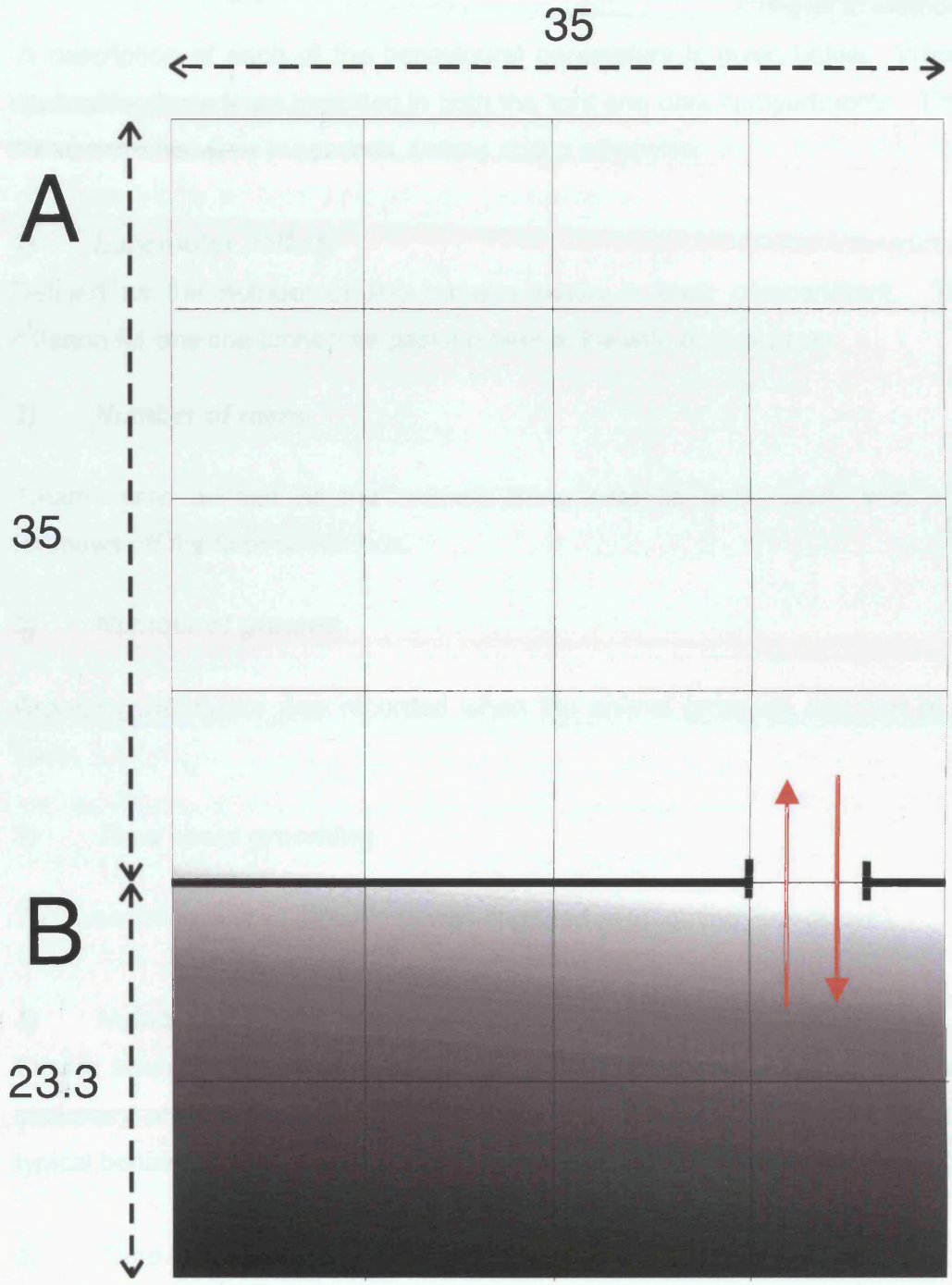


Figure 2.4. The light/dark exploration box (aerial perspective). A= light compartment; B= dark compartment. Dimensions (cm) shown are for the box used with rats. See section 2.7 for a description of box dimensions and experimental procedure for the light/dark exploration test in rats and mice.

A description of each of the behavioural parameters is given below. Where applicable, these were recorded in both the light and dark compartments. Time measurements were in seconds, unless stated otherwise.

1) Locomotor activity

Defined as the number of line crosses made in each compartment. The criterion for one line cross was passing over a line with all four paws.

2) Number of rears

'Rears' were defined as the animals lifting onto its back paws, with both forepaws off the floor on the box.

3) Number of grooms

Grooming behaviour was recorded when the animal groomed any part of its body.

4) Time spent grooming

This was the total time the animal was engaged in grooming (see above).

5) Number of stretch attend postures (SAP)

Stretch attend postures were defined as a flat body- posture with all four feet stationary and the animal stretching forwards. This behaviour is part of the typical behaviour sequelae of rats and mice when exploring novel surroundings.

6) Time to leave light zone

Time to leave was the time taken for the animal to leave the light compartment after initially being placed there. The animal was considered to have left the light compartment when all four paws where in the dark compartment.

7) Time to return to light zone

Time to return was the time between the animal leaving the light compartment when initially placed there, and its first return to the light compartment. A return (both for this behaviour and the total number of returns) was defined as an entry into the light compartment with the entire head and ears.

8) Number of returns to light

This was the total number of times the animal passed from the dark compartment to the light compartment (see above).

9) Time spent in light zone

This was the total time spent in the light, novel arena.

10) TZ activity/min in light zone

'Test Zone activity/minute' was the number of line crosses per minute in the light, novel compartment.

2.7. Light/dark exploration box-experimental procedure:

2.7.1 Experiments using rats

The rat exploration box (Figure 2.4; length 58.3 cm; width 35 cm; depth 35 cm) had a small 'dark zone' (length 23.3 cm; 30 lux) and a larger 'light zone' (length 35 cm; 2000 lux). The walls and floor of the dark zone were black and the walls and floor of the light zone transparent Plexiglas (McQuade *et al*, 1999). The floors of both zones were divided into $12cm^2$ squares (*i.e.* 6 in the dark zone; 9 in the light zone) to allow scoring of locomotor activity. A door (height 12 cm, width 8 cm) separated the two zones, with a removable partition to restrict movement between the zones.

Experiments were performed either in the morning (AM group, 09:00-13:30h) or the afternoon, (14:00-18:30h). A modified light/dark exploration box protocol was used. This involved an extended period of testing (120 min) to provide a time frame similar to that of the microdialysis experiments of Chapters 3 and 4. On the day of testing, rats were placed in the dark zone of the exploration box with the partition closed. After a 1 h habituation period, either fluoxetine or vehicle was administered. Subjects were put back in the dark zone for a further 90 min. They were then transferred to the novel compartment and the door between both compartments was removed. Subjects were placed in the centre of the novel compartment, facing away from the entrance to the dark arena. Behaviour was recorded on VHS film for 2 h for subsequent scoring. (See Figure 2.5.)

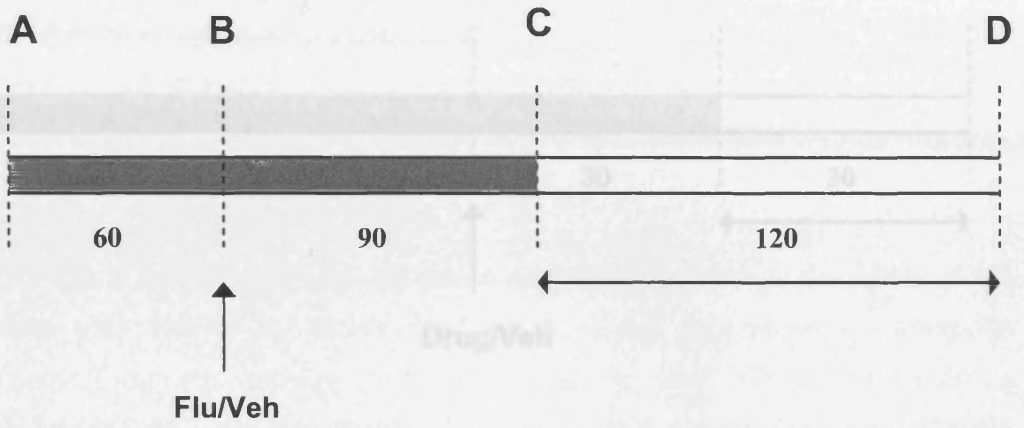


Figure 2.5. Representation of timing of light/dark exploration box testing using rats. A = rat placed in dark zone of box with door closed for 60 min. B= rat receives fluoxetine or vehicle injection, placed back in dark zone for a further 90 min. C= rat transferred to novel, brightly lit zone for 120 min while behaviour in both zones recorded for scoring. Animal had free access to both compartments during this period. D= end of experiment.

2.7.2. Experiments using mice

Adult, male C57BL/6 mice were used. The exploration box (length 45 cm; width 20 cm; depth 25 cm) had a small 'dark zone' (length 15 cm; 4 lux) and a larger 'light zone' (length 30 cm; 20 lux). The walls and floor of the dark zone were black and the walls and floor of the light zone were white. The floors of both zones were divided into 5 x 5 cm squares (*i.e.* 12 in the dark zone; 24 in the light zone) to allow scoring of locomotor activity. A door (height 10.5 cm, width 6.5 cm) separated the two zones, with a removable partition to restrict movement between the zones.

Mice were placed in the dark zone of the box with the partition in place for 1 h. They then received either drug or vehicle and were placed back in the dark zone for a further 30 min (see Figure 2.6). The door separating the light and dark zones was removed and mice were transferred to the centre of the light zone, facing away from the door. Their behaviour recorded on VHS film for later analysis. At the end of each experiment the mice were removed and killed by CO_2 overdose and cervical dislocation.

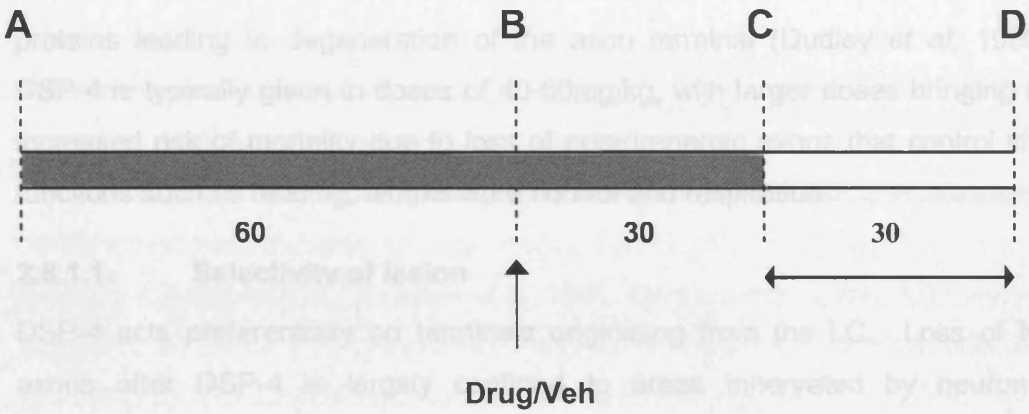


Figure 2.6. Representation of timing of light dark exploration box testing using mice. A = mouse placed in dark zone of box with door closed for 60 min. B= mouse receives drug or vehicle injection, placed back in dark zone for a further 30 min. C= mouse transferred to novel, brightly lit zone for 30 min while behaviour in both zones recorded for scoring. Mice had free access to both compartments during this period. D= end of experiment

2.8. Selective monoamine lesions

Experiment using fluoxetine in rats involved the use of monoamine-depleting, drug-induced lesions (Chapters 3, 4 & 5). These depletions were induced to determine the contribution of NA and 5-HT transmission to the *in vivo* effects of fluoxetine. Two agents were used: DSP-4 to deplete tissue stores of NA: *p*CPA to deplete tissue stores of 5-HT. A description of these agents is given below.

2.8.1. Depletion of NA stores using DSP-4.

The β-chloroalkylamine N-(2-chloroethly)-N-ethyl-2-bromobenzylamine (DSP-4) is a neurotoxic compound that selectively destroys noradrenergic axon terminals. A single administration of DSP-4 causes a rapid and extensive loss of NA terminals in the brain and periphery (Ross, 1976; Ross & Renyi, 1976). DSP-4 crosses the blood-brain barrier and can therefore be administered by intra-peritoneal injection. This is an advantage over existing noradrenergic toxins, such as 6-hydroxydopamine, which must be administered by i.c.v. infusion. DSP-4 is taken into NA neurone terminals *via* the NA transporter (Fritschy et al, 1990; Hallman & Jonsson, 1984).

Once inside the neurone, the toxin forms irreversible covalent bonds with cell proteins leading to degeneration of the axon terminal (Dudley *et al*, 1990). DSP-4 is typically given in doses of 40-60mg/kg, with larger doses bringing an increased risk of mortality due to loss of noradrenergic axons that control vital functions such as feeding, temperature control and respiration.

2.8.1.1. Selectivity of lesion

DSP-4 acts preferentially on terminals originating from the LC. Loss of NA axons after DSP-4 is largely confined to areas innervated by neurones projecting from this nucleus, (Fritschy & Grzanna, 1989). Thus, areas such as the hypothalamus are less affected by DSP-4. DSP-4 inhibits uptake of [^{3}H]-NA into cortical synaptosomes more potently (Ki ~ 180nM) than uptake into hypothalamic synaptosomes (Ki ~ 460nM), (Zaczek *et al* 1990). This may explain why LC efferents are more susceptible to the effects of DSP-4. Nevertheless, noradrenergic terminals in the hypothalamus are affected at higher concentrations of DSP-4 (Heal *et al*, 1993).

Effects of DSP-4 on other cell types are minimal. Some lesioning of 5-HT axon terminals has been reported, (Jonsson *et al*, 1981), but these are negligible in comparison with NA neurones. Ross & Renyi (1976) demonstrated that high doses of DSP-4 (100mg/kg) have no effect on uptake of [^{3}H]-DA in slices of striatum or [^{3}H]-5-HT uptake in slices of midbrain. Also, Harro *et al* (1992), who found no changes in [^{14}C]-5-HT or [^{14}C]-DA uptake in the frontal cortex, hippocampus, or striatum. Some authors however, use 5-HT uptake inhibitor (Heal *et al*, 1993), to protect 5-HT neurones from the effects of DSP-4. No significant destruction of DA neurones has been reported. This selectivity for NA neurones is a definite advantage of DSP-4 over 6-hydroxydopamine, which also affects DA axons.

2.8.1.2. Assessment of NA lesion

Many different measures are used to assess the extent of NA depletion caused by DSP-4. For example, immunohistochemical staining for NA itself, (Fritschy & Grzanna, 1991), for the enzyme marker of noradrenergic neurones, dopamine-β-hydroylase (DβH) (Fritschy & Grzanna, 1991; Schuerger *et al* 1995), measuring brain region tissue content of NA by HPLC, (Jonsson *et al*, 1981;

Berridge & Dunn, 1990; Finnegan *et al*, 1990; Cornwell-Jones *et al*, 1992; Lategan *et al*, 1992; Riekkinen *et al*, 1992; Heal *et al*, 1993; Mishra *et al*, 1994; Wiecezorek & Romaniuk, 1994; Wolfman *et al*, 1994; Zagrodzka *et al*, 1994; Abe *et al*, 1997; Al-Zahrani *et al*,1997; Fontana *et al*, 1999; Harro *et al*, 1999; Puolivali *et al*, 2000; Shirokawa *et al*, 2000; Prieto & Giralt, 2001), fluorescence histochemistry of NA fibres, (Jonsson *et al*, 1981;), [^{3}H]-NA uptake *in vitro* brain slices or synaptosomes, (Jonsson *et al*, 1981; Daoust *et al*, 1990), NA turnover, (Logue *et al*, 1985), [^{3}H]-desipramine binding, (Hallman & Jonsson, 1984) and NA efflux by *in vivo* microdialysis, (Kask *et al*,1997; Hughes & Stanford, 1996, 1998). Other less common measurements are weight loss, (Harro *et al*, 1995) and loss of post-decapitation reflex, (Delini-Stula *et al*, 1984). The consensus reached from these approaches is that DSP-4 causes a rapid and marked destruction of NA axon terminals.

2.8.1.3. Staining of noradrenergic fibres

Loss of NA staining is observed within several hours after DSP-4 and loss of DβH staining is maximal at 4-5 days (Fritschy *et al*, 1990). This suggests that DSP-4 causes axons to loose transmitter rapidly before destroying them. Some large, swollen axons remain visible after anti-NA and anti-DβH staining, even after the majority of stained fibres are lost. This suggests lesioning of the terminal field and therefore accumulation of these markers in proximal axons. After 7-14 days, these damaged axons also degenerate, leaving only residual axons of normal appearance. DβH staining has shown that noradrenergic cell bodies in the LC are also lost 6 months after exposure to 50mg/kg DSP-4, reaching an average of 57% after one year, (Fritschy & Grzanna, 1992).

Regeneration of lesioned axons has been demonstrated (Fritschy & Grzanna, 1992). Normal staining density of DβH in the forebrain is restored one year after DSP-4 administration, by regeneration of LC axons, despite 50-70% cell loss in the nucleus. The regeneration demonstrates the homeostatic ability of these cells.

2.8.1.4. Tissue Content of NA

Many studies have measured brain tissue content of NA using HPLC with electrochemical detection after DSP-4 exposure. In general, these demonstrate a marked reduction in NA. Jonsson *et al,* (1981) showed that this effect was rapid, occurring with 6 h after exposure, with most pronounced effects in the cerebral cortex and spinal cord. A marked reduction is seen in the cerebellum, also. Measurements of tissue content of NA provide an excellent means to verify the extent of DSP-4 lesion. However, they do not provide information about changes in noradrenergic transmission.

2.8.1.5. Receptor changes

As one would expect, DSP-4 leads to changes in noradrenergic receptor populations. Changes indicative of reduced NA tone are most common, *i.e.* up-regulation of postsynaptic α- and β-receptors. However, these changes are generally dependent on the time after lesion. Binding studies also use radioligands, which may or may not be of desired specificity. For example, Using [^{3}H]-dihydroalprenalol and [^{3}H]-WB4101 as ligands for β- and α-receptor binding respectfully, Jonsson *et al,* (1981) reported that 14 days after DSP-4 administration there was an up-regulation of β-adrenoceptor binding to membranes derived from homogenates of rat cerebral cortex. After 16 h, a significant decrease in α-adrenoceptor binding was observed. After 4 and 14 days a small, insignificant increase in α-receptor binding was observed. The initial decrease in binding was attributed to binding of DSP-4 to α-receptors.

Heal *et al,* (1993) looked at α_2-adrenoceptor binding after DSP-4 administration. At 3 days after exposure to the toxin, α_2-adrenoceptor binding was reduced in the cortex, hippocampus, cerebellum and hypothalamus. This loss of binding was supposedly due to a loss of presynaptic α_2-adrenoceptors following destruction of the axon terminals. Fifteen days after the lesion however, α_2-adrenoceptor binding showed some recovery.

Wolfmann *et al,* (1994) looked at changes in α_1, α_2 and β-adrenoceptor binding at various times after DSP-4 administration. They found that cortical α_1 and α_2-receptor binding was significantly increased 7days after a lesion, but had

returned to control values by 90 and 365 days. In contrast, β-adrenoceptor binding was increase at 7 days and 1 year after DSP-4 exposure.

All experiments were carried out 5 days after treatment with DSP-4 as this corresponds to the optimum loss of NA axons terminals (as shown by loss of DβH staining, Fritschy *et al*, 1990), with the no corresponding sprouting of surviving terminals.

2.8.1.6. Protocol of DSP-4 administration

DSP-4 was dissolved in 0.9% saline. Subjects received 40mg/kg (in 1ml/kg solution i.p.) 5 days prior to testing (either microdialysis or exploration box). For dialysis experiments, surgery was performed on day 4 after DSP-4 pretreatment.

2.8.2. Depletion of 5-HT tissue content by *p*CPA.

2.8.2.1 Mode of action

para-Chlorophenylalanine (*p*CPA) is an amino acid that potently and selectively depletes the brain of 5-HT. Its effects were first demonstrated in mice, rats and dogs (Koe & Weissman, 1966). Unlike DSP-4, *p*CPA is not a neurotoxin and does not destroy 5-HT axon terminals or cell bodies. *p*CPA depletes 5-HT stores by inhibiting the enzyme tryptophan hydroxylase (Jequier *et al*, 1967). Tryptophan hydroxylase catalyses the rate-limiting step in 5-HT biosynthesis, converting the amino acid 5-hydroxytryptohan (5-HTP) to the amine 5-hydroxtryptamine (5-HT). *In vitro*, *p*CPA is a competitive inhibitor of tryptophan hydroxylase. However, *in vivo*, *p*CPA blocks the enzyme irreversibly.

*p*CPA crosses the blood brain barrier and so can be administered systemically. 4 h after treatment with *p*CPA, both competitive and non-competitive inhibition of tryptophan hydroxylase occurs *in vivo*. After 2 days, however, the effects are irreversible (Jequier *et al*, 1967). This relatively slow time course of *p*CPA suggests that an active metabolite is responsible for its effects. This could be *para*-chlorophenylpyruvic acid, which is also an effective 5-HT depletor. Within 2 weeks, the effects of *p*CPA are completely reversed (Koe, 1971). This is due

to synthesis of new tryptophan hydroxylase enzyme, its transport to axon terminals and restoration of 5-HT synthesis.

2.8.2.2. Effects on 5-HT transmission

In addition to reducing the brain content, *p*CPA lowers the extracellular concentration of 5-HT *in vivo*, as demonstrated by microdialysis. O'Connell *et al*, (1991) demonstrated reduced 5-HT efflux in the frontal cortex of rats pretreated with a single dose of *p*CPA (150 mg/kg). This treatment reduced tissue stores and dialysis concentration of 5-HT by around 50%. The rise in 5-HT efflux following perfusion with a high concentration (100μM) of K^+ was also diminished suggesting that *p*CPA reduces impulse-dependent release of 5-HT. Pozzi *et al*, (1999), reported reduced tissue stores of 5-HT in the rat prefrontal cortex (87%) after a single oral dose of *p*PCA (300mg). This treatment had no effect on NA or dopamine. These animals also had a 44% reduction in the extracellular concentration of 5-HT.

An important finding with respect to the present experiments is that *p*CPA completely prevents the increase in 5-HT efflux produced by systemic fluoxetine or citalopram (Pozzi *et al*, 1999). The increase in 5-HT efflux after local infusion of both these drugs in the frontal cortex was also attenuated by *p*CPA. A diminished effect of fluoxetine on 5-HT efflux was also found by Yan *et al*, (1994). These studies support the case for using *p*CPA to determine the contribution of 5-HT transmission on the effects of fluoxetine, both on NA efflux and on behaviour. Since *p*CPA reduces 5-HT transmission *in vivo*, this should diminish any 5-HT dependent effects of fluoxetine.

2.8.2.3. Selectivity of depletion

*p*CPA is considered to be relatively selective for depletion of brain 5-HT, with minimal effects on content of NA (Koe & Weissman, 1966). Although *p*CPA inhibits tyrosine hydroxylase *in vitro* (with 40 times lower potency than the NA synthesis inhibitor, α-methyltyrosine), this effect is not seen *in vivo,* after 3 daily administrations of 100mg/kg (Koe & Weissman, 1966).

However, more recent work suggests that *p*CPA could have effects on NA transmission. Thus, Reader *et al*, (1986) reported depletion of NA content

(-24%) in the cerebral cortex of rats after 2 daily treatments of *p*CPA (400mg/kg). Géranton *et al*, (2004) found that NA content in the hypothalamus, but not the frontal cortex, was reduced by *p*CPA. Increased tyrosine hydroxylase activity in the LC of *p*CPA pretreated rats has also been reported, (McRae-Degrueurce *et al*, 1982). An increase in tyrosine hydroxylase activity indicates elevated synthesis of NA. This will occur if there is increased demand on NA transmission. In support of this, the spontaneous firing activity of LC noradrenergic neurones is also elevated by *p*CPA (Reader *et al*, 1986; Ferron, 1988).

However, these effects on NA transmission are not always replicated, since Shiekhattar & Aston-Jones, (1993) found no difference in LC firing rate in *p*CPA and saline pretreated rats. Also, *p*CPA pretreatment has no effect on spontaneous efflux of NA (Géranton *et al*, 2004; Mateo *et al*, 2000; Chapters 3 & 4), but markedly reduces 5-HT efflux (see above).

2.8.2.4. Protocol of *p*CPA administration.

Early studies show that, for several hours after *p*CPA pretreatment, brain catecholamine content is also reduced (Koe *et al*, 1971). However, after 2-3 days, effects of tryptophan hydroxylase predominate, with a marked depletion of 5-HT stores (Koe, 1971). Administration of large does of *p*CPA can be debilitating, due to effects in peripheral organs. Therefore, repeated administration of smaller doses is desirable. Experiments here used two administrations of (250mg/kg) *p*CPA: one at 48 h and one at 24 h prior to experimenting. This protocol will allow enough time for a maximum depletion of 5-HT biosynthesis, (see above) and minimize adverse effects outside the brain.

2.9. Statistical analysis

2.9.1. Analysis of Microdialysis data

Statistical analysis was performed on raw data routinely. For each experimental treatment group, the mean and standard error of the mean were calculated. Net changes in NA efflux were also calculated. Thus, the mean of the last three 'basal' samples was subtracted from all samples in the time course. This normalizes treatment groups with respect to basal efflux, facilitating drug-effect comparisons.

Analysis of all microdialysis data was performed using two-way analysis of variance (ANOVA) with repeated measure using SPSS version 12. 'Time' was used as the 'within subject' factor. The 'between subjects' factor depended on the particular experiment, details of which are given in the results chapters. Where necessary, the Greenhouse-Geisser correction was applied to compensate for violation of the variance-covariance matrix, a condition necessary for use of repeated measures ANOVA.

When comparing NA efflux after local infusion of drugs with basal efflux, data were divided into time 'bins'. Each time bin was of three consecutive time points, corresponding to either basal efflux, or the last three samples during local drug infusion. Bin was included as a second 'within subjects' factor.

2.9.2. Analysis of Behavioural data.

Behavioural data were analyzed for significance, using two-way or one-way ANOVA. The methods section of Chapters 5 & 7 give details of the treatment groups and comparisons made. In general, where a main effect was revealed by two-way ANOVA, this would be followed by one-way ANOVA. In all behavioural experiments, there were four treatment groups. Post-Hoc multiple comparisons using the LSD test were performed to explore any difference between individual treatment groups.

Levene's test of homogeneity of variance was performed routinely. Where this was significant, non-parametric analysis was performed using the Mann-Whitney test, as stated. If Levene's test was significant for a behaviour where an interaction between treatments was suspected, two-way ANOVA was performed on the log10 or square-root transformed data, as stated.

Analysis of Covariance (ANCOVA) was performed in all behavioural analysis to determine whether any differences in behavioural measures could be explained by differences in locomotor activity. This analysis was also used to disclose any effect that may otherwise be obscured by difference in locomotor activity.

For all experiments the criterion for significance was $P \leq 0.05$

Chapter 3

Comparison of the Effects of Systemic Fluoxetine on Noradrenaline Efflux in the Frontal Cortex and Hypothalamus of Freely-Moving Rats

3.1. Introduction

In vitro studies demonstrate that fluoxetine inhibits reuptake of 5-HT with greater potency that that of NA (see Chapter 1). Based on this evidence, the possibility that modulation of NA transmission contributes to the clinical effects of fluoxetine has been neglected, largely. However, interpreting effects from *in vitro* studies could be misleading, as microdialysis studies in rats demonstrate that fluoxetine increases NA efflux *in vivo*. If fluoxetine, and possibly other SSRIs, augment NA transmission, as well as that of 5-HT, this could contribute to their therapeutic mode of action.

When given systemically, reports of the effect of fluoxetine on NA efflux are markedly inconsistent. Several studies report increased efflux, (Perry & Fuller, 1997; Gobert *et al*, 1997; Zhang *et al*, 2000; Hatanaka *et al*, 2000; Bymaster *et al*, 2002; Zocchi *et al*, 2003; Koch *et al*, 2004), while others report no change, (Page & Abercrombie, 1997; Li *et al*, 2002; Beyer *et al*, 2002; Koch *et al*, 2004). Moreover, even studies from the same laboratory demonstrate this inconsistency, (Li *et al*, 2002; Perry & Fuller, 1997). So far, no explanation has been given for these disparate findings.

Although the number of studies is limited, fluoxetine's effects on NA efflux may depend on brain region. Increased efflux has been reported in the frontal cortex, but not the hypothalamus (Koch *et al*, 2004; but see Perry & Fuller, 1997), hippocampus, (Page & Abercrombie, 1997), or nucleus accumbens, (Koch *et al*, 2004). However, given that variable effects of fluoxetine have been reported in the same brain area (see Chapter 1: Table 1.1), it is not known whether these differences reflect region- or drug effect-dependent variation.

Here, dual-probe microdialysis was used to compare the effect of systemic fluoxetine in the frontal cortex and hypothalamus. This is the first time fluoxetine's effects on NA efflux have been compared simultaneously in two brain regions. These were chosen because they receive noradrenergic innveration from different brainstem sources: NA cells of LC innervate the frontal cortex while the hypothalamus is innervated predominantly by neurones projecting from the lateral tegmental nuclei (Moore and Bloom, 1979).

Microdialysis has shown that noradrenergic neurones of these areas can respond differently to a drug challenge. For example, both systemic and local infusion of *d*-amphetamine increases NA efflux to a greater extent in the hypothalamus than frontal cortex, (Géranton *et al*, 2003). Also, systemic injection of the mixed NA/5-HT uptake inhibitor, sibutramine, causes a rapid increase in NA efflux of short duration in the hypothalamus, but a sustained increase in the frontal cortex, (Wortley *et al*, 1999). Performing microdialysis simultaneously in these two areas will establish whether fluoxetine's effects are similarly region-dependent.

Fluoxetine could increase NA efflux by increasing the extracellular concentration of 5-HT, thereby activating heteroceptors on noradrenergic neurones (see Chapter 1). To test this, fluoxetine was administered to rats pretreated with the 5-HT synthesis inhibitor, *p*CPA (Koe & Weissman, 1966). This amino acid depletes tissues stores of 5-HT (Reader *et al*, 1986) and lowers its extracellular concentration, (O'Connell *et al*, 1991; Yan *et al*, 1994; Pozzi *et al*, 1999). If the effects of fluoxetine are secondary to an increase in the extracellular concentration of 5-HT, they should be diminished in *p*CPA pretreated rats. However, if fluoxetine's effects on NA are due to a direct action at noradrenergic neurones, these will occur irrespective of *p*CPA pretreatment.

Fluoxetine inhibits uptake of [^{3}H]-NA into synaptosomes from the cortical (Richelson & Pfenning, 1984) and hypothalamic tissue (Thomas *et al*, 1987). Therefore, fluoxetine could elevate NA efflux by inhibiting its uptake from the extracellular space. The NA transporter on noradrenergic axon terminals in the most likely site of action. However, inhibition of NA uptake at other sites is a possibility. For example, the ability of low concentrations of the NA reuptake

inhibitor desipramine (DMI) to inhibit uptake of [^{3}H]-NA into rat cortical synaptosomes was diminished by selective destruction of noradrenergic axon terminals using the neurotoxin, DSP-4 (Hughes & Stanford, 1996). This agent depletes tissue stores of NA, (Johnsson *et al*, 1981; Fritschy & Grzanna, 1989) reduces DβH staining (Ross, 1976) and reduces the number of NA uptake sites labeled by [^{3}H]-DMI (Lee *et al*, 1982) or [^{3}H]-nisoxetine (Cheetham *et al*, 1996). However, a higher concentration of DMI (50μM) inhibits NA uptake to the same extent into synaptosomes from tissue of DSP-4 lesioned and unlesioned rats (Hughes & Stanford, 1996). This is evidence for a second, low-affinity DMI binding site, which is not present on NA neurones, since it is not affected by DSP-4 pretreatment.

Infact, inhibition of [^{3}H]-NA uptake into cortical synaptosomes by a low concentration of fluoxetine is *greater* in DSP-4 pretreated rats, (Hughes & Stanford, 1996). This suggests that fluoxetine binds to uptake sites for NA that survive a DSP-4 lesion of NA axon terminals. DSP-4 pretreatment was used here to investigate the site of fluoxetine's effects. If DSP-4 pretreatment blunts the effects of fluoxetine on NA efflux, noradrenergic axon terminals are likely to be the site of its action. However, if fluoxetine increases NA efflux in both saline and DSP-4 pretreated rats, then another site, not present on NA axon terminals, must mediate its effects.

3.2. Aims

- To determine whether systemic administration of fluoxetine increases central NA efflux in both the frontal cortex and hypothalamus of freely moving rats. (Experiments 1-3.)
- To determine whether the effects of systemic fluoxetine on NA efflux depend on its effects on 5-HT transmission. (Experiment 2).
- To determine whether the effects of systemic fluoxetine on NA efflux are due to an action at NA axon terminals. (Experiment 3).

3.3. Methods

3.3.1 *In vivo* microdialysis

Experiments were performed on freely-moving rats (240-320g on day of surgery). For experiments using *p*CPA, animals received either 250 mg/kg *p*CPA i.p. or 1ml/kg 0.9% saline 48 hours and 24 hours before experimenting. A 2 day pretreatment with *p*CPA was used to allow maximum depletion of brain 5-HT (Koe & Weissman, 1966). For experiments using DSP-4, animals received either 40mg/kg DSP-4 or 1ml/kg 0.9% saline (i.p.) 5 days before microdialysis. A 5 day pretreatment was chosen as this corresponds to the optimum period for loss of dopamine-β-hydroxylase staining of NA axon terminals, but does not allow sufficient time for axonal regeneration, (Fritschy & Grzanna, 1990; see Chapter 2). Rats were implanted with microdialysis probes in both the frontal cortex and hypothalamus on the day before experimenting. See Chapter 2 (section 2.3) for details of the probe design and surgical procedure.

Fluoxetine was dissolved in distilled H_20 to make a 10mg/ml solution. This was administered in a volume of 1ml/kg (*i.e.* 10mg/kg). The dose of fluoxetine was chosen based on studies where an increase in NA efflux has been reported (see Chapter 1). Once basal efflux was established and at least 3 basal samples were taken fluoxetine or 1ml/kg H_2O was administered systemically by i.p. injection. The last three basal samples were designated T_{-40} - T_0, with injection of fluoxetine immediately after T_0. Sampling continued for a further 4 h after drug or vehicle injection. At the end of each experiment animals were killed immediately by halothane overdose and cervical dislocation and their brains removed for analysis of monoamine content.

- Experiment 1 compared the effect of systemic fluoxetine with vehicle.
- Experiment 2 compared the effect of *p*CPA and saline pretreatment on the effect of systemic injection of fluoxetine.
- Experiment 3 compared the effect of DSP-4 and saline pretreatment on the effect of systemic injection of fluoxetine.

3.3.2. Analysis of brain region monoamine content.

To determine the extent of *p*CPA and DSP-4- induced reduction of brain region monoamine content, homogenates from the frontal cortex and hypothalamus were analyzed for concentration of NA, DA and 5-HT, using HPLC. See Chapter 2 (section 2.4.4) for a detailed description of the tissue preparation and analytical methods used.

3.3.3. Statistical analysis

The concentration of NA in the brain dialysates is expressed as fmol/20min without correction for probe recovery. In addition to the raw data, net changes were calculated by subtracting the mean of the last three basal samples from all samples in the time course, for each subject. Raw and net data for all subjects were pooled and the mean and standard error of the mean (s.e.m.) calculated.

All data were analyzed for significance of differences using two-way analysis of variance (ANOVA) with repeated measures using SPSS 7.5. For experiments comparing the effect of systemic fluoxetine with vehicle, 'time' was the 'within subjects' factor and 'drug' (*i.e.* fluoxetine or vehicle) was the 'between subjects' factor.

For microdialysis experiments using DSP-4 or *p*CPA, 'time' was a 'within subjects' factor and 'pretreatment' (*i.e.*, saline, DSP-4, or *p*CPA) was a 'between subjects' factor. For all experiments using repeated measures ANOVA, the Greenhouse Geisser 'ε' correction was performed where Mauchley's test of sphericity of the variance-covariance matrix was significant.

Brain region monoamine content was analyzed using two-way ANOVA with 'pretreatment' (saline, *p*CPA or DSP-4) and 'brain region' as main factors. Where an effect of 'pretreatment' was found, data for frontal cortex and hypothalamus was analyzed using one-way ANOVA with 'pretreatment' as the main factor.

3.4. Results

3.4.1. Experiment 1: Effect of systemic fluoxetine on NA efflux

Frontal Cortex

There was no difference in basal efflux of animals receiving vehicle or fluoxetine, (T_{-40}-T_0; fmoles/20min: vehicle, 15.5±2.6; fluoxetine, 14.3±1.5). Fluoxetine increased NA efflux relative to vehicle (Figure 3.1A): whereas vehicle injection did not affect efflux, fluoxetine increased it (T_{-40}-T_{180}: main effect of 'time': $F_{4,70}$=3.8, P=0.01; main effect of 'drug': $F_{1,18}$= 5.0, P=0.04; 'drug' x 'time' interaction: $F_{4,70}$= 2.9, P= 0.03). At each hour after injection, NA efflux was greater in animals given fluoxetine, (Table 1.1). Analysis of all time points after injection (T_{20}-T_{240}) revealed a main effect of 'drug' ($F_{1,17}$=7.3, P=0.02). Analysis of net changes in NA efflux revealed a similar pattern of results as the raw data, (Figure 3.2A and Table 3.2).

Hypothalamus

There was no difference in basal efflux of animals receiving vehicle or fluoxetine (vehicle, 15.0±0.8; fluoxetine, 17.9±1.4). Injection of fluoxetine caused a transient increase in NA efflux, relative to vehicle (Figure 3.1B). Thus, NA efflux was influenced by an interaction between main factors 'drug' and 'time', over the first 8 time points only (T_{-40}-T_{100}: 'drug' x 'time' interaction $F_{3,44}$=3.0, P=0.05: main effect of 'time' $F_{3,44}$=6.4, P=0.001, Figure 3.1B). Analysis of all time points after injection revealed a main effect of 'drug', ($F_{1,15}$=4.6, P=0.05) However, comparison of time points T_{120}-T_{240} (*i.e.* those time points after the period where a 'drug' x 'time' interaction was found) revealed no difference in NA efflux between fluoxetine and vehicle groups (*i.e.* no main effect of 'drug', $F_{1,15}$=3.5, P=0.08). Also, comparison of each hour after injection revealed no differences in NA efflux due to drug treatment, (Table 3.1.)

Analysis of net changes revealed the same transient effects of fluoxetine in the hypothalamus (Figure 3.2B). Again, comparison of time points T_{120}-T_{240} revealed no difference in NA efflux between fluoxetine and vehicle groups (*i.e.* no main effect of 'drug', $F_{1,15}$=1.7, P=0.2). Comparison of each hour after injection revealed that NA efflux was greater in fluoxetine treated animals during the first hour after injection, only (Table 3.2.).

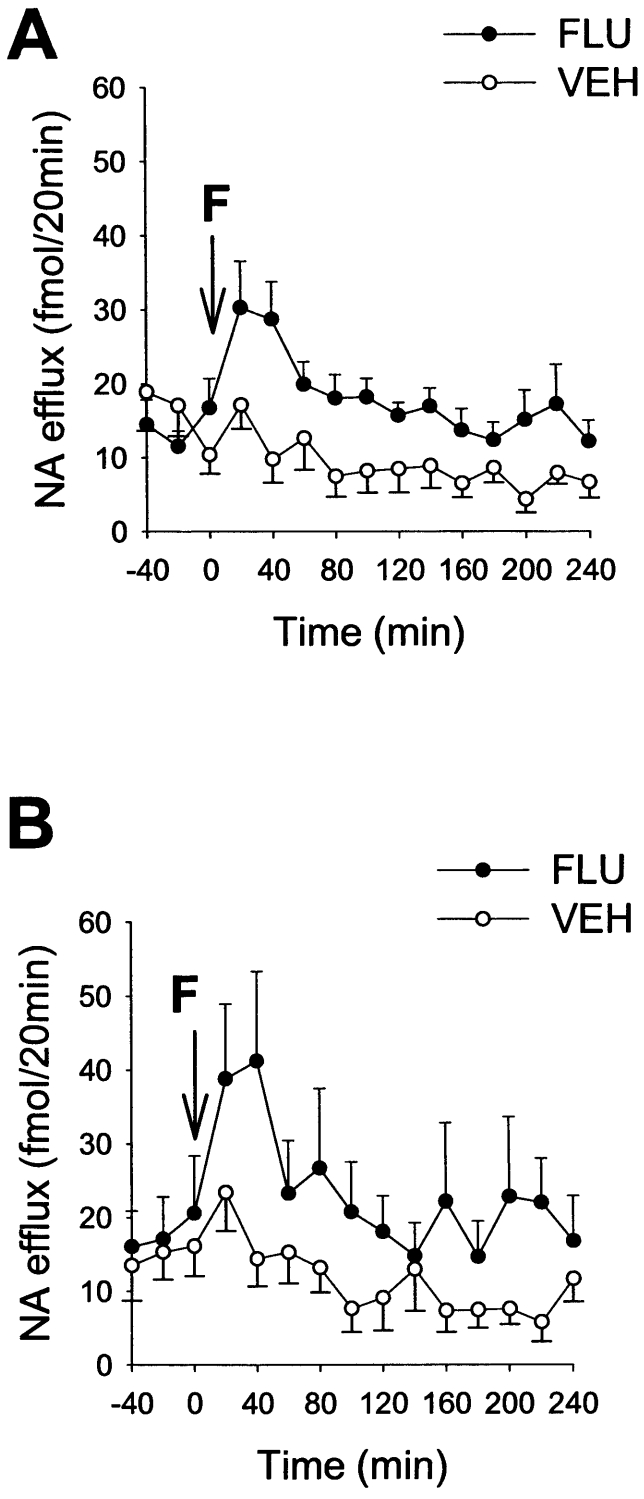


Figure 3.1. Effect of systemic fluoxetine or vehicle on NA efflux in A) frontal cortex and B) hypothalamus of freely moving rats. Plots show mean efflux ± s.e.m. Arrows indicate time of fluoxetine/vehicle injection. n=8-12 per group. See section 3.4.1. for details of statistical analysis.

	FRONTAL CORTEX		HYPOTHALAMUS	
T_{20}-T_{60}	**$F_{1,18}$=6.7**	***P*=0.02**	$F_{1,16}$=3.3	*P*=0.09
T_{80}-T_{120}	**$F_{1,18}$=6.7**	***P*=0.02**	$F_{1,18}$=2.6	*P*=0.13
T_{140}-T_{180}	**$F_{1,18}$=4.3**	***P*=0.05**	$F_{1,18}$=1.7	*P*=0.21
T_{200}-T_{240}	**$F_{1,17}$=4.6**	***P*=0.05**	$F_{1,15}$=4.1	*P*=0.06

Table 3.1. Statistics generated from two-way ANOVA comparing raw data of NA efflux at each hour after injection of fluoxetine or vehicle in the frontal cortex and hypothalamus, (main factor = 'drug treatment'). Significant differences are in bold. N=8-12. See Figure 3.1.

	FRONTAL CORTEX		HYPOTHALAMUS	
T_{20}-T_{60}	**$F_{1,18}$=5.4**	***P*=0.03**	**$F_{1,16}$=6.5**	***P*=0.02**
T_{80}-T_{120}	$F_{1,18}$=3.9	*P*=0.06	$F_{1,16}$=3.0	*P*=0.10
T_{140}-T_{180}	$F_{1,18}$=3.1	*P*=0.09	$F_{1,16}$=1.0	*P*=0.30
T_{200}-T_{240}	**$F_{1,17}$=4.5**	***P*=0.05**	$F_{1,15}$=2.2	*P*=0.20

Table 3.2. Statistics generated from two-way ANOVA comparing net data of NA efflux at each hour after injection of fluoxetine or vehicle in the frontal cortex and hypothalamus, (main factor = 'drug treatment'). Significant differences are in bold. N=8-12. See Figure 3.2.

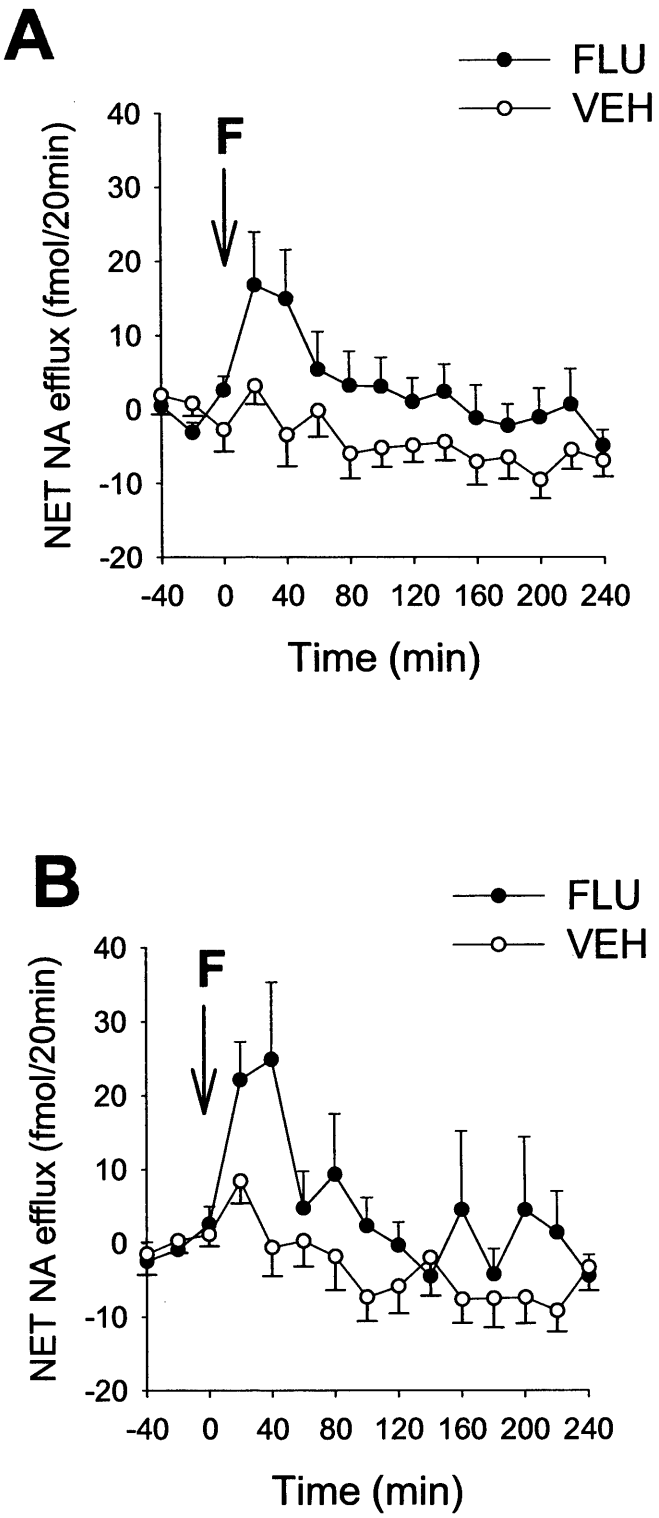


Figure 3. 2. Effect of systemic fluoxetine and vehicle on net changes in efflux in A) frontal cortex and B) hypothalamus of freely moving rats. Plots show mean change in efflux ± s.e.m. n=8-12 per group. Arrows indicate time of fluoxetine or vehicle injection. See section 3.4.1 for details of statistical analysis.

3.4.2. Experiment 2: Effect of *p*CPA pretreatment on the action of fluoxetine on NA efflux

3.4.2.1. Depletion of brain 5-HT content by *p*CPA.

*p*CPA pretreatment decreased 5-HT content in the frontal cortex (-72%, $F_{1,11}$=8.7, P=0.02) and hypothalamus (-90%, $F_{1,11}$= 190, P<0.001) compared with saline pretreated animals. The tissue concentration of NA in the hypothalamus was also reduced by *p*CPA (-45%, $F_{1,11}$=15, P=0.03). The concentration of NA in the frontal cortex, as well as that of DA in both brain regions, was unchanged by *p*CPA. Table 3.3.

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	***p*CPA**	**SAL**	***p*CPA**
NA	472±72	292±19	3060±314	**1691±153***
DA	376±861	155±2	719±284	375±73
5-HT	307±48	**86±85***	789±48	**78±19****

Table 3.3. Monoamine content in frontal cortex and hypothalamus of saline and *p*CPA-pretreated rats receiving systemic fluoxetine. Table shows mean content (ng/g wet tissue weight) ± s.e.m. n=7. Significant differences are in bold. *P<0.05; **P<0.005.

3.4.2.2. Effect of systemic fluoxetine on NA efflux in saline and *p*CPA pretreated rats.

Frontal cortex

There was no difference in basal NA efflux of saline and *p*CPA pretreated rats (fmoles/20min saline, 31.2±2.0; *p*CPA, 28.1±0.3). Unlike Experiment 1, fluoxetine did not increase NA efflux in either saline or *p*CPA pretreated rats (Figure 3.3A). Also, NA efflux did not differ in saline and *p*CPA pretreated animals at any hour injection of fluoxetine, (Table 3.4). Analysis of all time points revealed a main effect of 'time' ($F_{14,112}$=3.7, P<0.001), but no effect of 'pretreatment' and no 'time' x 'pretreatment' interaction. Analysis of net changes in NA efflux gave a similar pattern of results, with a main effect of 'time' (T_{-40}-T_{240}: $F_{14,126}$=2.2, P=0.01), but no effect of 'pretreatment', and no 'time' x 'pretreatment' interaction.

Hypothalamus

There was no difference in basal NA efflux of saline and *p*CPA pretreated rats (fmoles/20min; saline, 23.5±1.7; *p*CPA, 26.3±1.4). Unlike the previous experiment, fluoxetine injection had no effect on NA efflux in either saline or *p*CPA-pretreated rats (Figure 3.3B). Thus, there were no effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interaction (T_{-40} - T_{240}, Figure 3.3B). NA efflux was not different in saline or *p*CPA pretreated rats at any hour after fluoxetine injection (Table 3.4). Likewise, analysis of net changes also revealed no effect of fluoxetine in either group of rats, *i.e.* no effects of 'time', 'pretreatment' and no 'time' x pretreatment interaction.

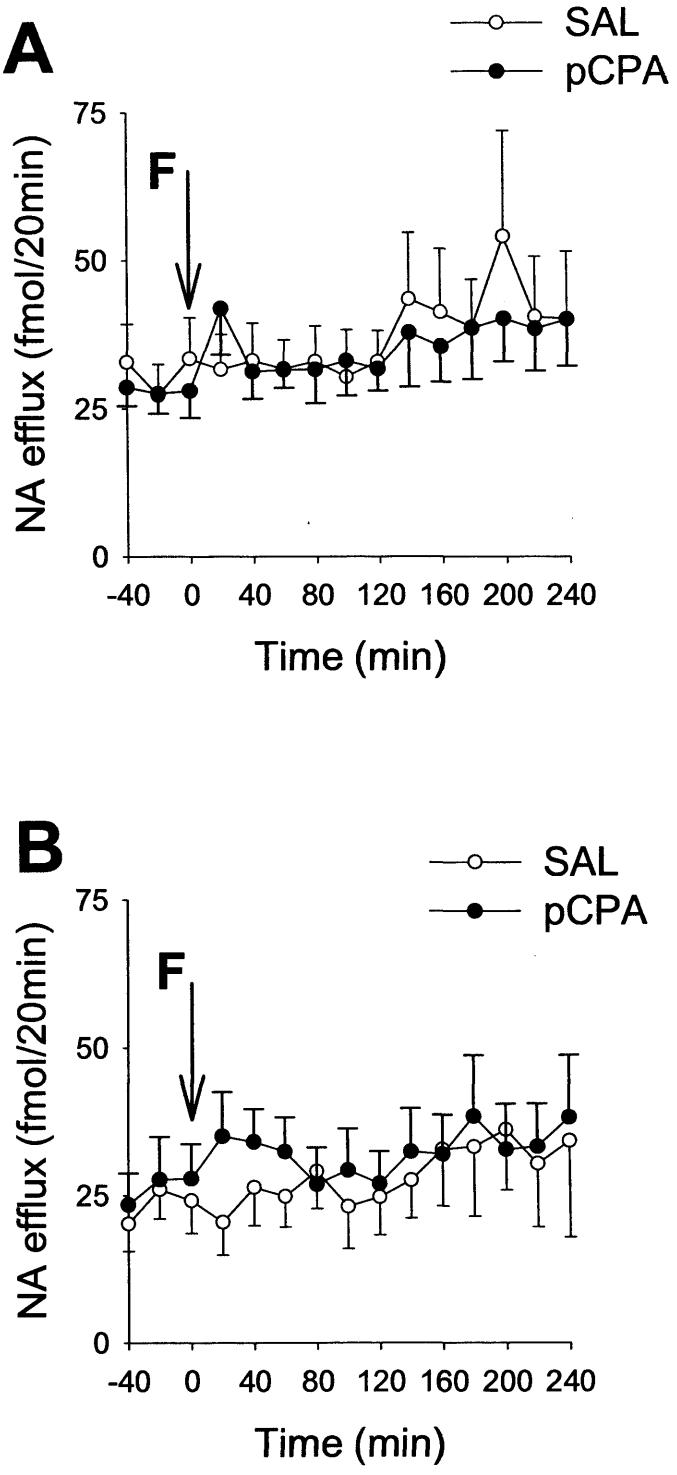


Figure 3.3. Comparison of systemic administration of fluoxetine on NA efflux in saline and *p*CPA-pretreated rats in A) frontal cortex B) hypothalamus. Plots show mean NA efflux ± s.e.m. N=7. Arrow indicates time of fluoxetine injection. See section 3.4.2. and Table 3.4 for details of statistical analysis.

	FRONTAL CORTEX	HYPOTHALAMUS
T_{20}-T_{60}	$F_{1,11}$=0.27, *P*=0.62	$F_{1,11}$=1.0, P=0.33
T_{80}-T_{120}	$F_{1,11}$=0.71, *P*=0.80	$F_{1,11}$=0.7, P=0.80
T_{140}-T_{180}	$F_{1,12}$=0.11, *P*=0.75	$F_{1,11}$=0.7, P=0.80
T_{200}-T_{240}	$F_{1,10}$=0.14, *P*=0.72	$F_{1,8}$=0.1, P=0.77

Table 3.4. Statistics generated from two-way ANOVA of raw data, showing no difference in NA efflux in either frontal cortex or hypothalamus of *p*CPA and saline pretreatment animals, at any hour after injection of fluoxetine. n=7 (main factor ='pretreatment').

3.4.3 Experiment 3: Effect of DSP-4 pretreatment on the action of systemic fluoxetine.

3.4.3.1. Depletion of brain NA content by DSP-4

DSP-4 reduced the tissue content of NA in the frontal cortex (-65%, $F_{1,11}$=5.2, *P*=0.05) and hypothalamus, (-47%, $F_{1,11}$=4.9, *P*=0.05). DSP-4 did not affect tissue content of DA or 5-HT in either brain region, (Table 3.5.).

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	**DSP-4**	**SAL**	**DSP-4**
NA	749±179	**261±115***	1531±148	**817±388***
DA	292±91	183±58	895±38	8803±250
5-HT	686±60	373±241	748±595	167±167

Table 3.5. Monoamine content in frontal cortex and hypothalamus of saline and DSP-4-pretreated rats receiving systemic fluoxetine. Values show mean content (ng/g wet tissue weight) ± s.e.m. n=8. Significant differences are in bold. *P<0.05; main factor = 'pretreatment'

3.4.3.2. Effect of systemic fluoxetine in DSP-4 and saline pretreated rats

Frontal Cortex

Basal efflux of NA was greater in DSP-4 pretreated animals than saline pretreated controls, (T_{-40}-T_0 fmoles/20min: saline, 7.85 ± 0.5; DSP-4, 17.29±1.0: main effect of 'pretreatment'; $F_{1,12}$=13.1, *P*=0.003 (Figure 3.4A). Fluoxetine had no effect on saline pretreated rats, but transiently increased NA efflux in DSP-4 pretreated rats during the first hour after injection, but thereafter caused a decrease, relative to saline basal efflux. Thus, there was an effect of 'time' ($F_{14,168}$=2.4, *P*=0.004), 'pretreatment' ($F_{1,12}$=6.8, *P*=0.02) and a 'time' x 'pretreatment' interaction ($F_{14,168}$=4.0, *P*<0.001) when all time points were analyzed (T_{-40} -T_{240}). Table 3.6 shows statistical comparison between saline and DSP-4 pretreated rats for each hour after in injection of fluoxetine.

Analysis of net changes in efflux also revealed that NA efflux was different in DSP-4 and saline pretreated animals each hour after fluoxetine administration (Table 3.7). In DSP-4 pretreated animals, NA efflux was greater during the first hour after fluoxetine was given. For the subsequent 3 hours of dialysis, however, NA efflux was reduced in DSP-4 pretreated animals (Figure 3.5A). Analysis of all time points revealed a main effect of time ($F_{14,196}$=2.4, *P*=0.004), and a 'time' x 'pretreatment' interaction ($F_{14,168}$=4.0, *P*<0.001), but no effect of 'pretreatment' ($F_{1,12}$=3.6, *P*=0.08, T_{-40} - T_{240},).

Hypothalamus

Basal NA efflux appeared greater in DSP-4 pretreated animals, (fmoles/20min, saline, 10.14±1.4; DSP-4, 18.57±0.3), but this failed to reach significance (no effect of 'pretreatment', $F_{1,12}$=3.2, *P*=0.1). However, extending the comparison from T_{-40}-T_{40} revealed greater NA efflux in DSP-4-pretreated rats ($F_{1,12}$=5.7; *P*=0.03. Figure 3.4B). Fluoxetine injection had no effect in saline pretreated rats, but decreased NA efflux in the DSP-4 pretreated group. Analysis of raw data revealed an effect of 'time' ($F_{14,168}$=3.5, *P*<0.001), and a 'time' x 'pretreatment' interaction ($F_{14,168}$=1.99, *P*=0.02), but no effect of 'pretreatment' (T_{-40}-T_{240}: $F_{14,168}$=1.3, *P*=0.3). There was no difference in NA efflux between DSP-4 and saline pretreated animals during any of the 4 hours after fluoxetine administration (Table 3.6).

Analysis of net changes also revealed a reduction in NA efflux in DSP-4 pretreated animals given fluoxetine. (Figure 3.5B). Thus, there was a main effect of 'time', (T_{-40}-T_{240}: $F_{14,168}$=3.5, P<0.001) and a 'time' x 'pretreatment' interaction, ($F_{14,168}$=2.0, P=0.02), but no effect of 'pretreatment'. There was no difference in NA efflux between saline and DSP-4 pretreated animals for the first hour after acute fluoxetine. During the second hour, (T_{80}-T_{120}), NA efflux was significantly reduced in the DSP-4 group only. During the third hour, this just missed the criterion for significance. However, reduced NA efflux in DSP-4 pretreated rats was restored during the final hour after fluoxetine injection, (see Table 3.7).

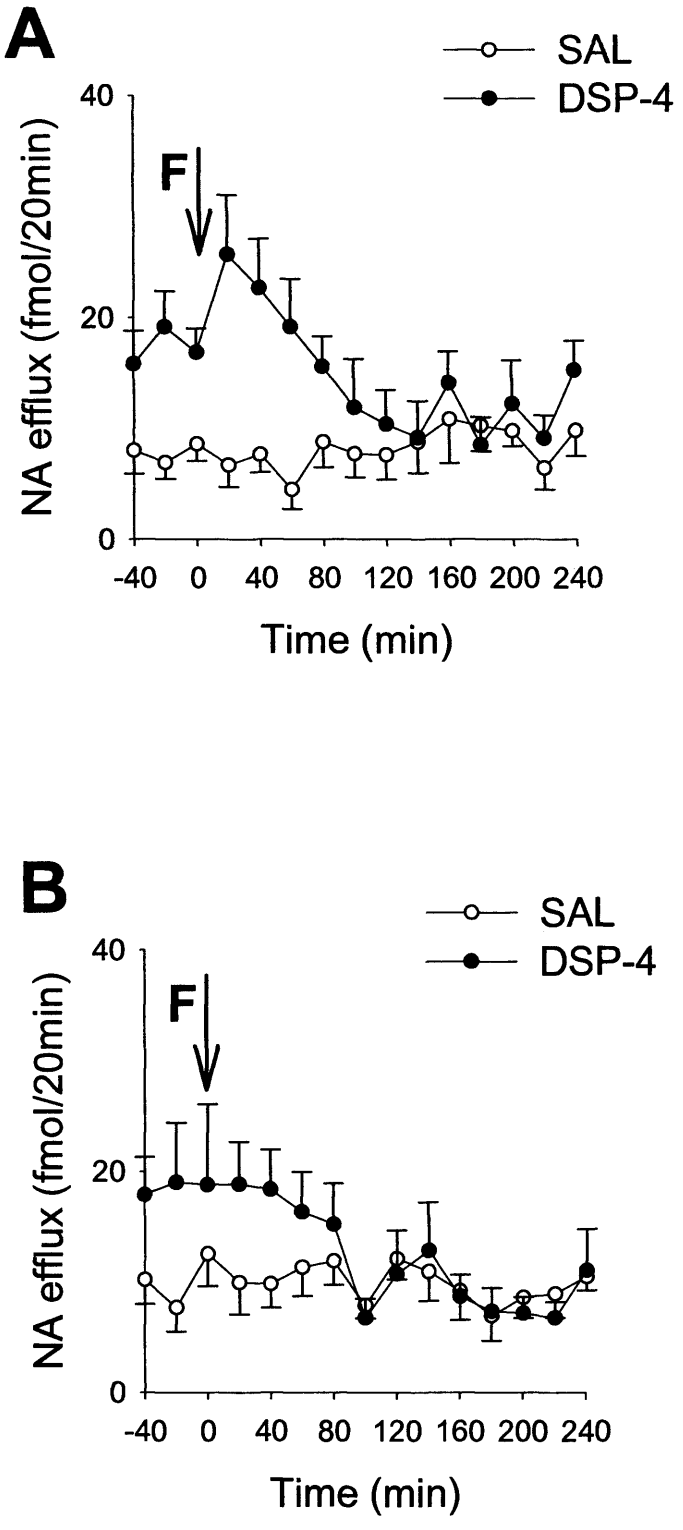


Figure 3.4. Comparison of raw data on the effects of systemic administration of fluoxetine on NA efflux in saline and DSP-4 pretreated freely moving rats in A) frontal cortex B) hypothalamus. N=8. Arrow indicates injection of fluoxetine. See section 3.4.3.2 and Table 3.6 for details of statistical analysis.

	FRONTAL CORTEX	HYPOTHALAMUS
T_{20}-T_{60}	**$F_{1,12}$=16.3, P=0.002**	$F_{1,12}$=3.7, P=0.10
T_{80}-T_{120}	$F_{1,12}$=1.9, P=0.19	$F_{1,12}$=0.009, P=0.93
T_{140}-T_{180}	$F_{1,12}$=0.03, P=0.86	$F_{1,12}$=0.03, P=0.86
T_{200}-T_{240}	$F_{1,12}$=2.3, P=0.16	$F_{1,12}$=0.18, P=0.68

Table 3.6. Statistics generated from two-way ANOVA using raw data, showing effect of DSP-4 pretreatment on NA efflux in frontal cortex or hypothalamus animals, at each hour after systemic fluoxetine injection (main factor ='pretreatment'). Significant differences are in bold. N=8.

	FRONTAL CORTEX	HYPOTHALAMUS
T_{20}-T_{60}	**$F_{1,12}$=5.1, P=0.04**	$F_{1,12}$=0.03, P=0.86
T_{80}-T_{120}	**$F_{1,12}$=6.4, P=0.03**	**$F_{1,12}$=5.1, P=0.04**
T_{140}-T_{180}	**$F_{1,12}$=9.5, P=0.01**	$F_{1,12}$=4.4, P=0.06
T_{200}-T_{240}	**$F_{1,12}$=5.5, P=0.04**	**$F_{1,12}$=7.3, P=0.02**

Table 3.7. Statistics generated from two-way ANOVA using net data, showing effect of DSP-4 pretreatment on NA efflux in frontal cortex or hypothalamus animals, at each hour after systemic fluoxetine injection (main factor ='pretreatment'). Significant differences are in bold. N=8

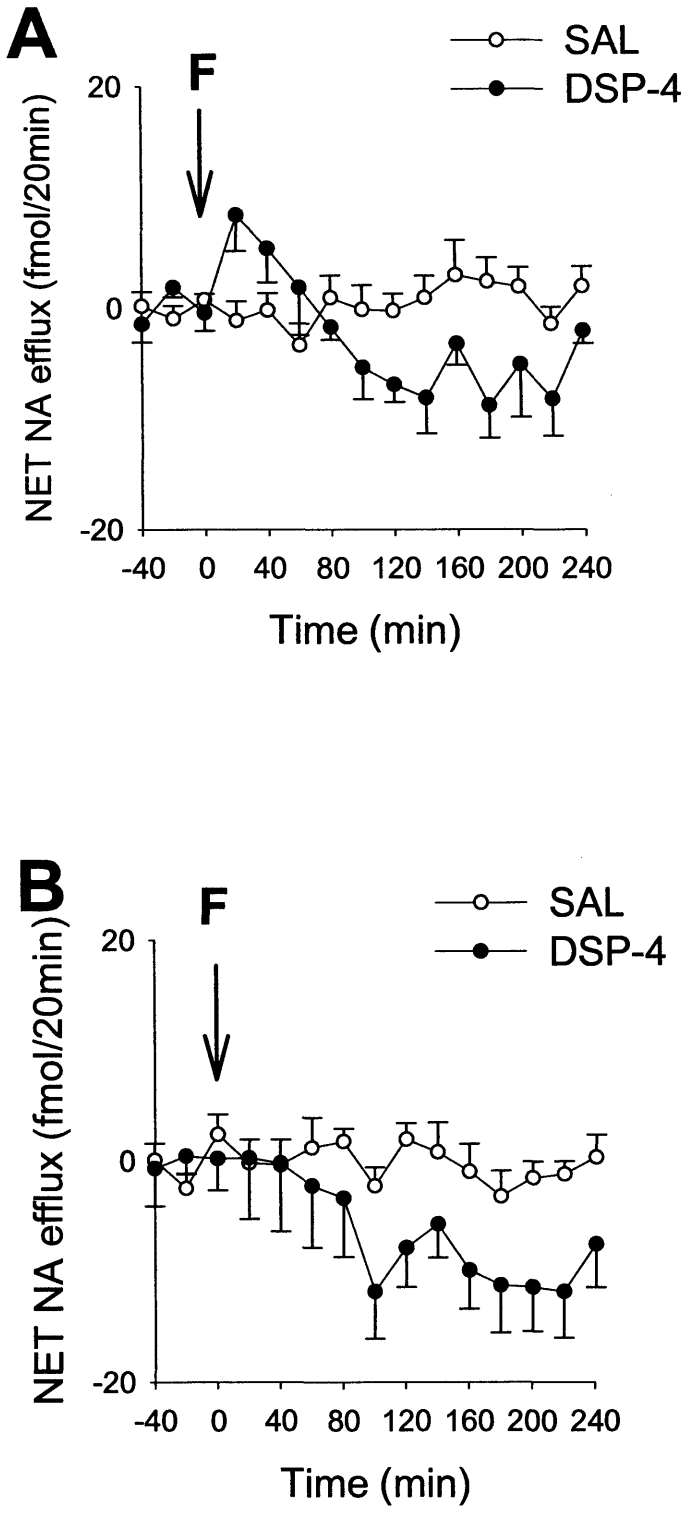


Figure 3.5. Net change in NA efflux in A) frontal cortex and B) hypothalamus in DSP-4 and saline pretreated rats, in response to systemic fluoxetine. N=8. Arrow indicates injection of fluoxetine. See section 3.4.3.2 and Table 3.7 for details of statistical analysis.

3.5. Discussion

The objectives of experiments described in this chapter were: 1) to determine if fluoxetine's effects on central NA efflux depend on brain region, by comparing them simultaneously in the rat frontal cortex and hypothalamus; 2) to establish whether these effects are dependent on intact 5-HT transmission; 3) to determine if any fluoxetine-induced changes in NA efflux are due to an action at noradrenergic axon terminals.

In Experiment 1 systemic injection of fluoxetine increased NA efflux in both the frontal cortex and hypothalamus, compared to vehicle injection. However, a regional difference was apparent: the effect in the frontal cortex lasted longer than that in the hypothalamus. In the frontal cortex, NA efflux after fluoxetine injection was greater than in vehicle treated rats for the remaining 4 hours of the experiment. In contrast, in the hypothalamus this difference lasted for 100 min only (*i.e.* until T_{120}, Figures 3.1B and 3.2B).

This pattern of results is strikingly similar to that reported for the mixed NA/5-HTuptake inhibitor, sibutramine. Using single-probe microdialysis in separate groups of freely-moving rats, Wortley *et al*, (1999) reported increased NA efflux in the frontal cortex and hypothalamus after systemic administration of sibutramine. However, the effect in the frontal cortex was sustained throughout the experiment, whereas that in the hypothalamus was of shorter duration. The different response to sibutramine and fluoxetine could reflect differences in regulation of NA transmission in these brain regions. For example, there is evidence that the NA transporter in the frontal cortex and hypothalamus differs with respect to binding of DSP-4, the catecholamine uptake inhibitor, mazindol, as well as NA itself (Zaczek *et al*, 1990). Also, the proportion of pre- to postsynaptic α_2-adrenoceptors is greater in the hypothalamus (Heal *et al,* 1993), which could result in greater feedback inhibition of NA in this brain region. Consistent with this, Wortley *et al*, (1999) reported a greater potentiation of the effect of sibutramine by the α_2-adrenoceptor antagonist, RX821002, in the hypothalamus than frontal cortex. This regional difference in the effects of RX821002 was seen when it

was administered systemically, or infused locally. Regional differences in the NA response to locally applied fluoxetine are investigated in Chapter 4.

In contrast to the results described above, fluoxetine did not increase NA efflux in saline-pretreated animals in Experiments 2 and 3. This adds to the empirical evidence that fluoxetine has a variable, unpredictable effect on NA efflux when given acutely by the systemic route. Previous microdialysis studies have found either increased NA efflux, (*e.g.*, Jordan *et al*, 1994; Gobert *et al*, 1997; Hatanaka *et al*, 2000; Koch *et al*, 2004; Perry & Fuller, 1997) or no effect (Li *et al*, 2002; Beyer *et al*, 2002) of fluoxetine. Even studies of the same brain region, or from the same laboratory, (Perry & Fuller, 1997; Li *et al*, 2002) report this variability, which has never been formally acknowledged. Thus, the variable effects of fluoxetine in the present experiments reflect the inconsistent reports in the literature. However, this is the first time a variable effect of fluoxetine of NA efflux has been explicitly noted within a single study.

The variable NA response to fluoxetine bears striking similarity to the inconsistent reports gathered from the much larger body of evidence from microdialysis studies of the effects of SSRIs on 5-HT efflux. These too demonstrate either increased (Jordan *et al*, 1994; Hajos-Korcsok *et al*, 2000; Gobert & Millan, 1999) or unchanged (Hérvas & Artigas, 1998; Gartside *et al*, 1995, 1999; Sharp *et al*, 1997; Invernizzi *et al*, 1992) 5-HT efflux after administration of SSRIs. This effect is true also of the tricyclic antidepressant and preferential 5-HT uptake inhibitor, clomipramine (Adell & Artigas, 1991). This variable effect of acute SSRIs is now known to be due to activation of autoreceptors that diminish cell firing and release of 5-HT. Thus, co-administration of SSRIs with 5-HT_{1A} antagonists, (e.g. WAY100635, Hérvas & Artigas, 1998; Romero *et al*, 1996; Gartside *et al*, 1995) consistently increases 5-HT efflux in the frontal cortex (for reviews see Hjorth *et al*, 2000; Kinney *et al*, 2000; Romero *et al*, 1996; Gardier *et al*, 1996).

Interestingly, a variable response to systemic administration of uptake inhibitors extends to drugs regarded as selective for the NA transporter: for instance,

desipramine (DMI) either increases (Mateo *et al*, 1998; Yan *et al*, 1993) or has no effect (Nutt *et al*, 1997) on NA efflux when given systemically. This is due to activation of somatodendritic and terminal α_2-adrenoceptors (for review see Invernizzi & Garattini, 2004). Thus, co-administration of DMI with α_2-adrenoceptor antagonists (*e.g.* idazoxan) produces a greater elevation of NA efflux than DMI alone (Dennis *et al*, 1987; Thomas & Holman, 1991; Nutt *et al*, 1997). Also, the α_2-adrenoceptor antagonist, atipamezole, potentiates the increase in NA efflux produced by administration of sibutramine in the frontal cortex and hypothalamus (Wortley *et al*, 1999). A similar mechanism appears to constrain the effect of systemic fluoxetine on NA efflux since, in the frontal cortex, this is potentiated by pretreatment with atipamezole (Gobert *et al*, 1997).

The effectiveness of α_2-adrenoceptor antagonists to potentiate the effects of NA reuptake inhibitors is due to increased activation of autoreceptors when clearance of NA is reduced. The NA transporter removes NA from the extracellular space and normally renders α_2-adrenoceptors inactive, since the receptors seem to be located beyond the site of release and are not normally exposed to NA (Callado & Stamford 2000). When uptake is blocked (or overwhelmed during periods of high NA release rate), α_2-adrenoceptors are activated, rendering the extracellular concentration of NA sensitive to α_2-adrenoceptor antagonists. Presumably, whether or not there is an increase in NA efflux after administration of fluoxetine would depend on the tonic rate of NA release. Therefore, the neurochemical status of the animals at the time of the experiment would influence the drug's effect.

Given that 5-HT modulates NA transmission *in vivo* (see Chapter 1), it is possible that fluoxetine's effects on NA are secondary to its ability to elevate the concentration of extracellular 5-HT. To investigate this, Experiment 2 used *p*CPA pretreatment to determine whether fluoxetine increases NA efflux in rats with diminished 5-HT transmission. However, given the variable nature of fluoxetine's effects on NA efflux (see above) the contribution of 5-HT could not be determined. No effect of fluoxetine was seen in either saline or *p*CPA pretreated rats.

Nevertheless, an interesting observation from Experiment 2 was the reduction in hypothalamic NA whole tissue content, as well as that of 5-HT. This effect has been reported previously (Reader *et al*, 1986; Géranton *et al*, 2004) and could be due to depletion of NA stores in this brain region following loss of an inhibitory 5-HT-mediated input. The dose of *p*CPA used here depletes tissue stores of 5-HT and reduces its extracellular concentration (O'Connell *et al,* 1991; Yan *et al*, 1994; Pozzi *et al*, 1999). Despite this reduction in tissue NA stores, NA efflux was not changed by *p*CPA, in either brain region. Other studies have also shown that *p*CPA pretreatment does not change spontaneous efflux of NA (Géranton *et al*, 2004; Mateo *et al*, 2000). Yet, this is at odds with experiments measuring the firing rate of LC neurones, (Reader *et al*, 1986; Ferron, 1988; but see: Shiekhattar & Aston-Jones 1993) and LC tyrosine hydroxylase activity (McRae-Degrueurce *et al*, 1982), which are both increased by *p*CPA pretreatment. These parameters were not measured in the present study, but the results here add to the body of evidence that *p*CPA pretreatment does not change spontaneous efflux of NA. Therefore, studies using *p*CPA demonstrate dissociation between the rate of NA synthesis, LC firing rate and efflux at axon terminals.

Experiment 3 compared the effect of systemic fluoxetine in animals with a partial lesion of NA axon terminals, induced by DSP-4. This neurotoxin selectively destroys NA axon terminals, but leaves cell bodies intact (Ross, 1976; Fritschy & Grzanna, 1989). The principal finding from this experiment was that fluoxetine markedly reduced NA efflux in both the frontal cortex and hypothalamus of DSP-4 pretreated rats only. A reduction in NA efflux will occur following an increase in its reuptake and/or a reduction it its rate of release. Reduced NA release is more likely, since fluoxetine binds to the NA transporter and inhibits uptake of [^{3}H]-NA into both cortical (Richelson & Pfenning, 1984) and hypothalamic (Thomas *et al*, 1987) synaptosomes (reviewed by Stanford, 1996).

The site of action of fluoxetine that leads to decreased NA release cannot be determined with any certainty, since it was administered systemically. However, it is unlikely to be a target on NA axon terminals, since these are

extensively lesioned by DSP-4. An effect on NA neurones, upstream of the terminals, is more likely to mediate the reduction in NA efflux. This could involve activation of somatodendritic α_2-adrenoceptors on NA neurones. Thus, if fluoxetine increases the extracellular concentration of NA in the LC, as well as frontal cortex and hypothalamus, this would decrease LC cell firing and release (Van Gaalen *et al*, 1997). In support of this, Mateo *et al*, (1998) reported increased NA efflux in the LC, and simultaneously decreased efflux in the cingulate cortex after systemic administration of a low dose (1mg/kg) of DMI. The reduction of NA efflux in the cortex was prevented by intra-LC infusion of RX821002, demonstrating an α_2-adrenoceptor-mediated inhibition of NA release.

Therefore, the effects of fluoxetine administration on NA transmission are similar to the known effects of NA uptake-inhibiting drugs and are consistent with the known regulatory process of NA neurones (Cedarbaum & Aghajanian, 1976). Experiments here also suggest that the hypothalamic NA innervation, from cells of the lateral tegmental brainstem nuclei, possess similar regulatory processes. If this is the case, local administration of fluoxetine to the frontal cortex and hypothalamus should increase NA efflux since the inhibitory effects upstream of axon terminals will not be activated. This prediction was tested in the next experiments (Chapter 4).

An important finding from experiments using DSP-4 was the marked increase in spontaneous NA efflux in both the frontal cortex and hypothalamus. Despite reduced tissue content of NA in the frontal cortex (-67%) and hypothalamus (-42%), NA efflux was elevated approximately 2-fold in both these areas. There are few studies of NA efflux in DSP-4 pretreated animals. The majority of studies using this toxin use measurements of whole tissue stores of NA or immunohistochemical measurements as indicators of a lesion (see Chapter 1). However, Hughes & Stanford (1996, 1998) similarly reported a 2-fold increase in NA efflux, in the frontal cortex of rats, using the same protocol for administration of DSP-4 as that used here. This increase is thought to reflect a compensatory increased firing rate of surviving NA neurones, coupled with the loss of NA uptake sites on lesioned axon terminals. Studies by Kask *et al*,

(1997), using DSP-4, and Abercrombie & Zigmond, (1989) using 6-hydroxydopamine (6-OHDA), reported no decrease in NA efflux in the frontal cortex or hippocampus respectively, despite both these treatments causing more than 50% reduction in tissue content of NA. The above studies, and the results presented here, demonstrate that, although tissue content of transmitter may be a useful indication of the extent of a neurotoxin lesion, caution should be taken when extrapolating this to effects on neurotransmission, which will be determined by the extracellular concentration of transmitter.

It should be noted that considerable variation was found in baseline (saline pretreated) tissue concentrations of NA and 5-HT between experiments 2 and 3 (see Tables 3.3 and 3.5). The precise explanation for this wide variability is unclear. Preparation of samples for analysis by HPLC-ED requires homogenization, centrifugation and extraction of the supernatant fluid. This extraction was performed once only for each sample. It may be that if several repetitions of the extraction were performed, the values for monoamine concentration would be more consistent across experiments. Additionally, unavoidable discrepancies in the time between collection of samples and analysis may have contributed to this variability. Similar discrepancies were noted between basal concentrations of tissue monoamine between experiments (see Chapters 4 and 5). However, brain tissue concentration was used solely as a demonstration of an effect of treatment (i.e. DPS-4 or *p*CPA) rather than to quantify absolute concentration of transmitter.

This is the first microdialysis study to report increase NA efflux in the hypothalamus of DSP-4 pretreated rats. DSP-4 preferentially destroys axon terminals in areas innervated by NA cells of the LC (Jonsson *et al*, 1981; Fritschy & Grzanna 1989). This is thought to reflect the greater affinity of DSP-4 for the NA transporter of these LC neurones (cortex K_i=179nM; hypothalamus K_i=460nM, Zaczek *et al*, 1990). However, if the DSP-4 dose is increased, LTA neurones are also lesioned. Thus, Heal *et al*, (1993) reported an 83% reduction in hypothalamic tissue content of NA after pretreatment with 100mg/kg DSP-4. Therefore, DSP-4 can destroy NA axons in the hypothalamus, albeit with less efficacy than those in the cortex, as was the

case here (-65% reduction of tissue content in the frontal cortex, -47% in the hypothalamus).

3.6. Conclusion

Systemic fluoxetine produce a variable, unpredictable increase in NA efflux in the frontal cortex and hypothalamus. Thus, using the same drug dose, strain of animals and route of administration, fluoxetine either increased or had no effect on NA efflux in the frontal cortex and hypothalamus. This indicates that the disparity between studies reported in the literature is a result of drug-effect variation, rather than inter-study variation. Likewise, although there was a difference in the duration of fluoxetine's effects in the frontal cortex and hypothalamus, a variable response was seen in both these regions. This suggests that, with respect to the frontal cortex and hypothalamus at least, the disparate effects of fluoxetine on NA efflux reported in the literature are due primarily to drug-effect variation, as opposed to brain-region variation. In this respect, the effects of systemic fluoxetine on NA efflux are strikingly similar to the reported effects of SSRIs on 5-HT transmission.

Unfortunately, given the nature of fluoxetine's effect on NA efflux, the contribution of 5-HT transmission could not be determined.

Finally, systemic fluoxetine reduced NA efflux in the frontal cortex and hypothalamus after a DSP-4 lesion. This suggests an inhibitory effect of fluoxetine on NA transmission upstream of axon terminals. This affects neurones innervating both the frontal cortex and hypothalamus.

To determine whether this inhibition masks an opposing effect of fluoxetine at axon terminals experiments in Chapter 4 use local infusion of fluoxetine to determine its effects on NA efflux in the frontal cortex and hypothalamus. The contribution of 5-HT transmission is also determined.

Chapter 4

Comparison of the Effects of Local Infusion of Fluoxetine or Citalopram on Noradrenaline Efflux in the Frontal Cortex and Hypothalamus of Freely-Moving Rats.

4.1. Introduction

Experiments described in Chapter 3 demonstrate that, when given systemically, fluoxetine either increased (Experiment 1) or had no apparent effect (Experiments 2 & 3) on NA efflux in the frontal cortex and hypothalamus. Such inconsistency in fluoxetine's effects is borne out by previously published microdialysis studies. Overall, these inconsistent reports suggest that that the effects of systemic fluoxetine on NA (and 5-HT) efflux in forebrain areas are truly variable. Whereas this variability has been explicitly acknowledged for the effects of acute SSRIs on 5-HT efflux, until now it has been overlooked for their effects on NA efflux.

Variable effects of reuptake inhibitors on 5-HT efflux are due to opposing effects at the somatodendritic and terminal level. Experiments in Chapter 3 suggest this could also be the case for fluoxetine's effects on NA. Thus, when the contribution of its effects at NA axon terminals is diminished (by DSP-4), systemic fluoxetine *decreased* NA efflux in the frontal cortex and hypothalamus. Therefore, if fluoxetine has opposing effects at NA cell bodies and terminals, it should increase NA efflux when applied locally to both these brain regions.

To test this hypothesis, the effects of fluoxetine at NA axon terminals was determined by infusing the drug directly to the frontal cortex and hypothalamus, *via* a microdialysis probe. This technique of 'reverse dialysis' can be thought of as equivalent to experiments using tissue slice preparations *in situ*. It is used widely to determine the effects of drugs at sites within the brain.

Few studies have applied this method to the study of fluoxetine's effect on NA efflux. However, those that have consistently report increased NA efflux in the frontal cortex, at least. Jordan *et al*, (1994) infused fluoxetine in the frontal cortex of freely-moving rats. 100μM fluoxetine elevated NA efflux in the prefrontal cortex, (+200% of baseline). Similarly, Hughes and Stanford (1996) reported increased NA efflux in the frontal cortex of both anaesthetized and freely-moving rats during local infusion of 5 and 50μM fluoxetine. Only one study has examined the effects of local infusion of fluoxetine to the hypothalamus (Perry & Fuller, 1997). They reported only a minor effect of 10μM fluoxetine on NA efflux. To date, no study has compared simultaneously the effects of local fluoxetine infusion on NA efflux in two brain regions.

Whether or not the effects of fluoxetine on NA efflux are due to 5-HT dependent mechanisms remains undetermined. Local infusion of fluoxetine could increase NA efflux by elevating the extracellular concentration of 5-HT, which causes secondary activation 5-HT heteroceptors (see Chapter 1). Experiments in this chapter use the 5-HT synthesis inhibitor *p*CPA to deplete stores of this neurotransmitter. If the effects of fluoxetine on NA efflux are secondary to an increase in extracellular 5-HT, they will be diminished in *p*CPA pretreated animals. However, if the effects are due to direct inhibition of NA uptake, they will not be affected by *p*CPA pretreatment.

If the augmentation of NA efflux by local infusion of fluoxetine occurs solely as a result of its actions on 5-HT transmission, this should be replicated by local infusion of other SSRIs. Citalopram is the most selective SSRI for inhibition of 5-HT versus NA uptake *in vitro* (see Stanford, 1996). Experiment 2 of this chapter tested the effects of local infusion of citalopram on NA efflux. Since this compound is a more potent inhibitor of 5-HT uptake than fluoxetine (see Chapter 1), if the effects of fluoxetine on NA uptake are secondary to an action on 5-HT transmission, citalopram should also increase NA efflux. If the effects of local infusion of fluoxetine are due to inhibition of NA uptake, citalopram should not produce as great an increase in NA efflux, given its lower binding affinity for the NA transporter (Thomas *et al*, 1987).

The effect of DSP-4 pretreatment on local infusion of fluoxetine was also examined in this chapter. Experiments in Chapter 3 showed that systemic fluoxetine decreased NA efflux in the frontal cortex and hypothalamus of animals pretreated with DSP-4. This neurotoxin partially destroys NA axon terminals but leaves cell bodies intact (Fritschy & Grzanna, 1989). The inhibitory effect of systemic fluoxetine on NA efflux is unlikely to be due to an action at NA axon terminals since these are destroyed by DSP-4. If this hypothesis is correct, the effects of local infusion of fluoxetine on NA efflux in the frontal cortex and hypothalamus will be diminished by DSP-4 pretreatment. However, if fluoxetine increases NA efflux by acting on a site not on NA terminals, its effects should remain in rats given DSP-4.

4.2. Aims

- To compare the effect of local infusion of fluoxetine on NA efflux in the frontal cortex and hypothalamus, two brain areas receiving noradrenergic innervation from different brainstem sources and with differences in regulation of NA transmission. (Experiments 1 & 3).

- To determine whether the effects of local fluoxetine infusion on NA efflux are 5-HT dependent, using the 5-HT synthesis inhibitor, *p*CPA. (Experiment 1).

- To determine whether infusion of the more selective SSRI, citalopram, elevates NA efflux to a greater extent than fluoxetine. If so, this will suggest the effects of fluoxetine are secondary to inhibition of 5-HT reuptake. (Experiment 2)

- To determine whether the effects of local infusion of fluoxetine on NA efflux in the frontal cortex and/or hypothalamus are diminished by selective destruction of NA axon terminals using DSP-4 (Experiment 3).

4.3. Methods

4.3.1. Microdialysis

Experiments were performed on freely-moving rats (240-320g on day of surgery). For experiments using *p*CPA, animals received either 250 mg/kg *p*CPA i.p. or 1 ml/kg 0.9% saline 2 days and 1 day before dialysis experiments. For experiments using DSP-4, subjects were pretreated with either 40mg/kg DSP-4 i.p. or 1ml/kg 0.9% saline i.p. 5 days before dialysis experiments. Microdialysis probes were implanted to both the frontal cortex and hypothalamus on the day before experimenting. See Chapter 2 (section 2.3) for details of probe design and surgical procedure.

Fluoxetine or citalopram were dissolved in Ringer's solution to make 0.5, 5.0 and 50μM solutions. Once basal NA efflux was stable and at least 3 basal samples taken, fluoxetine or citalopram was administered by changing the perfusion solution for Ringer's containing the drug. Each concentration was perfused for 80min, in the order 0.5, 5.0 then 50μM. The last three basal samples were designated T_{-40}-T_0, with perfusion of fluoxetine or citalopram starting immediately after T_0. Microdialysis sampling continued for a further 4 h. At the end of each experiment animals were killed immediately by halothane overdose and cervical dislocation.

4.3.2. Measurement of brain region monoamine content

To determine the extent of *p*CPA and DSP-4- induced reduction of whole tissue monoamine content, homogenates from the frontal cortex and hypothalamus were analyzed for their content of NA, dopamine and 5-HT, using HPLC. See Chapter 2 (section 2.4.4) for a detailed description of the tissue preparation and analytical methods used.

4.3.3. Statistical analysis

Data for monoamine content were analyzed using two-way ANOVA with 'brain region' as a 'within subjects' factor and 'pretreatment' (*i.e. p*CPA vs saline, or DSP-4 vs saline) as a 'between subjects' factor. The total concentration of monoamines in the frontal cortex and hypothalamus is expressed as mean ng/g wet tissue, (± s.e.m).

All data from microdialysis experiments were analyzed for significance using two-way ANOVA with repeated measures. The concentration of NA in brain dialysates is expressed as fmol/20min without correction for recovery. In addition to raw data, net changes were calculated by subtracting the mean of the last three basal samples from all samples in the time course. Data were pooled and the mean and s.e.m. calculated.

- Experiment 1 compared the effect of *p*CPA-pretreatment on the action of locally infusion of fluoxetine.
- Experiment 2 compared the effect of *p*CPA pretreatment on the action of local infusion of citalopram.
- Experiment 3 compared the effect of DSP-4 pretreatment on local infusion of fluoxetine.

For each of these experiments, 'time' was a 'within subjects' factor and 'pretreatment' (*i.e. p*CPA vs saline or DSP-4 vs saline) a 'between subjects' factor.

The data were divided into four 'bins' with three consecutive samples per bin. Bins of data were compared for differences in both within and between treatment groups. 'Bin 1' represents basal efflux (*i.e.* T_{-40}-T_0). For bins representing NA efflux during drug infusion, the last three samples during infusion of each concentration of drug were used. Therefore, bin 2 =0.5 μM (T_{40}-T_{80}), bin 3 =5.0μM (T_{120}-$_{160}$), and bin 4 =50 μM (T_{200}-T_{240}). The Greenhouse-Geisser 'ε' correction was performed where Mauchley's test of sphericity was significant.

4.4 Results

4.4.1 Experiment 1: Effect of local infusion of fluoxetine on NA efflux in *p*CPA and saline pretreated rats

4.4.1.1 Effect of *p*CPA pretreatment on monoamine content

*p*CPA pretreatment reduced tissue content of 5-HT in the frontal cortex (-84%, $F_{1,12}$=114, P<0.001) and hypothalamus (-85%, $F_{1,13}$=33, P<0.001, Table 4.1). In the frontal cortex, changes in other monoamines did not reach significance (NA: $F_{1,12}$=0.23, P=0.64; DA: $F_{1,12}$=1.0, P=0.34). However, in the hypothalamus, the tissue content of NA (-34%, $F_{1,13}$=6.0, P=0.03), but not that of DA ($F_{1,13}$=0.35, P=0.56) was reduced by *p*CPA.

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	*p*CPA	SAL	*p*CPA
NA	277±39	241±29	2234±203	**1560±217***
DA	517±140	395±114	653±153	547±115
5-HT	270±19	**41±9.5****	697±108	**111±22****

Table 4.1. Monoamine content in frontal cortex and hypothalamus of saline and *p*CPA-pretreated rats receiving local infusion of fluoxetine. Data show mean content (ng/g wet tissue weight) ± s.e.m. n=7-9. Significant differences are in bold. *P<0.05; **P<0.005 *c.f.* saline.

4.4.1.2. Effect of fluoxetine on NA efflux

Frontal cortex

There was no difference in basal NA efflux of saline and *p*CPA pretreated rats (fmoles/20min; T_{-40}-T_0: saline, 16.7 ± 1.4, *p*CPA, 19.3 ± 1.3). Local infusion of 0.5μM fluoxetine had no effect of NA efflux in the frontal cortex in either *p*CPA or saline pretreated animals (Figure 4.1A). Thus, comparison of bin 1 and bin 2 (T_{40}-T_{80}) showed no effect of 'bin', 'pretreatment' and no 'bin' x 'pretreatment interaction'

Infusion of 5.0μM fluoxetine increased NA efflux (*c.f.* basal efflux) in saline pretreated animals only (see Figure 4.1A). Thus, comparison of bin 1 and bin 3 (T_{120}-T_{160}) showed a main effect of 'bin', ($F_{1,26}$=6.9, *P*=0.01) a 'bin' x 'pretreatment' interaction, ($F_{1,26}$=6.7, *P*=0.02), but no effect of 'pretreatment' ($F_{1,26}$=0.6, *P*=0.4). Analysis of saline pretreated animals alone revealed that NA efflux was increased after 5.0μM fluoxetine, relative to basal (main effect of 'bin' $F_{1,16}$=13.1, *P*=0.02). 5.0μM fluoxetine did not increase NA efflux in *p*CPA pretreated animals relative to basal, (no main effect of 'bin').

Infusion of 50μM fluoxetine increased NA efflux in both saline and *p*CPA pretreated animals, compared with basal efflux (Figure 4.1A). Thus, comparison of bin 1 and bin 4 (T_{200}-T_{240}) showed a main effect of 'bin', ($F_{1,20}$=19.6, *P*<0.001), but no 'bin' x 'pretreatment' interaction and no effect of 'pretreatment'.

Analysis of raw over all time points analyzed (T_{-40}-T_{240}) revealed a main effect of 'time', ($F_{4,266}$=3.3, *P*=0.01), but no 'time' x 'pretreatment' interaction and no effect of 'pretreatment'. NA efflux was not different in saline and *p*CPA pretreated animals at each hour during fluoxetine infusion (see Table 4.2).

Analysis of net changes in NA efflux revealed a similar pattern of results as analysis of raw data, with the addition of a main effect of 'pretreatment' at T_{80}-T_{120} ($F_{1,26}$=5,7, *P*=0.03). Figure 4.2A and Table 4.3.

Hypothalamus

There was no difference in basal NA efflux between saline and *p*CPA pretreated rats (fmoles/20min; T_{-40}-T_0: saline, 16.8 ± 1.0; *p*CPA , 17.3 ± 0.4 Figure 4.1B).

Neither 0.5μM nor 5.0μM fluoxetine increase NA efflux in either saline or *p*CPA pretreated rats. Thus, comparison of time bins (T_{40}-T_{80}) and (T_{120}-T_{160}) with basal efflux (T_{-40}-T_0) revealed no effects of 'bin', 'time' or 'bin' x 'time interactions, (Figure 4.1B).

50μM fluoxetine elevated NA efflux in saline and *p*CPA pretreated rats. Thus, analysis of time points T_{140}-T_{240} (*i.e.* immediately before and during infusion of 50μM fluoxetine) revealed a main effect of 'time', ($F_{5,80}$=4.5, *P*=0.001), but no effect of 'pretreatment' and no 'time' x 'pretreatment' (see Figure 4.1B). This effect was present when data for saline pretreated rats was analyzed alone (main effect of time, $F_{5,55}$=4.0, *P*=0.004), but not *p*CPA pretreated rats (no effect of 'time'). Analysis of raw data for each hour during fluoxetine administration found no main effects of 'pretreatment'. Table 4.2.

Analysis of net changes revealed the same pattern of results as analysis of raw data. Figure 4.2B and Table 4.3.

	FRONTAL CORTEX		HYPOTHALAMUS	
T_{20}-T_{60}	$F_{1,21}$=0.16,	*P*=0.7	$F_{1,17}$=0.004,	*P*=0.95
T_{80}-T_{120}	$F_{1,21}$=2.3,	*P*=0.14	$F_{1,16}$=0.74,	*P*=0.4
T_{140}-T_{180}	$F_{1,21}$=2.6,	*P*=0.12	$F_{1,13}$=1.1,	*P*=0.3
T_{200}-T_{240}	$F_{1,15}$=0.004,	*P*=0.95	$F_{1,14}$=0.86,	*P*=0.37

Table 4.2 Statistics generated from two-way ANOVA using raw data, showing effect of *p*CPA pretreatment on NA efflux in frontal cortex or hypothalamus animals, during local infusion of fluoxetine (main factor 'pretreatment').

	FRONTAL CORTEX		HYPOTHALAMUS	
T_{20}-T_{60}	$F_{1,21}$=0.78,	*P*=0.39	$F_{1,21}$=0.3,	*P*=0.59
T_{80}-T_{120}	**$F_{1,21}$=5.4,**	***P*=0.03**	$F_{1,18}$=0.85,	*P*=0.31
T_{140}-T_{180}	$F_{1,21}$=3.1,	*P*=0.09	$F_{1,16}$=1.4,	*P*=0.26
T_{200}-T_{240}	$F_{1,19}$=0.12,	*P*=0.73	$F_{1,17}$=0.64,	*P*=0.44

Table 4.3 Statistics generated from two-way ANOVA using net data, showing effect of *p*CPA pretreatment on NA efflux in frontal cortex or hypothalamus animals, during local infusion of fluoxetine (main factor 'pretreatment'). Significant differences in bold.

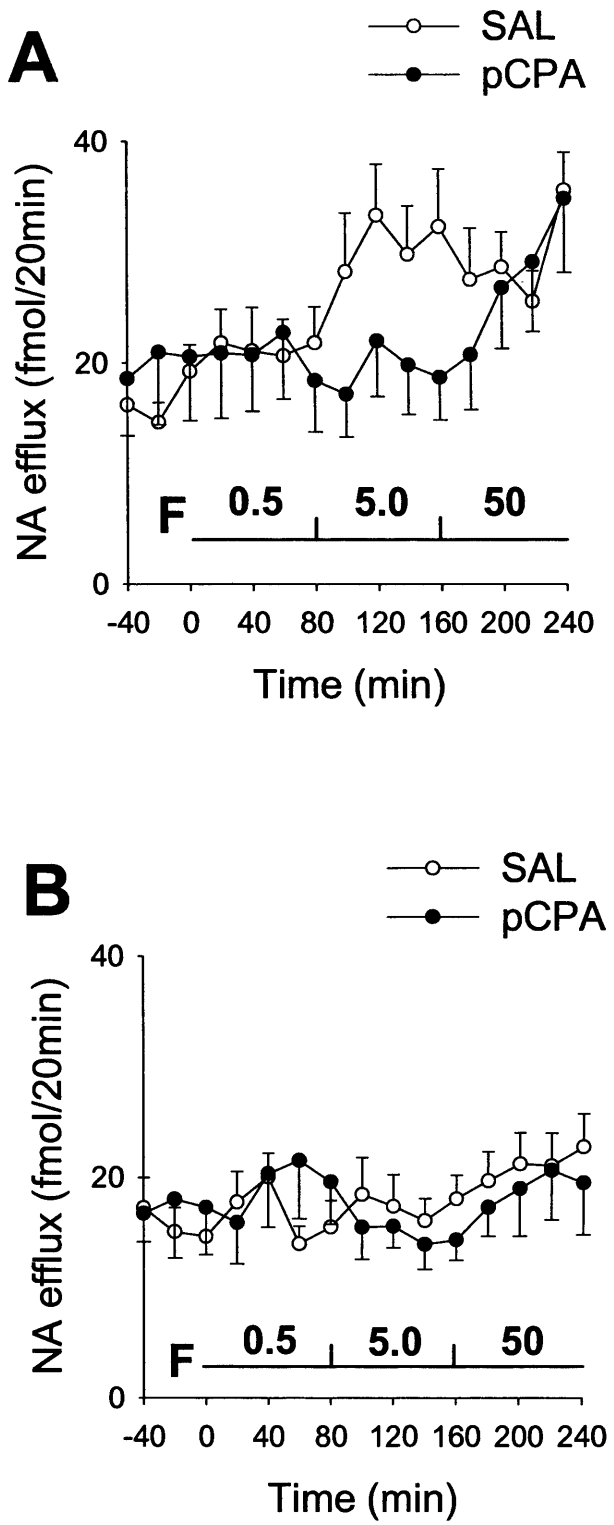


Figure 4.1. Effect of local infusion of fluoxetine on NA efflux (raw data) in A) frontal cortex and B) hypothalamus of saline and *p*CPA pretreared animals. Graphs show mean ± s.e.m. (n= 9-17 each time point). Infusion of 0.5, 5.0 and 50μM fluoxetine for 80 min each as marked by bar. See section 4.4.1 for details of statistical analysis.

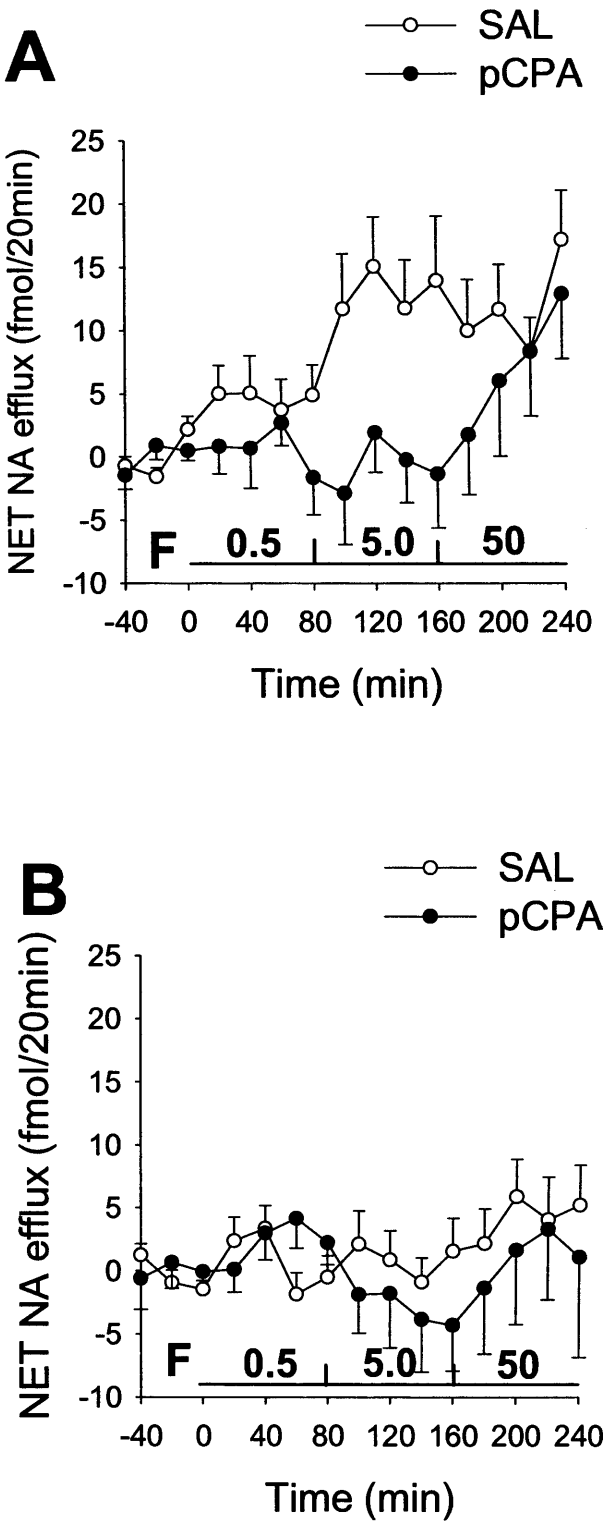


Figure 4.2. Effect of local infusion of fluoxetine on net changes in NA efflux in A) frontal cortex and B) hypothalamus of saline and *p*CPA pretreated animals. Graphs show mean ± s.e.m. (n= 9-17). Infusion of 0.5, 5.0 and 50μM fluoxetine for 80 mins each as marked by bar. See section 4.4.1 for details of statistical analysis.

4.4.2. Experiment 2: Local infusion of citalopram in the frontal cortex and hypothalamus of *p*CPA and saline pretreated rats.

4.4.2.1. Effect of *p*CPA pretreatment on monoamine content.

*p*CPA pretreatment reduced tissue content of 5-HT in the frontal cortex (-70%, $F_{1,13}$=24, P<0.001) and hypothalamus (-71%, $F_{1,13}$=6.8, P<0.02; Table 4.4). In the frontal cortex, there were no changes in other monoamines (NA: $F_{1,13}$=1.5, P=0.2; DA: $F_{1,13}$<0.001, P=0.99). In the hypothalamus, tissue content of DA was unchanged ($F_{1,13}$<0.001, P=0.99) and the apparent reduction of NA failed to reach the criterion for significance ($F_{1,13}$=3.3, P=0.09).

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	*p*CPA	SAL	*p*CPA
NA	414±150	225±33	341±160	129±48
DA	303±24	62±40	208±33	217±18
5-HT	908±85	**269±100****	712±131	**210±140***

Table 4.4. Monoamine content in frontal cortex and hypothalamus of saline and *p*CPA-pretreated rats receiving local infusion of citalopram. Table shows mean content (ng/g wet tissue weight) ±s.e.m. n=7-11. Significant differences are in bold. *P<0.05; **P<0.005 *c.f.* saline pretreated rats

4.4.2.2. Effect of local citalopram infusion on NA efflux

Frontal Cortex.

There was no difference in basal NA efflux between saline and *p*CPA pretreated rats (fmoles/20min; T_{-40}-T_0: saline, 17.6 ± 0.8; *p*CPA, 16.1± 2.4). Local infusion of citalopram did not change NA efflux in either saline or *p*CPA pretreated rats (Figure 4.3A). Analysis of raw data over all time points (T_{-40}-T_{240}) revealed no effects of 'time', 'pretreatment', and no 'time' x 'pretreatment' interaction.

Infusion of 0.5μM citalopram had no effect on NA efflux in either saline or *p*CPA pretreated animals. Thus comparison of bin 1 (T_{-40}-T_0) with bin 2 (T_{40}-T_{80}) found no effect of 'bin', 'pretreatment', and no 'bin' x 'pretreatment' interaction.

Infusion of 5.0μM citalopram did not change NA efflux in either group of rats, (Figure 4.3A). Thus comparison of bin 1 with bin 3 (T_{120}-T_{160}) revealed no effect of 'bin', 'pretreatment', and no 'bin' x 'pretreatment' interaction.

50μM citalopram had no effect on NA efflux, (Figure 4.3A). Thus, comparison of bin 1 with bin 4 (T_{200}-T_{240}) revealed no effects of 'bin', 'pretreatment' and no 'bin x 'pretreatment' interaction.

Hypothalamus

There was no difference in basal NA efflux between saline and *p*CPA pretreated rats (fmoles/20min; T_{-40}-T_0: saline, 18.7.5 ± 0.3; *p*CPA, 16.5 ± 0.8) Local infusion of citalopram did not change efflux in saline- or *p*CPA-pretreated rats (Figure 4.3B). Analysis of raw data over all time points, (T_{-40}-T_{240}) revealed no effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interaction.

Infusion of 0.5μM citalopram had no effect on NA efflux in either group of rats. Thus, comparison of bin 1 with bin 2 (T_{40}-T_{80}) revealed no effects of 'bin', 'pretreatment', and no 'bin' x 'pretreatment' interaction.

Infusion of 5.0μM citalopram did not change NA efflux in either group of animals (Figure 4.3B). Thus, comparison of bin 1 with bin 3 (T_{120}-T_{160})

revealed no effects of 'bin', 'pretreatment' and no 'bin' x 'pretreatment' interaction.

Infusion of 50μM citalopram did not modify NA efflux in either saline or *p*CPA pretreated animals (Figure 4.3B). Thus comparison of bin1 with bin 4 (T_{200}-T_{240}) revealed no effects of 'bin', 'pretreatment' and no 'bin' x 'pretreatment interaction. Analysis of raw data revealed no difference in NA efflux between saline and *p*CPA pretreated in either brain regions during each hour of citalopram infusion, (Table 4.5.).

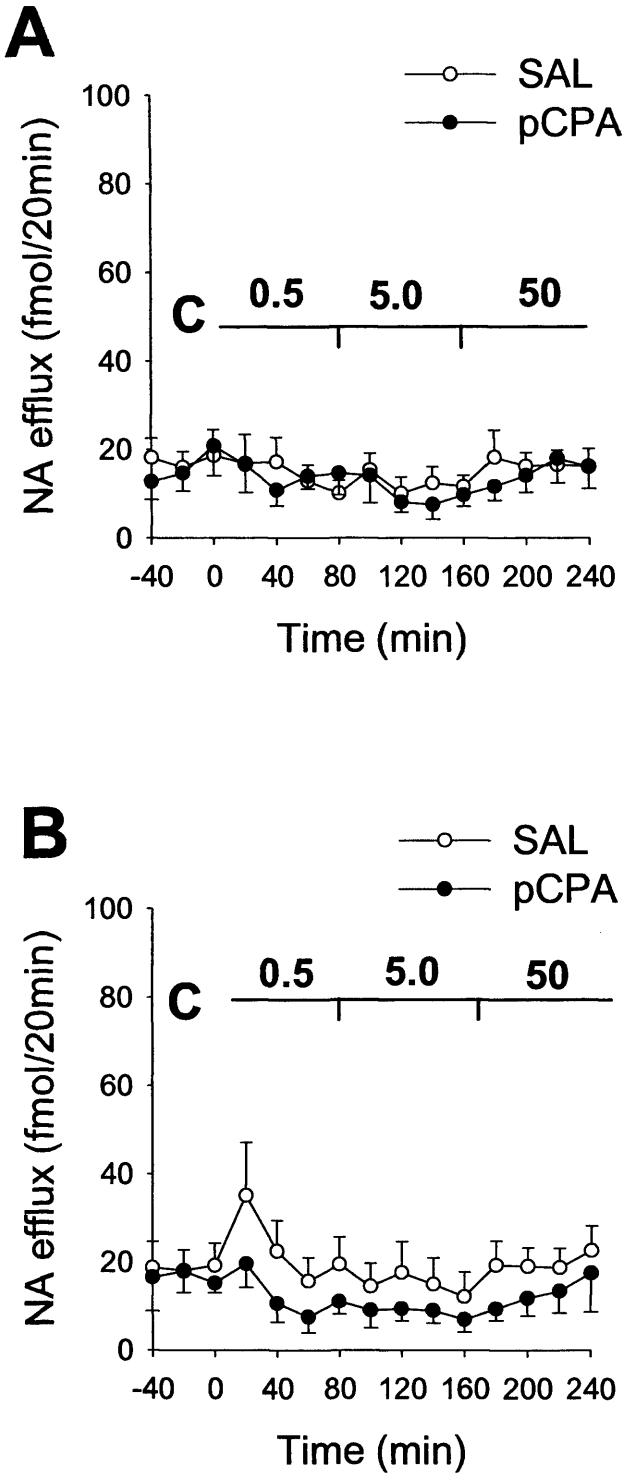


Figure 4.3. Effect of local infusion of citalopram on NA efflux (raw data) in A) frontal cortex and B) hypothalamus of saline and *p*CPA pretreated animals. Graphs show mean ± s.e.m. (n = 7-10 each time point). 0.5, 5.0 and 50μM citalopram were infused for 80 min each, as marked by bar. See section 4.4.2.2 for details of statistical analysis.

	FRONTAL CORTEX	HYPOTHALAMUS
T_{20}-T_{60}	$F_{1,16}$=0.7, P=0.43	$F_{1,16}$=2.0, P=0.18
T_{80}-T_{120}	$F_{1,16}$=0.3, P=0.62	$F_{1,16}$=1.4, P=0.25
T_{140}-T_{180}	$F_{1,16}$=1.5, P=0.24	$F_{1,16}$=1.8, P=0.20
T_{200}-T_{240}	$F_{1,16}$=0.2, P=0.7	$F_{1,16}$=1.0, P=0.33

Table 4.5. Statistics generated from two-way ANOVA using raw data, showing effect of *p*CPA pretreatment on NA efflux in frontal cortex or hypothalamus animals, during local infusion of citalopram (main factor 'pretreatment').

4.4.3. Experiment 3: Local infusion of fluoxetine to DSP-4 and saline pretreated rats

4.4.3.1. Effect of DSP-4 pretreatment on monoamine content

DSP-4 pretreatment reduced tissue content of NA in the frontal cortex (-39%, $F_{1,17}$=5.6, P=0.03). In the hypothalamus, the apparent reduction in NA content did not reach the criterion for significance, ($F_{1,17}$=3.3, P=0.09). DSP-4 did not reduce tissue content of 5-HT or dopamine in either brain region. Table 4.6

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	**DSP-4**	**SAL**	**DSP-4**
NA	338±39	**206±38***	838±107	528±127
DA	286±133	166±32	154±41	148±38
5-HT	117±80	152±105	181±151	129±129

Table 4.6. Monoamine content in frontal cortex and hypothalamus of saline and DSP-4-pretreated rats receiving local infusion of fluoxetine. Table shows mean content (ng/g wet tissue weight) ± s.e.m. n=11. Significant differences are in bold. *P<0.05 *c.f.* saline pretreated rats

4.4.3.2. Effect of local fluoxetine infusion of NA efflux

Frontal Cortex

Basal efflux of NA was greater in DSP-4 pretreated animals, (saline, 12.8 ± 0.5 fmoles/20min; DSP-4, 22.0 ± 0.8; effect of 'pretreatment', $F_{1,23}$=4.6, P=0.04; Figure 4.4A). NA efflux increased after fluoxetine infusion, in saline, but not DSP-4 pretreated rats. Analysis of all time points (T_{-40}-T_{240}) revealed significant effects of 'time', ($F_{6,149}$=4.7, P<0.001), a 'time' x 'pretreatment' interaction, ($F_{6,148}$=4.3, P<0.001), but no main effect of 'pretreatment'.

Infusion of 0.5μM fluoxetine had no effect on NA efflux in either saline or DSP-4 pretreated rats, (Figure 4.4A). Thus, comparison of bin 1 (basal efflux; T_{-40}-T_0) with bin 2 (T_{40}-T_{80}) found no effect of 'bin', no 'bin' x 'pretreatment' interaction, but a main effect of 'pretreatment', ($F_{1,23}$=4.7, P=0.04), reflecting the greater efflux of NA in DSP-4 pretreated rats.

Infusion of 5.0μM fluoxetine increased NA efflux in saline pretreated animals only, (Figure 4.4A). Thus, comparison of bin 1 with bin 3 (T_{120}-T_{160}) revealed no main effect of 'bin', or 'pretreatment', but a 'bin' x 'pretreatment' interaction, ($F_{1,23}$=7.1, P=0.01). The lack of effect of 'bin' may have been due to the difference in basal between DSP-4 and saline pretreated rats. Analysis of data for saline pretreated, but not DSP-4 pretreated rats revealed a main effect of 'bin', $F_{1,13}$=13.4, P=0.003), *i.e.* NA efflux was increased in the saline group, with respect to basal efflux.

Infusion of 50μM fluoxetine increased NA efflux in saline pretreated animals only, (Figure 4.4A). Thus, comparison of bin 1 with bin 4 (T_{200}-T_{1240}) revealed a main effect of 'bin', ($F_{1,23}$=13.7, P=0.001) a 'bin' x 'pretreatment' interaction, ($F_{1,23}$=9.8, P=0.05) but no effect of 'pretreatment'. Analysis of data from saline pretreated rats only revealed a main effect of 'bin', ($F_{1,13}$=41.7, P<0.001).

The loss of effect of DSP-4 'pretreatment' during 5.0μM and 50μM is due to the increase in NA efflux in saline pretreated animals, with no corresponding increase in efflux in the DSP-4 pretreated group. Analysis of raw data revealed no difference in NA efflux between saline and DSP-4 pretreated animals at any

hour during fluoxetine infusion, in either the frontal cortex or hypothalamus (Table 4.7)

Analysis of net changes in NA efflux revealed similar pattern of results, with a main effect of 'time' ($F_{7,155}$=4.7, P<0.001), 'pretreatment', ($F_{1,23}$=4.2, P=0.05; and a 'time' x 'pretreatment' interaction, ($F_{7,155}$=4.7, P<0.001: T_{-40}-T_{240}, Figure 4.5A).

0.5μM fluoxetine had no effect on NA efflux in either group of rats. Thus, comparison of bin 1 (T_{-40}-T_0) with bin 2 (T_{40}-T_{80}) found no effects of 'bin', 'pretreatment', and no 'bin' x 'pretreatment' interaction, (Figure 4.5A).

5.0μM fluoxetine increased NA efflux in saline pretreated animals only. Thus, comparison of bin1 with bin 3 (T_{120}-T_{160}) revealed a main effect of 'bin', ($F_{1,23}$=4.6, P=0.04), 'pretreatment', ($F_{1,23}$=6.5, P=0.02), and a 'bin' x 'pretreatment' interaction, ($F_{1,23}$=8.3, P=0.01, Figure 4.5A).

Infusion of 50μM fluoxetine increased NA efflux in saline pretreated animals only, (Figure 4.5A). Thus, comparison of bin 1 with bin 4 (T_{200}-T_{240}) revealed a main effect of 'bin', ($F_{1,23}$=13.8, P=0.001), 'pretreatment', ($F_{1,23}$=8.4, P=0.008) and a 'bin' x 'pretreatment' interaction, ($F_{1,23}$=8.4, P=0.008). Analysis of time points during infusion of 5.0 and 50μM fluoxetine (T_{100}-T_{240}) revealed a main effect of 'time', ($F_{7,161}$=2.1, P=0.05), 'pretreatment', ($F_{1,23}$=6.4, P=0.02) and a 'time' x 'pretreatment' interaction, ($F_{7,161}$=4.3, P<0.0001), indicating that fluoxetine elevated NA efflux in saline pretreated animals only.

Hypothalamus

DSP-4 pretreatment had no effect on basal NA efflux (saline, 17.9 ± 0.4; DSP-4, 17.7 ± 0.7; no effect of 'pretreatment'). NA efflux was increased by infusion of fluoxetine (Figure 4.4B). Thus, analysis of raw data over all time points (T_{-40}-T_{240}) revealed a main effect of 'time', ($F_{7,132}$=3.2, P=0.004), but no effect of 'pretreatment', and no 'time' x 'pretreatment' interaction.

Infusion of 0.5μM fluoxetine had no effect on NA efflux in saline or DSP-4 pretreated rats. Thus, comparison of bin 1 with bin 2 (T_{40}-T_{80}) found no effect

of 'bin', no effect of 'pretreatment' and no 'bin' x 'pretreatment' interaction, (Figure 4.4B).

Infusion of 5.0μM fluoxetine had no effect on NA efflux in either group of animals. Thus, comparison of bin 1 with bin 3 (T_{120}-T_{160}) revealed no effects of 'bin', 'pretreatment', and no 'bin' x 'pretreatment' interaction. Figure 4.4B

Infusion of 50μM fluoxetine increased NA efflux, (Figure 4.4B). Thus, comparison of bin 1 with bin 4 (T_{200}-T_{1240}) revealed a significant effect of 'bin', ($F_{1,20}$=8.3, *P*=0.01) but no effect of 'pretreatment', and no 'bin' x 'pretreatment' interaction. Analysis of data from DSP-4 pretreated rats only revealed a main effect of 'bin', $F_{1,10}$=11.9, *P*=0.006). However, when saline pretreated rats only were analyzed, there was no main effect of 'bin', ($F_{1,10}$=1.2, *P*=0.3).

Analysis of net changes in NA efflux revealed the same pattern of results as analysis of raw data. Figure 4.5B and Table 4.8.

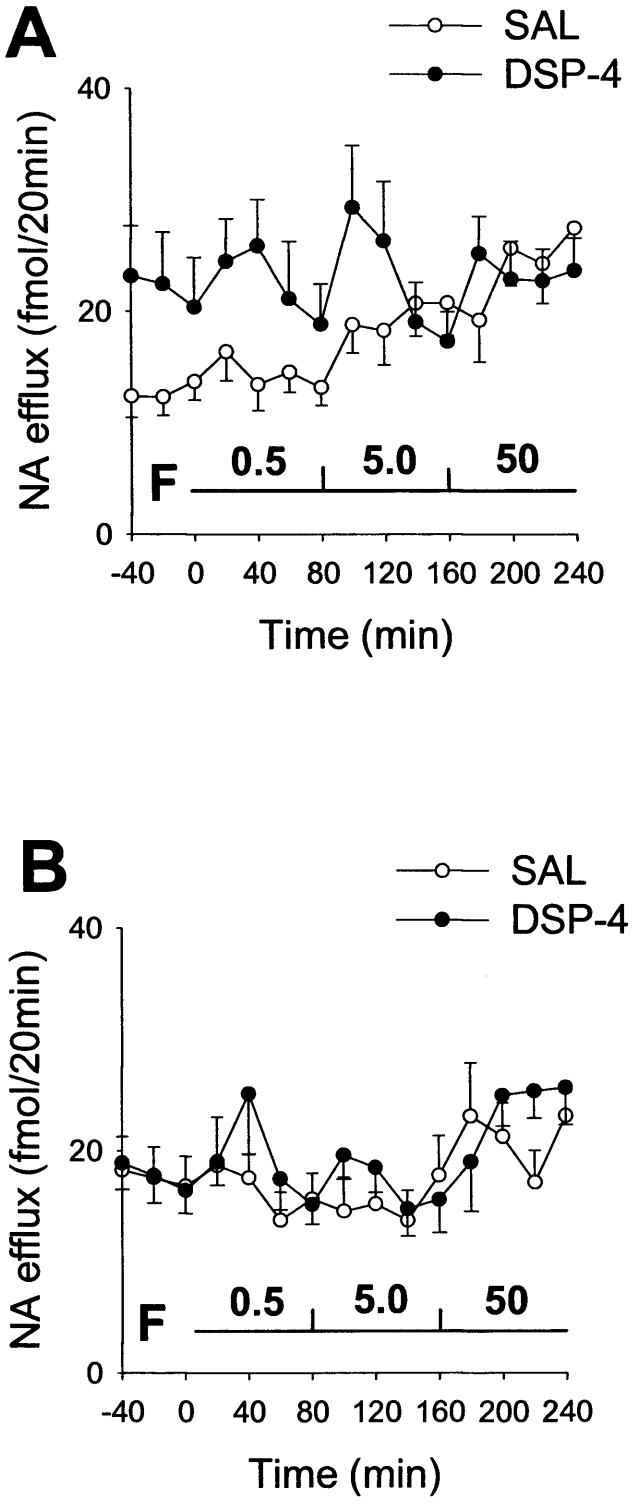


Figure 4.4. Effect of local infusion of fluoxetine in A) frontal cortex and B) hypothalamus of animals receiving saline or DSP-4 pretreatment. Graphs show mean ± s.e.m. (n = 11-14). 0.5, 5.0 and 50μM fluoxetine infused for 80 min each as marked by bar. See section 4.4.3.2 for details of statistical analysis

	FRONTAL CORTEX		HYPOTHALAMUS	
T_{20}-T_{60}	$F_{1,23}$=3.7,	*P*=0.07	$F_{1,20}$=0.63,	*P*=0.44
T_{80}-T_{120}	$F_{1,23}$=2.7,	*P*=0.12	$F_{1,20}$=0.9,	*P*=0.36
T_{140}-T_{180}	$F_{1,23}$=0.21,	*P*=0.66	$F_{1,20}$=0.09,	*P*=0.77
T_{200}-T_{240}	$F_{1,13}$=0.84,	*P*=0.37	$F_{1,20}$=01.4,	*P*=0.25

Table 4.7 Statistics generated from two-way ANOVA using raw data, showing effect of DSP-4 pretreatment on NA efflux in frontal cortex or hypothalamus animals, during local infusion of fluoxetine (main factor 'pretreatment').

	FRONTAL CORTEX		HYPOTHALAMUS	
T_{20}-T_{60}	$F_{1,23}$=0.1,	*P*=0.75	$F_{1,21}$=1.4,	*P*=0.24
T_{80}-T_{120}	$F_{1,23}$=0.2,	*P*=0.69	$F_{1,21}$=0.3,	*P*=0.60
T_{140}-T_{180}	**$F_{1,23}$=7.5,**	***P*=0.01**	$F_{1,21}$=0.39,	*P*=0.54
T_{200}-T_{240}	**$F_{1,23}$=9.4,**	***P*=0.005**	$F_{1,20}$=2.5,	*P*=0.13

Table 4.8 Statistics generated from two-way ANOVA using NET data, showing effect of DSP-4 'pretreatment' on NA efflux in frontal cortex or hypothalamus, during local infusion of fluoxetine (main factor 'pretreatment').

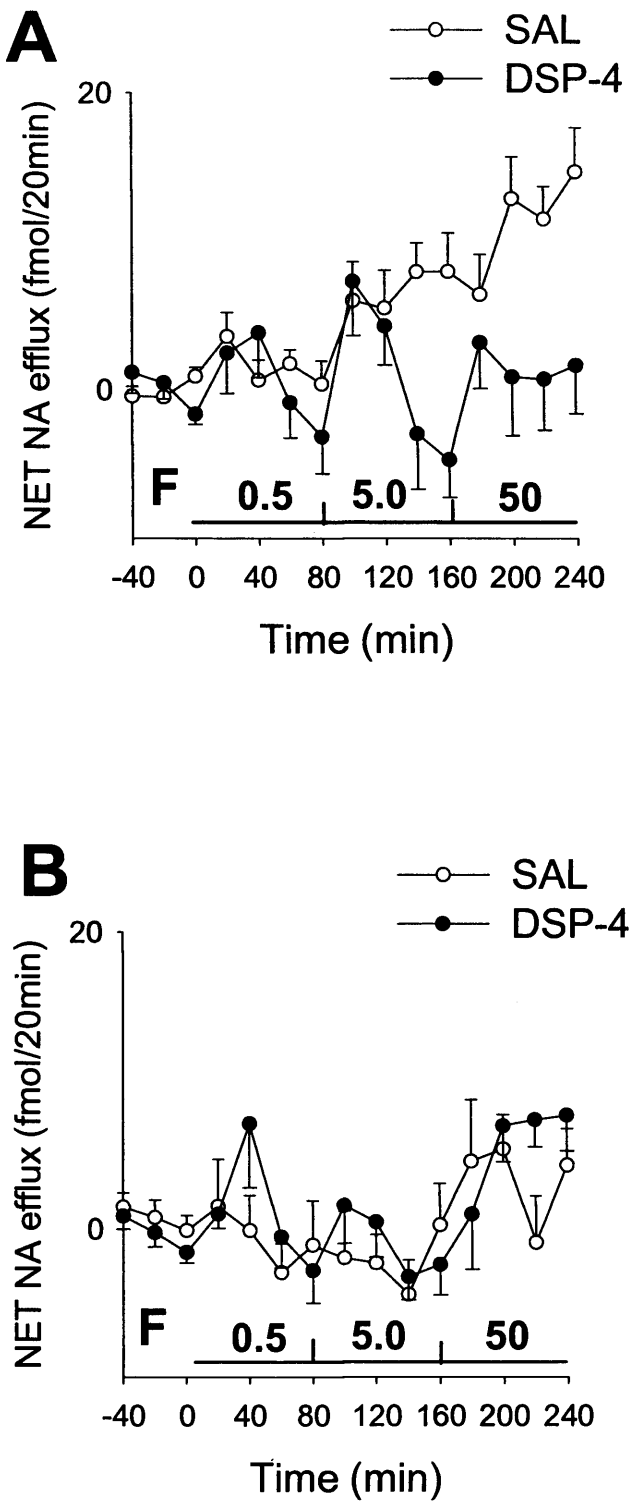


Figure 4.5. Effect of local infusion of fluoxetine on net changes in NA efflux in frontal cortex and B) hypothalamus of saline and DSP-4 pretreated animals. Graphs show mean ± s.e.m. (n= 11-14). 0.5, 5.0 and 50 μM fluoxetine were infused for 80 min each as marked by bar. See section 4.4.3.2 for details of statistical analysis

4.5. Discussion

The aim of experiments reported in this chapter was to determine the effects of local infusion of fluoxetine on NA efflux in the frontal cortex and hypothalamus, to establish the contribution of 5-HT to these effects and to determine whether an action at NA axon terminals is involved. This is the first study to use dual probe microdialysis to compare the effects of local administration of an SSRI in the frontal cortex and hypothalamus and to determine the contribution of 5-HT and NA axon terminals to these effects.

In the frontal cortex, infusions of 5.0 and 50μM fluoxetine increased NA efflux in saline pretreated rats (Experiments 1 and 3). This confirms that, when applied directly in the vicinity of noradrenergic axon terminals of LC neurones, fluoxetine elevates NA efflux. However, in DSP-4 pretreated rats, neither concentration of fluoxetine was effective. DSP-4 diminished tissue content and elevated efflux of NA in the frontal cortex, suggesting a marked lesion of NA axon terminals had occurred. Thus, intact NA neurones are required for fluoxetine to modulate NA transmission in this brain region.

Infusion of drugs that inhibit NA reuptake such as DMI (Dalley & Stanford, 1995), imipramine (Jordan *et al*, 1994), BTS 54354 (Géranton *et al*, 2004), reboxetine (Owens & Whitton, 2003), cocaine, (Thomas *et al*, 1994) and zotepine (Rowley *et al*, 1998), elevate the extracellular concentration of NA when infused to the frontal cortex of freely moving rats. Unlike the variable effect produced by systemic administration, the results here and of previous studies (Jordan *et al*, 1996; Hughes & Stanford, 1996; 1998) consistently report increased NA efflux when fluoxetine is infused to the frontal cortex. Thus the route of administration seems critical in determining fluoxetine's effects in the forebrain (see below).

Experiments described in Chapter 3 showed a decrease in NA efflux in the frontal cortex when fluoxetine was administered to rats with a partial lesion of NA axon terminals. This decrease was not seen in rats with intact NA axon terminals. Taken together with results of this chapter, it appears that fluoxetine has opposing effects on NA transmission: an enhancing effect at axon terminals (see above) and an inhibitory effect at a site upstream of these

(Chapter 3). This is entirely consistent with established regulatory mechanisms of central NA transmission (Cedarbaum & Aghajanian, 1976). Thus, an increase is the extracellular concentration of NA at the somatodendritic level will activate α_2-adrenoceptors, inhibiting cell firing (Mateo *et al*, 1998) and release of NA in the forebrain (Thomas *et al*, 1994; Van Gaalen *et al*, 1997; Mateo *et al*, 1998). This effect becomes apparent when the opposing actions of fluoxetine at the axon terminals are diminished by pretreatment with DSP-4.

The effects of fluoxetine on NA transmission in the frontal cortex are strikingly similar to those reported for SSRIs on 5-HT transmission (see Chapter 3), suggesting that fluoxetine modulates both transmitters in a similar way. However, in the case of 5-HT, a lack of effect of systemic fluoxetine on efflux in the forebrain is attributed to activation of somatodendritic and terminal autoreceptors (Hérvas & Artigas, 1998; Romero *et al*, 1996; Gartside *et al*, 1995; see Chapter 3). The results presented here suggest that systemic fluoxetine modulates NA transmission *in vivo* but this may go undetected by microdialysis due to the balance of opposing effects on NA axon terminals and soma. If fluoxetine modulates NA transmission, in a similar way to that of 5-HT, a lack of effect on NA efflux should not been taken as evidence for selectivity of this drug for effects on 5-HT versus NA *in vivo*, as has been assumed in the past (Beyer *et al*, 2002).

For this hypothesis to be correct two conditions must be met. Firstly, co-administration of α_2-adrenoceptor antagonists with fluoxetine will result in consistent increases in forebrain NA efflux, as opposed to the variable effect reported on administration of fluoxetine alone. Secondly, local infusion of fluoxetine to the LC will elevate NA efflux there, while simultaneously decreasing LC cell firing and release of NA the cortex. This reduction in LC firing and release will be blocked by intra-LC infusion of an α_2-adrenoceptor antagonist.

Although published studies are limited, evidence that the first of these conditions may have been met comes from the study of Gobert *et al*, (1997). They reported potentiation of the fluoxetine-induced increase in frontal cortex NA efflux by co-administration of the α_2-antagonist, atipamezole. Co-

administration of buspirone also potentiates the effects of fluoxetine on NA efflux, an effect attributed to the α_2-adrenoceptor antagonist properties of its metabolite, 1-(2-pyrimidinyl-piperazine), (1PP; Gobert *et al*, 1999). However, these authors consistently report elevated NA efflux in the frontal cortex after systemic fluoxetine and have not so far reported a lack of effect of this drug. For the effects of systemic fluoxetine on NA efflux to be the same as those on 5-HT, ideally, α_2-adrenoceptor antagonist administration should disclose an effect that is not otherwise evident.

The second condition has been demonstrated by Mateo *et al,* (2000). Local infusion of the SSRI citalopram, elevates NA efflux in the LC, simultaneously decreases the firing rate of LC neurones and decreases NA efflux in the cingulate cortex. The effects of citalopram on LC firing and release of NA were prevented by intra-LC infusion of the α_2-adrenoceptor antagonist, RS79948. It would be interesting to known if this effect generalizes to other SSRIs.

The question remains whether the effect of fluoxetine on NA efflux is secondary to increase extracellular 5-HT concentration. Using *p*CPA pretreatment (Experiment 1), the effect of the lower concentration of fluoxetine (5.0μM) was abolished and is therefore 5-HT dependent. However, at the higher concentration (50μM) the effect was present in both saline and *p*CPA pretreated rats. The 5-HT independent effect of 50μM fluoxetine on NA efflux could be due to direct inhibition of NA reuptake. It is not possible to determine the exact concentration of fluoxetine in the area of the brain surrounding the probe. However, estimates of probe efficiency suggest only around 10% of drugs in the perfusate will enter the brain. Therefore, 50μM fluoxetine in the probe approximates to around 5μM in the extracellular fluid immediately surrounding the probe. This is within the range of inhibition of [^{3}H]-NA uptake by fluoxetine into cortical synaptosomes (0.28μM, Richelson & Pfenning, 1984; see Stanford, 1996).

The NA response to 50μM fluoxetine infusion was greater in the frontal cortex than hypothalamus. As was noted in Chapter 3, these brain areas differ with respect to NA transporter pharmacology, (Zaczek *et al*, 1990). For example, fluoxetine inhibits NA uptake with greater affinity in synaptosomes prepared

from cortical tissue (K_i=0.28μM, Richelson & Pfenning, 1984) than from the hypothalamus (K_i=0.5μM; Thomas *et al*, 1987). This greater affinity for the cortical NA transporter could contribute to the greater magnitude of fluoxetine's effect on NA efflux in the frontal cortex. Alternatively, the greater proportion of presynaptic α_2-adrenoceptors (Heal *et al*, 1993) and their greater inhibitory control of NA transmission in the hypothalamus (Wortley *et al*, 1999) may be involved.

Since the effect of 5μM fluoxetine on NA efflux in the frontal cortex was 5-HT dependent, this should occur after local infusion of the more potent inhibitor of 5-HT reuptake, citalopram (Experiment 2). However, local infusion of this drug did not increase NA efflux even at the highest concentration (50μM). This demonstrates that while 5-HT is necessary for the increase in NA efflux seen with 5μM fluoxetine, it is not sufficient to induce an increase in NA efflux *per se*. This is consistent with the finding that local infusion of exogenous 5-HT (5μM) to the frontal cortex did not increase NA efflux, (Hughes & Stanford, 1998). Also, systemically administered fluvoxamine, despite causing a marked increase in 5-HT efflux in the frontal cortex, had no effect on NA efflux in the same brain region, (Jordan *et al*, 1994).

It is seems that merely increasing the extracellular concentration of 5-HT does not elevate NA efflux in the frontal cortex, (although 5-HT efflux was not measure here). However, the effects of 5μM fluoxetine infusion are clearly dependent on 5-HT, since they are abolished by *p*CPA pretreatment. It can be deduced that, although 5-HT is necessary for the effect of 5μM fluoxetine, an additional property of this drug, not shared by citalopram (and therefore not obviously related to inhibition of 5-HT reuptake), is required for its effect on NA efflux to occur. Fluoxetine binds to 5-HT_{2C} receptors, with greater affinity than citalopram, where it acts as an antagonist, (Palvimaki *et al*, 1996; Ni & Miledi, 1997). Fluoxetine also inhibits [^{3}H]-GABA binding to the $GABA_A$ receptor (Tunnicliff *et al*, 1999) and inhibits K^+ channel currents in cultured hippocampal neurones (Choi *et al*, 2004). Whether or not any of these properties explains the 5-HT-dependent effects of fluoxetine on NA efflux not shared by citalopram is as yet, unknown. It is interesting that a property of fluoxetine, additional to its

established effects on 5-HT uptake, could contribute to effects on another neurotransmitter system relevant to the treatment of depression (NA).

The effects of local infusion of fluoxetine in the hypothalamus differ from those in the frontal cortex in several important respects. Firstly, the lower concentration (5.0μM) had no effect in this region. This is in agreement with the study of Perry & Fuller (1997), who reported only a modest increase in NA efflux in the hypothalamus after infusion of 10μM fluoxetine. Therefore, whereas 5μM fluoxetine increased NA efflux in the frontal cortex by a 5-HT dependent mechanism, this effect was not seen in the hypothalamus. This echoes the findings of Géranton *et al* (2004), who also demonstrated a 5-HT dependent increase of NA in the frontal cortex, but not the hypothalamus, following infusion of a mixed reuptake inhibitor. Thus, local infusion of the NA/5-HT reuptake inhibitor, BTS 54354, elevated NA efflux in both the frontal cortex and hypothalamus. However, the effect in the frontal cortex, but not the hypothalamus, was abolished by *p*CPA and is, therefore, 5-HT dependent. Thus, the study of Géranton *et al*, using BTS 54354, and experiments here, using fluoxetine (5μM), demonstrate a 5-HT-dependent modulation of NA transmission in the frontal cortex, which is not seen in the hypothalamus. Further support for this comes from the finding that 10μM fluoxetine, infused to the hypothalamus, does not increase NA efflux in this brain region, despite increasing 5-HT efflux 7-fold (Perry & Fuller, 1997).

Unlike experiments in Chapter 3, DSP-4 pretreatment did not elevate spontaneous NA efflux in the hypothalamus. It is interesting that, in this experiment, DSP-4 not deplete tissue stores in the hypothalamus, either. Previous studies have reported variation in the extent of a DSP-4 induced lesion of NA tissue content (Hughes & Stanford, 1996; Hughes & Stanford, 1998). This suggests that the increase in NA efflux in DSP-4 pretreated rats requires appreciable damage to nerve terminals. This absence of a lesion would explain why DSP-4 did not prevent the effects of 50μM fluoxetine in the hypothalamus.

Considerable variation was seen in measurements of monoamine tissue content (see Tables 4.1, 4.4., 4.6). The explanation for this variability is

unclear, but may be related to the method of sample preparation or discrepancies in the time between collection of samples and analysis. Similar discrepancies were found in Chapters 3 and 5. However, brain tissue concentration was used solely as a demonstration of treatment effect, rather than absolute concentration of transmitter.

4.6. Conclusion

A 5-HT-dependent increase in NA efflux at a low concentration (5μM) of fluoxetine was seen in the frontal cortex of saline, but not *p*CPA, pretreated rats. Local infusion of citalopram did not elevate NA efflux in either brain region, demonstrating that, although 5-HT dependent, the effect of 5μM fluoxetine is not explained simply by its ability to block 5-HT uptake. Other effects of fluoxetine (antagonism of 5-HT_{2C}, $GABA_A$ receptors or K^+ channel conductance) may be involved, but this remains to be tested.

Infusion of 50μM fluoxetine elevates NA efflux in both the frontal cortex and hypothalamus. The effects of this concentration were not 5-HT-dependent and are most likely due to inhibition of NA reuptake. The response to local fluoxetine was greater in the frontal cortex, which may reflect regional differences in regulation of NA transmission but further work is needed to identify the factors responsible for this difference.

DSP-4 pretreatment prevented the effects of fluoxetine (5 and 50μM) in the frontal cortex. DSP-4 pretreatment did not prevent the effects of fluoxetine in the hypothalamus, but no effect on tissue content or efflux of NA was seen in this region, suggesting there was no appreciable lesion of NA axon terminals.

Results from local and systemic fluoxetine studies suggest opposing effects at axon terminals and soma (see also Chapter 3). It is proposed that co-administration of an α_2-adrenoceptor antagonist in microdialysis studies would result in more consistent effects of systemic fluoxetine (and other SSRIs) on forebrain NA efflux. Experiments in Chapter 5 use the light/dark exploration box to determine whether an action on central NA transmission contributes to the effect of fluoxetine on behavioural.

Chapter 5

Noradrenaline and 5-HT-related effects of fluoxetine: analysis of behaviour of DSP-4 and *p*CPA pretreated rats in the light/dark exploration box.

5.1. Introduction

Experiments described in Chapters 3 and 4 demonstrate modulation of central NA transmission by fluoxetine. The aim of this chapter was to establish whether this modulation could influence behaviour in a modified light/dark exploration box. This procedure was used because the behavioural response of rodents to spatial novelty involves changes in central NA transmission (see below). Moreover, drugs that target NA transmission modulate behaviour in tests where exposure to spatial novelty is a component (*e.g.* Haller *et al*, 1997; Mason *et al*, 1998; Stone *et al*, 2004).

5.1.1. Spatial Novelty

Rodents respond to novel situations and environments with a characteristic pattern of behaviour. When faced with an unfamiliar environment, they display a behavioural adaptation that facilitates investigation for any potential benefit (*e.g.* food, mate, resources, etc: "approach") and assessment of any potential threats (risk assessment: "avoidance"). This is known as exploration (Montgomery, 1955).

The response of rats and mice exposed to a novel enclosure, (spatial novelty) has been exploited in a number of behavioural tests. These ethological models (*e.g.* light/dark test, open field, elevated plus-maze) were used originally because behaviour in these tests is changed consistently by established anxiolytics drugs, (*e.g.* benzodiazepines and barbiturates; Crawley & Goodwin, 1980; Crawley, 1981). Drugs such as diazepam increase the proportion of time that rats and mice spend exploring and

reduce avoidance of the situation threat. This has been described as an 'anxiolytic' effect, but its relationship with the symptoms of human anxiety disorders is far from clear.

5.1.2. Spatial novelty and NA

The behavioural response of rodents on exposure to spatial novelty is paralleled by changes in central NA transmission. Using *in vivo* microdialysis in freely-moving rats, Dalley & Stanford, (1995) reported increased NA efflux in the frontal cortex when rats were exposed to a novel cage. Efflux increased incrementally, in parallel with transfer of the rats to test situations of increasing salience. For example, transfer to a novel arena that was 5-fold more brightly illuminated the animal's homecage increased NA efflux, relative to basal. The most marked increases in efflux (compared to efflux in handled controls) were seen when rats were placed in a novel, brightly-lit chamber that also contained an unfamiliar rat. Likewise, Mason *et al*, (1998) reported elevated NA efflux in the frontal cortex of rats placed in a novel environment (circular enclosure with equal illumination as the animal's homecage). Later studies demonstrated increased NA efflux in the hypothalamus, as well as frontal cortex, of freely-moving rats placed in a novel, brightly-lit arena (McQuade et al, 1999). All these studies demonstrate that central NA transmission is augmented when rats are exposed to environments that are known to induce a behavioural response.

That central NA transmission is responsive to changes in an animal's environment is in line with electrophysiological and behavioural studies. In rats, (Aston-Jones & Bloom, 1981) and primates (Rajkowski *et al*, 1994), the firing activity of neurones in the LC is closely paralleled by the behavioural state. Thus, tonic LC firing activity is greatest when rats are engaged in 'exploratory alertness'. This led to the idea that NA neurones are important in maintaining vigilance, defined as "as state of surveillance of the external environment and readiness to respond to unexpected stimuli", (Aston-Jones & Bloom, 1981). This aptly describes the behaviour of rodents in a novel, brightly-lit environment, *i.e.* stretch attend postures, thigmotaxis, rearing, sniffing and locomotor exploration of the environment occur. On this basis, it should be possible to use behavioural responses to spatial novelty as an

'assay' for changes in central NA transmission. To this end, a modified light/dark exploration test is used here, specifically to determine whether fluoxetine has behavioural effects that could be due to an action on central NA function.

In support of this approach, several studies have reported modulation of behaviour in novel environments by drugs that target NA transmission. Thus, the α_2-adrenoceptor antagonist, idazoxan, increased locomotor activity of rats in a novel, open field, (Haller *et al*, 1997). This effect was blocked by either an α_1 or β adrenoceptor antagonist, (prazosin and propranolol respectively), suggesting a postsynaptic-adrenergic mediated behavioural change. Mason *et al*, (1998) reported increased NA efflux in the frontal cortex of freely moving rats on exposure to spatial novelty (see above). The anxiogenic drugs, yohimbine and FG7142, both modulated the increase in NA efflux (yohimbine prevented it, FG7142 enhanced it). These drugs also modulated the locomotor activity in the novel enclosure *i.e.* yohimbine enhanced locomotor activity in the novel enclosure, while FG7142 reduced it. These studies demonstrate that drugs that modulate central NA transmission have a detectable effect on the behavioural response to novelty. This supports the presumption that, if fluoxetine has effects on NA transmission, this will influence the behavioural response to novelty.

5.1.3. Spatial novelty and 5-HT

Behaviour in response to spatial novelty is not responsive to changes in noradrenergic function alone. 5-HT is also implicated in such behaviours. This is important for the present study, where effects of an SSRI are to be determined. Several studies demonstrate 5-HT modulation of behaviour in the light/dark test. Cheng *et al*, (1994) augmented 5-HT transmission in mice by administering the 5-HT precursor, 5-hydroxytryptophan (5-HTP). This raised 5-HT concentration in the frontal cortex, hippocampus and striatum. However, this was assessed by post-mortem tissue content and so does not indicate effects on 5-HT transmission *in vivo*. Nevertheless, these animals spent less time in the light, novel zone, reared less in the light and had a reduced latency to leave the light for the first time. The effects of 5-HTP treatment were antagonized by the non-selective 5-HT_2 receptor

antagonist, methsergide, and reversed by the 5-$HT_{2A/2C}$ antagonist, ritanserin, the 5-HT_{1A} agonist, 8-OHDPAT, and the 5-HT_3 receptor antagonists, ondansetron and tropisetron. A similar pattern of all these effects was reported by Artaiz *et al*, (1998). However, Sanchez (1995), reported an increase in activity in light after 5-HTP administration.

The above studies demonstrate that drugs that modulate 5-HT transmission modify the response of mice to a novel environment. Less attention has been paid to the effect of 5-HT manipulation in rats than in mice. However, Miura *et al*, (2002) demonstrated increased 5-HT turnover (defined as increased 5-HIAA/5-HT tissue content ratio in the prefrontal cortex and hippocampus) in rats exposed to 'novelty stress' (20 min placement in a novel semi-transparent plastic arena). Noguchi *et al*, (2001) exposed rats to a novel environment (communication box): 30 min exposure augmented 5-HT turnover in the medial prefrontal, anterior cingulate and prelimbic cortices, again defined as an increase in 5-HT/5HIAA ratio.

Thus, 5-HT transmission is important in the behavioural response to novelty. Therefore, effects of fluoxetine in the light/dark test could be due to modulation of 5-HT, rather than (or in addition to) any effects of NA transmission. For this reason, the present experiments used both DSP-4 and *p*CPA pretreatments. If modulation of behaviour by fluoxetine in the light/dark test involves an action at NA neurones, then behavioural effects of this drug will be identified that are modulated by DSP-4 pretreatment, but not *p*CPA.

In some respects this approach is similar to studies employing the modified rat forced swim test (Detke *et al*, 1995; see Cryan *et al*, 2005). All antidepressants reduced the time that rats spend immobilized when confined to a cylinder of water. Antidepressant drugs that target 5-HT transmission primarily increase the proportion of time rats spend swimming, whereas drugs that target NA transmission increase climbing at the side of the tank. Thus, the neurochemical substrates of antidepressant drugs can be distinguished by their divergent effects on behaviour. However, the forced swim test has produced some inconsistent results, *e.g.* fluoxetine does not

increase climbing behaviour, despite its effects on NA transmission *in vivo* (see Chapters 1 & 3). Also, duloxetine selectively increases climbing behaviour, (indicating effects on NA transmission primarily), despite its greater affinity for the 5-HT transporter. Likewise, paroxetine increases swimming, with no effect on climbing, even at doses (40mg/kg) that are known to elevate NA efflux (Beyer *et al*, 2002).

Here, the light/dark exploration box was used since it employs a situation similar to that which modulates transmission of NA (and 5-HT) and is sensitive to drugs that target these systems. The aim was to detect effects of fluoxetine on NA, and possible 5-HT transmission also, as opposed to just the system on which fluoxetine acts predominantly (5-HT) as is the case with the modified rat forced swim test.

5.1.4. Effects of fluoxetine in the light/dark exploration test

Despite the role of NA and 5-HT systems in behavioural responses to spatial novelty, no clear-cut effects of fluoxetine in the light/dark test have been reported, hitherto. Rather, inconsistent effects have been found. Increased transitions between compartments, (Bourin *et al*, 1996), suppressed locomotor activity, (Belzung *et al*, 2001, Krzyanowska *et al*, 2001), decreased (Kshama *et al*, 1990) and increased, (de Angelis, 1996) time spent in the novel compartment, and no effect on behaviour (Sanchez & Meier, 1997) have all been reported.

These inconsistent effects could reflect the lack of standardization of protocols across experiments. Thus, species (rat or mouse) and strain of animals used, as well as differences in experimental apparatus vary across these studies. Another possibility is the variable effect of acute fluoxetine on 5-HT and NA transmission (see Chapter 3). Indeed, it is surprising that drugs with known variable effects should produce predictable changes in certain behavioural tests on acute administration (*e.g.* forced swim, tail suspension). Behaviour in the light/dark test is modulated by antidepressants in a more variable way.

A previous study from this laboratory demonstrated effects of fluoxetine on locomotor activity in both the light (novel) and dark (familiar) compartments of the same test apparatus as the that used here (Krzyanowska *et al*, 2001). For this reason, the light/dark exploration box was used to determine whether any of the behavioural effects of fluoxetine are due to an action on NA transmission.

Unlike most light/dark shuttle box experiments, which record only behaviours considered to related to "anxiolysis", a more extensive profile of behaviours was recorded in the present study, (see Chapter 2, section 2.6.1). This was to increase the likelihood of identifying NA-related behaviours, which will not necessarily be the same as those related to the effects of established anxiolytics.

5.1.5. Time of day of testing

Rodents exhibit circadian cycles of locomotor activity (Gorka *et al*, 1996), body temperature, (Weinert *et al*, 1999), corticosterone secretion, (Windle *et al*, 1998) and pain sensitivity (Konecka *et al*, 1998). Time-dependent biological rhythms may also determine behavioural responses. Thus, Valentinuzzi *et al*, (2000) found increased locomotor activity of CB7BL/6 mice in the open field when tested during the active (dark) phase. However, Jones & King (2001) found no variation due to time of day of testing of rat behaviours in a variety of procedures (elevated plus-maze, open field test, holeboard test).

The behavioural effects of drugs also may also depend on the time of the light/dark cycle. Lu & Nagayama (1996) reported greater behavioural responses to the 5-HT_{1A} agonist 8-OHDPAT during the mid-inactive phase (*i.e.* 12:00 h) with the lowest responses seen during the mid-active phase (00:00 h).

Time-dependent behavioural effects of fluoxetine have also been reported. Indeed, previous work from this laboratory (Krzyzanowska *et al,* 2001) demonstrated time-dependent effects of fluoxetine in the same test as that used here. Fluoxetine reduced locomotor activity during the morning but

increased it during the afternoon. Thus, time of day of testing may be a source of variation, both for baseline behaviour in the exploration box, as well as the behavioural effects of fluoxetine. Therefore, experiments described in this chapter were performed both in the morning and the afternoon and time of testing was included as an independent variable.

5.2. Aims

The aim of experiments in this chapter was to determine whether fluoxetine produces behavioural effects in rats that could be related to effects of NA transmission. The hypothesis to be tested is that fluoxetine modifies the behavioural response to a novel, brightly lit environment by modulation of central NA transmission. The second aim of this chapter was to discriminate any 5-HT related behavioural effects of fluoxetine. These effects should be selectively modulated by DSP-4 and *p*CPA pretreatment, respectively.

If NA-related behavioural effects of fluoxetine can be identified, this would support evidence from the microdialysis experiments of Chapters 3 & 4 that fluoxetine modulates NA transmission *in vivo*.

5.3. Methods

5.3.1. Animals and pretreatments

Adult, male Sprague Dawley rats (200-320g) were used throughout. For experiments using DSP-4, rats were administered either 0.9% saline, or a single dose of DSP-4 (40mg/kg) five days prior to testing. For experiments using *p*CPA, rats were administered either 0.9% saline or 250mg/kg *p*CPA, 48 and 24 h before testing. DSP-4 and *p*CPA were dissolved in 0.9% saline. All drugs were administered in a volume of 1ml/kg, by i.p. injection.

5.3.2. Treatment groups.

Three separate experiments were performed:

Experiment 1: Rats were randomly assigned to receive either DSP-4 or saline pretreatment. Immediately prior to testing, all subjects received vehicle injection.

Experiment 2: Rats were randomly assigned to receive either DSP-4 or saline pretreatment. Immediately prior to testing, all subjects received fluoxetine injection (10mg/kg i.p).

Experiment 3: Rats were randomly assigned to receive either *p*CPA or saline pretreatment. Immediately prior to testing, all subjects received fluoxetine injection, (10mg/kg i.p.)

5.3.3. Test Procedure.

See Chapter 2 (section 2.7.1) for a detailed description of the experimental protocol used. At the end of the experiments, rats were killed immediately by stunning and cervical dislocation, their brains removed and frozen for subsequent analysis of monoamine content.

5.3.4. Behavioural analysis

A complete list of the behavioural parameters recorder is given in Chapter 2 (section 2.6.1).

5.3.5. Analysis of brain monoamine content

See Chapter 2, (section 2.4.4) for a description of the method used to determine brain monoamine content.

5.3.6. Statistical Analysis

Each of the behaviours in the light/dark exploration box was analyzed using two-way ANOVA with 'time of day' and 'pretreatment' as 'between subjects' factors. If no significant effect of 'pretreatment' or 'time of day' was seen, data from these groups were pooled and one-way ANOVA performed to test the effect of the remaining factor.

When a main effect of either 'time of day' or 'pretreatment' was observed, data were analyzed using one-way ANOVA with 'treatment group' (n=4; *i.e.* pretreatment/time of day) as a between subjects factor, with post-hoc LSD test.

The Levene's test of equality of error variances was performed routinely. Where error variances across treatment groups were different, data were analyzed using the Mann-Whitney non-parametric test, or normalized using Log_{10} transformation, as stated. For these behaviours, one-way ANOVA and post-hoc analysis were not performed.

Analysis of covariance (ANCOVA) was routinely performed to investigate whether any changes in behaviour were independent of any main effect on locomotor activity.

Mead's resource equation was used to determine whether sample sizes of treatment groups were sufficient to detect significant differences should any exist.

The effects of DSP-4 and *p*CPA on brain monoamine content were analyzed using two-way ANOVA, with 'pretreatment' as a 'between subjects' factor and 'brain region' as a 'within subjects' factor. Where a main effect of either factor, or an interaction between these factors was evident, one-way ANOVA was performed with 'pretreatment' as the main factor.

5.4. Results

5.4.1. Effect of DSP-4 pretreatment on monoamine content in the frontal cortex and hypothalamus in animals destined for vehicle injection (Experiment 1).

DSP-4 pretreatment reduced NA content in the frontal cortex, (-54%, $F_{1,24}$=19.7, P<0.001) and hypothalamus, (-39%, $F_{1,22}$=6.0, P=0.02). There was no effect of DSP-4 on content of DA or 5-HT in either brain region (Table 5.1)

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	**DSP-4**	**SAL**	**DSP-4**
NA	488 ± 47	**230 ± 42****	2292 ± 177	**1388 173***
DA	266 ± 64	500 ± 282	286 ± 65	309 ± 78
5-HT	1010 ± 151	740 ± 81	1379 ± 311	917 ± 139

Table 5.1. Effect of DSP-4 pretreatment on monoamine content in the frontal cortex and hypothalamus. Displayed are mean ± s.e.m. concentration (ng/g wet tissue; n=11-14). Significant effects are in bold. **P<0.001; *P=0.02 *c.f.* saline pretreated rats

5.4.2. Effect of DSP-4 pretreatment on monoamine content in the frontal cortex and hypothalamus in animals destined for fluoxetine injection (Experiment 2).

DSP-4 pretreatment reduced NA content in the frontal cortex, (-60%, $F_{1,51}$=21, P<0.001) and hypothalamus, (-42%, $F_{1,51}$=6.4, P=0.02). There was no effect of DSP-4 on content of DA or 5-HT in brain region (Table 5.2)

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	**DSP-4**	**SAL**	**DSP-4**
NA	625±71	**250±41 ****	1354±131	**789±137***
DA	313±46	237+33	520±134	382±85
5-HT	399±141	427+88	591±150	463±112

Table 5.2. Effect of DSP-4 pretreatment on monoamine content in the frontal cortex and hypothalamus. Displayed are mean ± s.e.m. concentrations (ng/g wet tissue; n=24/26). Significant effects are in bold. **P<0.001; *P=0.02 *c.f.* saline pretreated rats.

5.4.3. Effect of *p*CPA pretreatment on monoamine content in the frontal cortex and hypothalamus in animals destined for fluoxetine injection (Experiment 3).

*p*CPA pretreatment reduced in 5-HT content in the frontal cortex, (-80%, $F_{1,54}$=6.7, *P*=0.01) and hypothalamus, (-72%, $F_{1,53}$=21, *P*<0.001). There was no effect of *p*CPA on content of NA or DA in either brain region (Table 5.3).

	FRONTAL CORTEX		HYPOTHALAMUS	
	SAL	***p*CPA**	**SAL**	***p*CPA**
NA	261±26	199±27	1052±186	733±179
DA	440±70	359±72	448±93	387±64
5-HT	272±85	**56±10***	840±127	**238±38****

Table 5.3. Effect of *p*CPA pretreatment on monoamine content in the frontal cortex and hypothalamus. Displayed are mean ± s.e.m. concentrations (ng/g wet tissue; n=25-28). Data were analyzed using one-way ANOVA with 'pretreatment' as a between subjects factors. Significant effects are in bold. *P=0.01; **P<0.001 *c.f.* saline pretreated rats

5.4.4. Experiment 1: Effect of DSP-4 pretreatment and time of day on behaviour of rats receiving acute vehicle

Neither DSP-4 pretreatment nor time of day alone had any effect on behaviour in the light/dark exploration box. Thus, neither two-way nor one-way ANOVA revealed any effects of 'pretreatment', 'time of day' or 'pretreatment' x 'time of day' interactions for any of the behaviours tested. For behaviours where Levene's test of homogeneity of variance was significant, data were analyzed using the Mann Whitney test, with 'pretreatment' or 'time of day' as grouping variables. Again, no significant effects were evident (see Figure 5.1 and Table 5.4).

Performing Mead's Resource Equation for individual behaviours gave values of 21-23, indicating that increasing the sample size would not increase the likelihood of revealing differences between treatment groups.

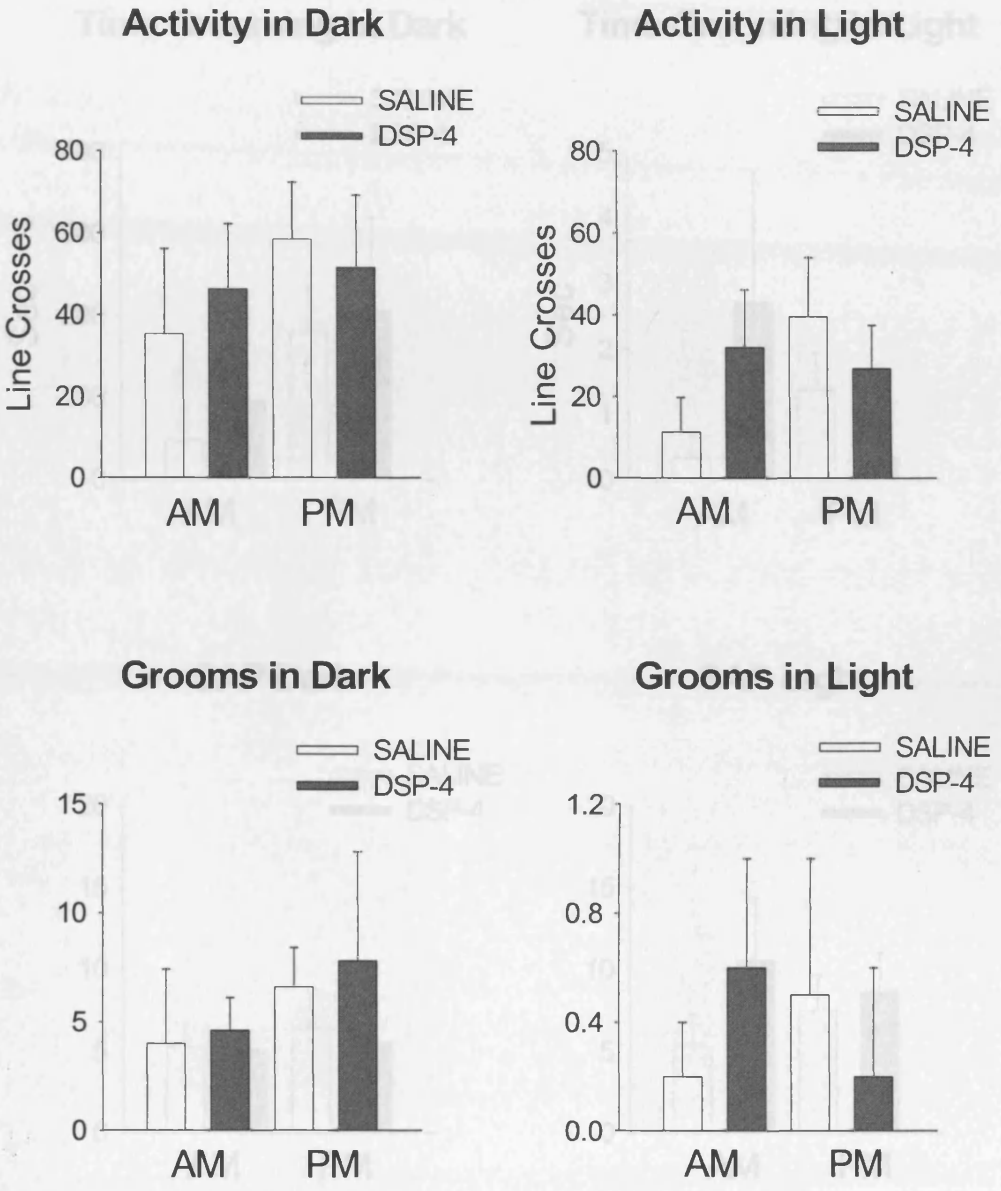


Figure 5.1. Effect of DSP-4 pretreatment and time of day on behaviour of rats receiving acute vehicle. Charts show mean ± s.e.m. n=6-8 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. [Continued over].

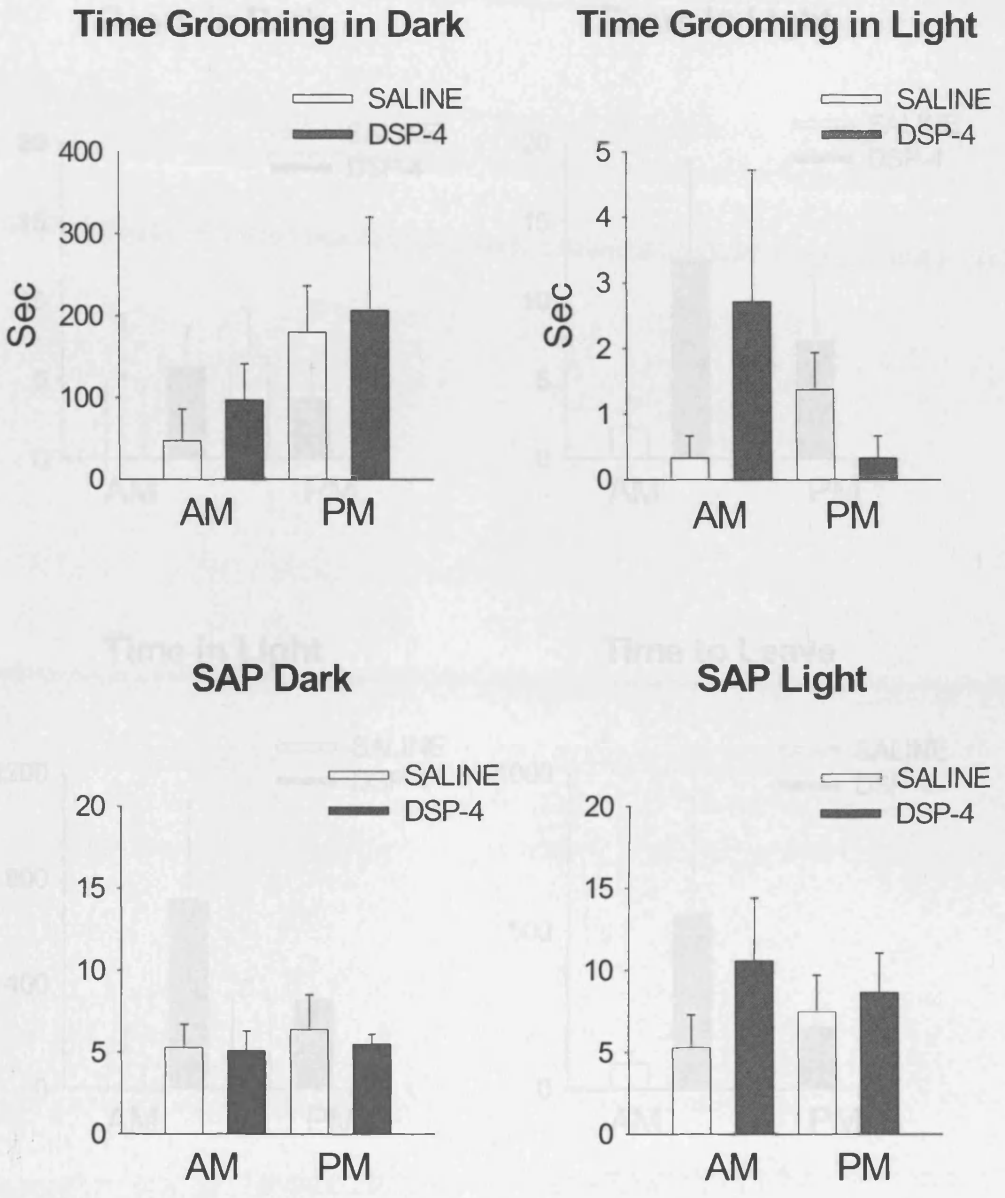


Figure 5.1. Effect of DSP-4 pretreatment and time of day on behaviour of rats receiving acute vehicle. Charts show mean ± s.e.m. n=6-8 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. SAP= Stretch Attend Posture. [Continued over].

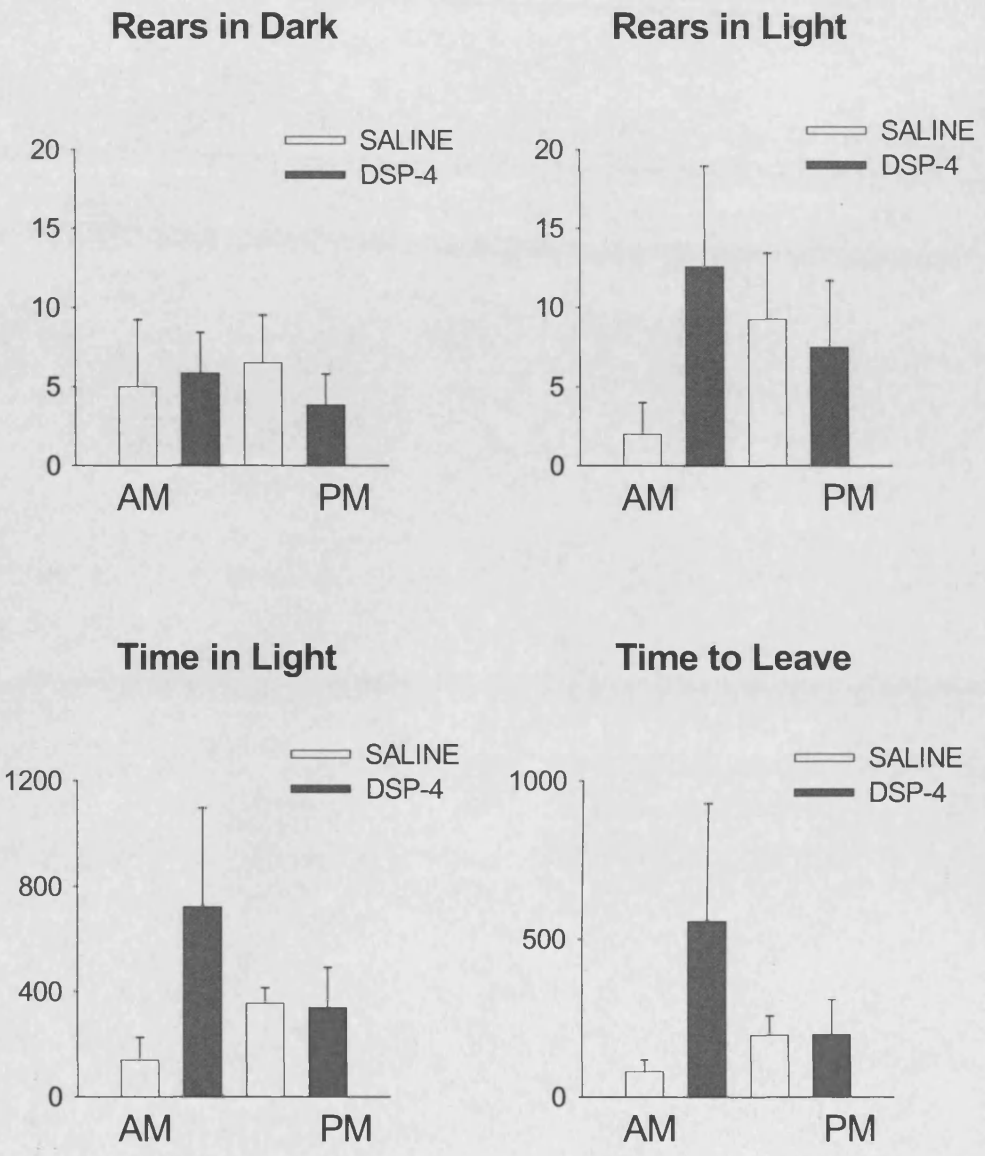


Figure 5.1. Effect of DSP-4 pretreatment and time of day on behaviour of rats receiving acute vehicle. Charts show mean ± s.e.m. n=6-8 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. [Continued over].

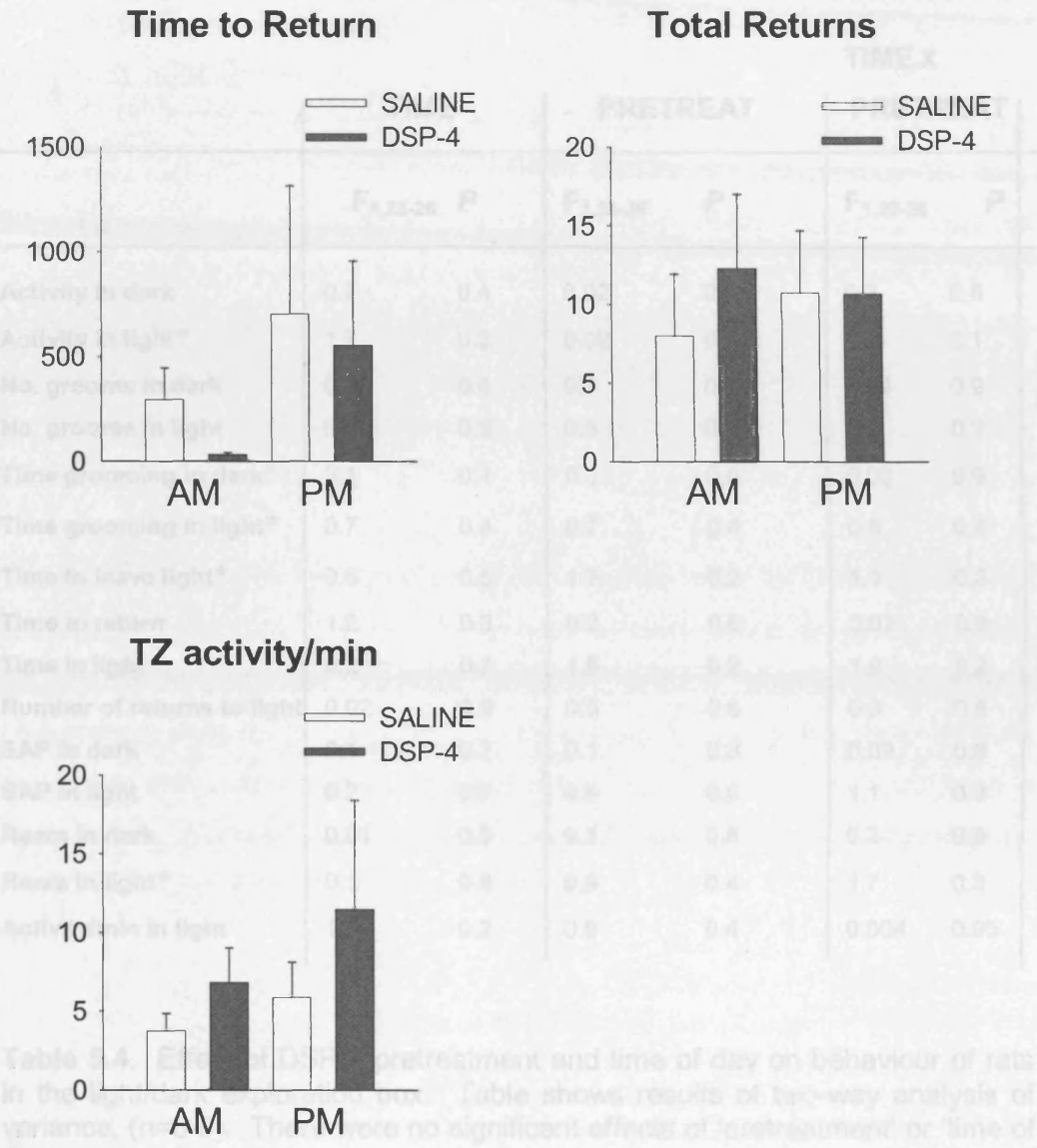


Figure 5.1. Effect of DSP-4 pretreatment and time of day on behaviour of rats receiving acute vehicle. Charts show mean ± s.e.m. n=6-8 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. TZ activity/min= Test Zone (light compartment) locomotor activity per minute.

	TIME		PRETREAT		TIME x PRETREAT	
	$F_{1,23-26}$	*P*	$F_{1,23-26}$	*P*	$F_{1,23-26}$	*P*
Activity in dark	0.7	0.4	0.02	0.9	0.3	0.6
Activity in light*	1.7	0.2	0.02	0.9	2.8	0.1
No. grooms in dark	0.7	0.4	0.1	0.7	0.04	0.9
No. grooms in light	0.6	0.5	0.6	0.5	2.2	0.2
Time grooming in dark*	3.1	0.1	0.3	0.6	0.02	0.9
Time grooming in light*	0.7	0.4	0.7	0.4	0.8	0.4
Time to leave light*	0.5	0.5	1.7	0.2	1.1	0.3
Time to return	1.2	0.3	0.2	0.6	0.02	0.9
Time in light	0.2	0.7	1.8	0.2	1.9	0.2
Number of returns to light	0.02	0.9	0.3	0.6	0.3	0.6
SAP in dark	0.1	0.7	0.1	0.8	0.02	0.9
SAP in light	0.2	0.7	0.6	0.5	1.1	0.3
Rears in dark	0.01	0.9	0.1	0.8	0.3	0.6
Rears in light*	0.1	0.8	0.9	0.4	1.7	0.3
Activity/min in light	1.5	0.2	0.9	0.4	0.004	0.95

Table 5.4. Effect of DSP-4 pretreatment and time of day on behaviour of rats in the light/dark exploration box. Table shows results of two-way analysis of variance, (n=6-8). There were no significant effects of 'pretreatment' or 'time of day'. Data from 'pretreatment' and 'time of day' groups were pooled and analyzed with one-way ANOVA, (n= 12/16). No significant differences were found. * denotes behaviours where Levene's test of homogeneity of variance was significant. Behaviours were analyzed using Mann Whitney test, (n=13/14). No significant effects of 'pretreatment' or 'time of day' were found.

5.4.5. Experiment 2: Effect of DSP-4 pretreatment and time of day in rats receiving acute fluoxetine

Locomotor 'activity in the dark' compartment was lower in the afternoon in both DSP-4 and saline pretreated rats (Figure 5.2). Thus, there was a main effect of 'time' ($F_{1,50}$=12.4, P=0.001), but no effect of DSP-4 'pretreatment' and no 'time' x 'pretreatment' interaction. Data for 'activity in dark' were also analyzed using the Mann-Whitney test, since the Levene's test was significant for this behaviour. Again, Mann-Whitney test revealed an effect of 'time', (P<0.001), but not DSP-4 'pretreatment'

Locomotor 'activity in the light' compartment was lower in DSP-4 pretreated animals (Figure 5.2). Thus, there was a main effect of 'pretreatment', ($F_{1,50}$=4.05 P=0.05), but no effect of 'time', and no 'time' x 'pretreatment' interaction. Data for 'activity in light', were analyzed using the Mann-Whitney test, since the Levene's test of homogeneity of variance was significant for this behaviour. Mann-Whitney analysis revealed an effect of 'pretreatment', (P=0.02), but no effect of 'time'.

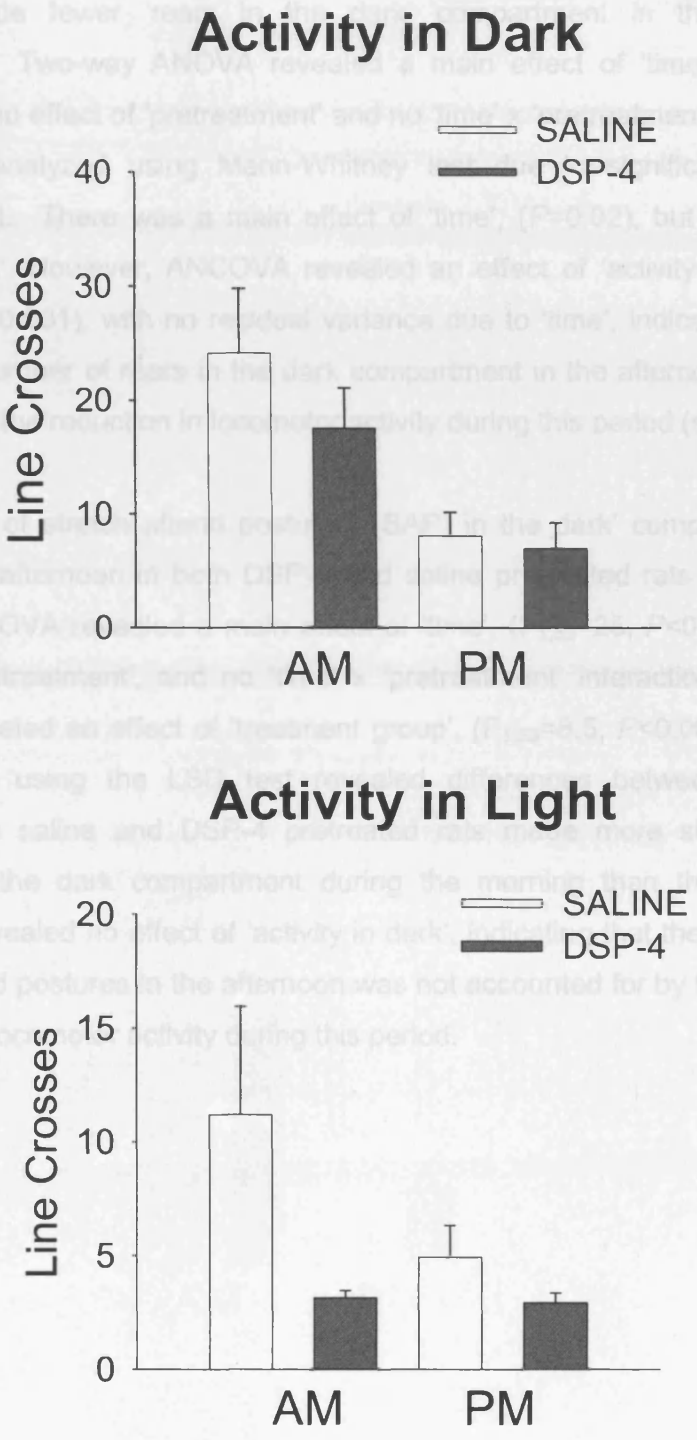


Figure 5.2. Effect of DSP-4 and time of day on locomotor activity in dark and light compartments. All subjects received acute fluoxetine. Bars charts show mean ± s.e.m. (n=13/14 per treatment group): note difference in scale. Activity in dark was lower in the afternoon (main effect of 'time of day', P=0.001). DSP-4 pretreatment reduced 'activity in the light' (main effect of 'pretreatment', P=0.02).

Animals made fewer 'rears in the dark' compartment in the afternoon (Figure 5.3). Two-way ANOVA revealed a main effect of 'time', ($F_{1,53}$=5.4, P=0.03) but no effect of 'pretreatment' and no 'time' x 'pretreatment' interaction. Data were analyzed using Mann-Whitney test due to significance of the Levene's test. There was a main effect of 'time', (P=0.02), but no effect of 'pretreatment'. However, ANCOVA revealed an effect of 'activity in the dark' ($F_{1,53}$=48, P<0.001), with no residual variance due to 'time', indicating that the decreased number of rears in the dark compartment in the afternoon could be explained by the reduction in locomotor activity during this period (see above).

The number of stretch attend postures '(SAP) in the dark' compartment was lower in the afternoon in both DSP-4 and saline pretreated rats (Figure 5.3). Two-way ANOVA revealed a main effect of 'time', ($F_{1,53}$=25, P<0.001), but no effect of 'pretreatment', and no 'time' x 'pretreatment' interaction. One-way ANOVA revealed an effect of 'treatment group', ($F_{1,53}$=8.5, P<0.001). Multiple comparisons using the LSD test revealed differences between treatment groups: both saline and DSP-4 pretreated rats made more stretch attend postures in the dark compartment during the morning than the afternoon. ANCOVA revealed no effect of 'activity in dark', indicating that the reduction in stretch attend postures in the afternoon was not accounted for by the observed reduction in locomotor activity during this period.

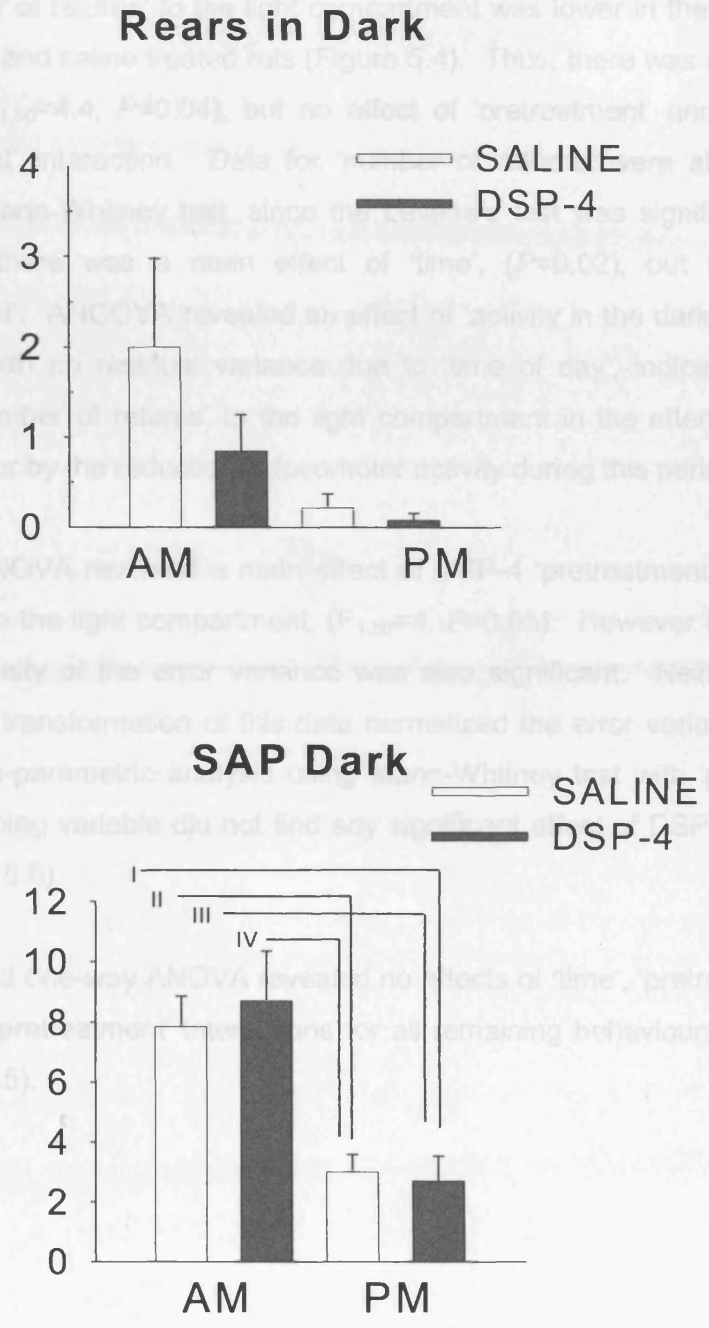


Figure 5.3. Effect of DSP-4 pretreatment and time of day on behaviour of rats receiving acute fluoxetine. Bar charts show mean ± s.e.m. n=13/14 per treatment group. Both 'rears in dark' and 'Stretch Attend Postures in dark' were both lower in the afternoon. Post-hoc LSD test **I**; P=0.001, **II**; P=0.002, **III**; P<0.001, **IV**; P<0.001

The 'number of returns' to the light compartment was lower in the afternoon of both DSP-4 and saline treated rats (Figure 5.4). Thus, there was a main effect of 'time', ($F_{1,50}$=4.4, P=0.04), but no effect of 'pretreatment' and no 'time' x 'pretreatment' interaction. Data for 'number of returns' were also analyzed using the Mann-Whitney test, since the Levene's test was significant for this behaviour: there was a main effect of 'time', (P=0.02), but no effect of 'pretreatment'. ANCOVA revealed an effect of 'activity in the dark', ($F_{1,53}$=109, P<0.001), with no residual variance due to 'time of day', indicating that the reduced 'number of returns' to the light compartment in the afternoon can be accounted for by the reduction in locomotor activity during this period.

Two-way ANOVA revealed a main effect of DSP-4 'pretreatment' on the total time spent in the light compartment, ($F_{1,50}$=4, P=0.05). However Levene's test of homogeneity of the error variance was also significant. Neither $\log_{10}$ nor square root transformation of this data normalized the error variance between groups. Non-parametric analysis using Mann-Whitney test with 'pretreatment' as the grouping variable did not find any significant effect of DSP-4 on time in light (Figure 5.5).

Two-way and one-way ANOVA revealed no effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interactions for all remaining behaviours (Figure 5.5 and Table 5.5).

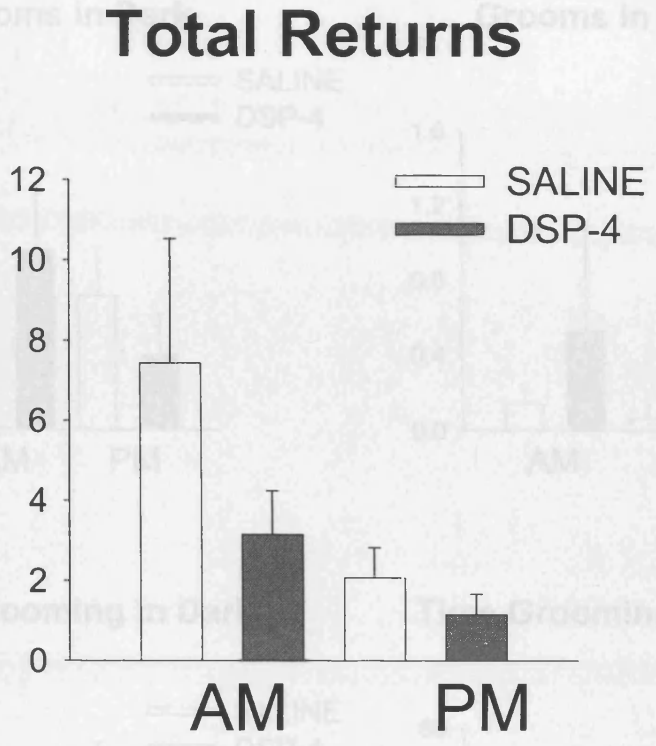


Figure 5.4. Effect of DSP-4 pretreatment and time of day on the `number of returns' to the light compartment made by rats receiving acute fluoxetine. Bar charts show mean ± s.e.m. n= 13/14 per treatment group. Main effect of 'time of day' *P*=0.02

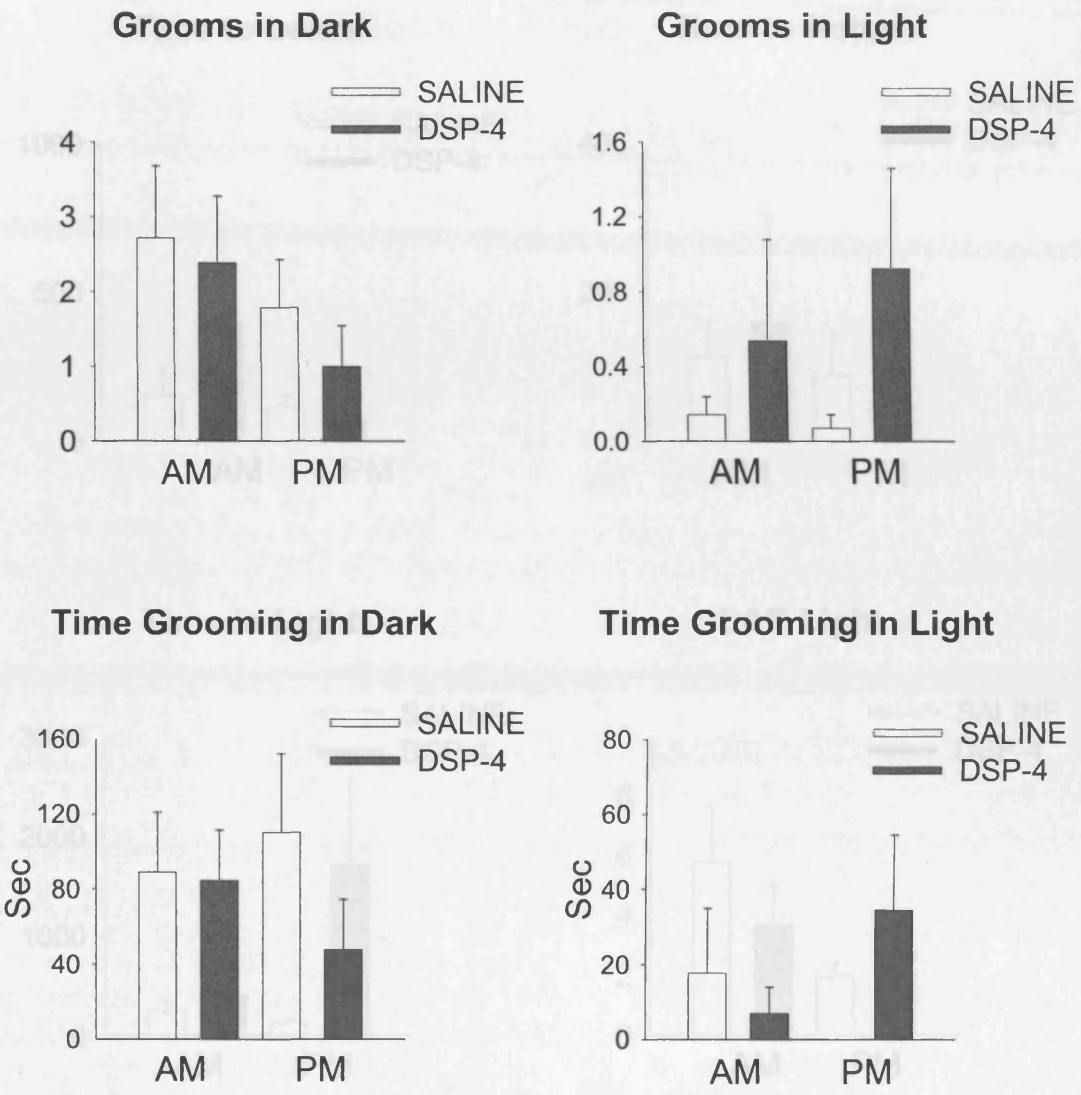


Figure 5.5. Behaviours unaffected by DSP-4 pretreatment or time of day in rats receiving acute fluoxetine. Bar charts show mean ± s.e.m. n=13/14 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. See Table 5.5 for statistical comparisons. [Continued over].

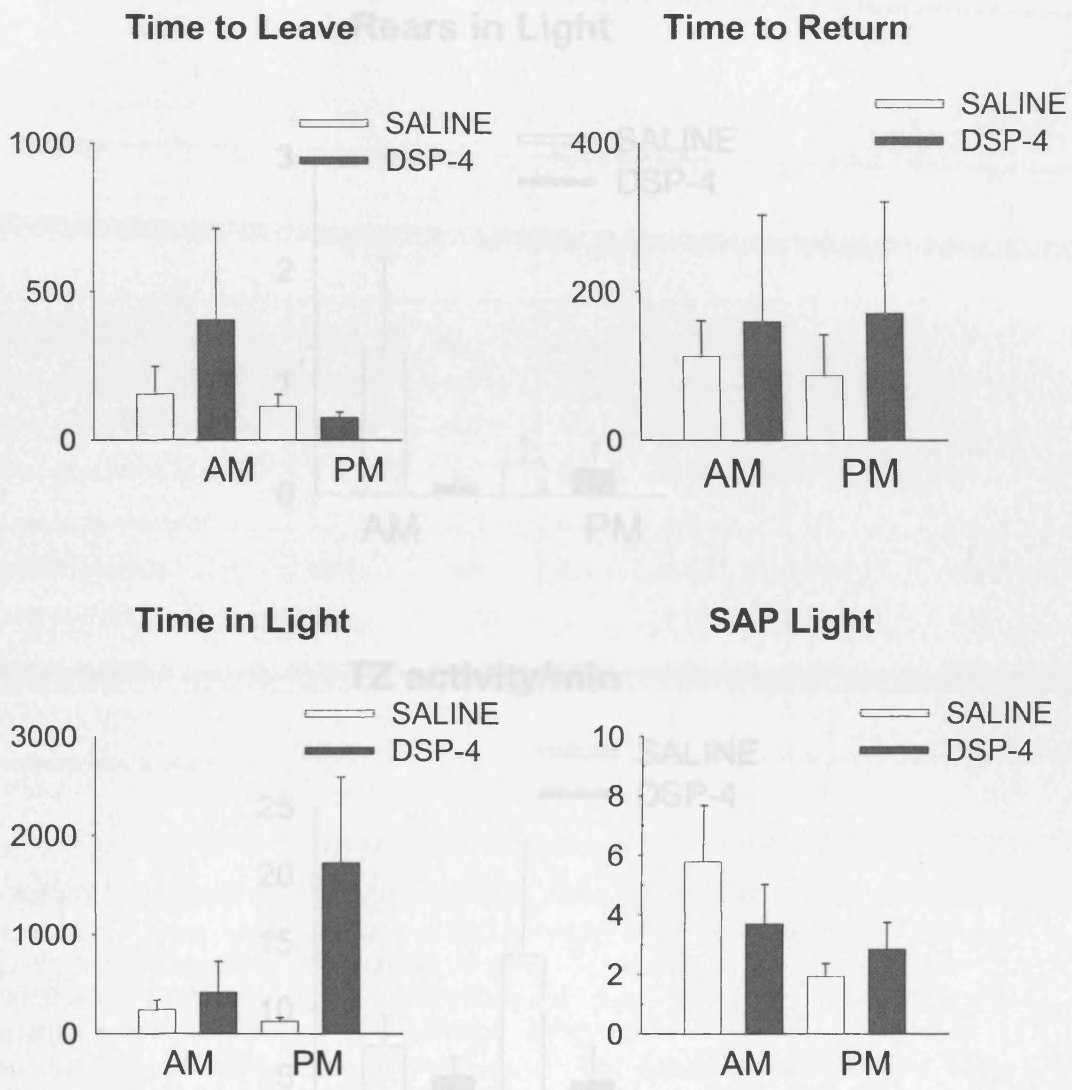


Figure 5.5. Behaviours unaffected by DSP-4 pretreatment or time of day in rats receiving acute fluoxetine. Bar charts show mean ± s.e.m. n=13/14 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. See Table 5.5 for statistical comparisons. [Continued over.]

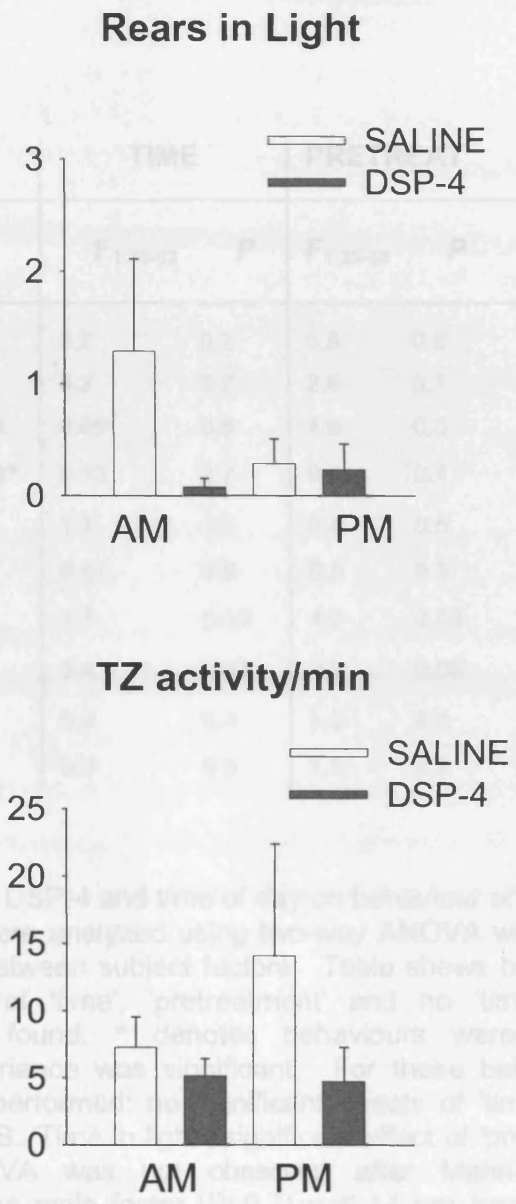


Figure 5.5. Behaviours unaffected by DSP-4 pretreatment or time of day in rats receiving acute fluoxetine. Bar charts show mean ± s.e.m. n=13/14 per treatment group. Neither two-way nor one-way ANOVA revealed any significant effects of 'pretreatment' or 'time of day'. TZ activity/min= Test Zone (light compartment) locomotor activity per minute. See Table 5.5 for statistical comparisons.

	TIME		PRETREAT		TIME x PRETREAT	
	$F_{1,35-53}$	*P*	$F_{1,35-53}$	*P*	$F_{1,35-53}$	*P*
No. grooms in dark	2.2	0.2	0.5	0.5	0.1	0.8
No. grooms in light*	0.2	0.7	2.8	0.1	0.4	0.5
Time grooming in dark	0.05	0.8	1.0	0.3	0.8	0.4
Time grooming in light*	0.13	0.7	0.8	0.4	2.8	0.1
Time to leave light*	1.2	0.3	0.4	0.5	0.7	0.4
Time to return	0.01	0.9	0.5	0.5	0.04	0.9
Time in light*	1.7	0.19	4.0	0.05	2.5	0.1
SAP in light*	3.4	0.07	0.2	0.65	1.4	0.2
Rears in light*	0.9	0.4	1.9	0.2	1.6	0.2
Activity/min in light	0.5	0.5	1.5	0.2	0.6	0.5

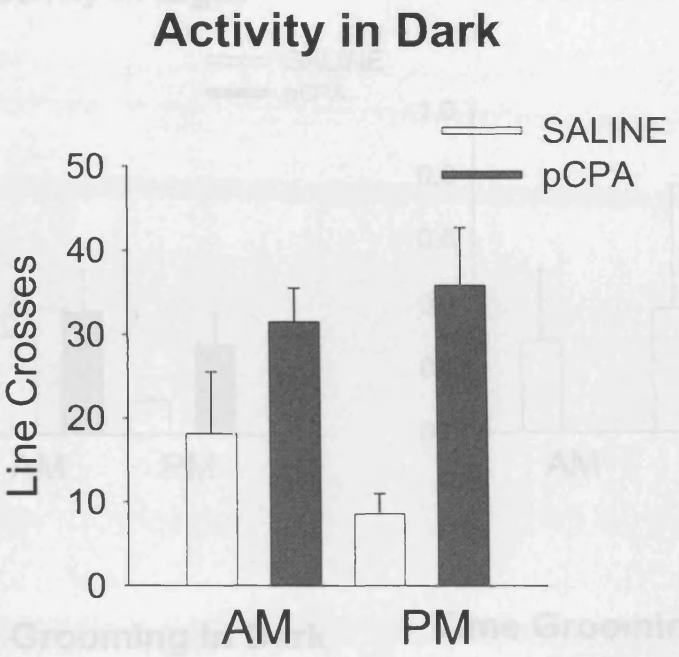
Table 5.5. Effect of DSP-4 and time of day on behaviour of rats receiving acute fluoxetine. Data were analyzed using two-way ANOVA with 'time of day' and 'pretreatment' as between subject factors. Table shows behaviours where no significant effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interactions were found. * denotes behaviours were Levene's test of homogeneity of variance was significant. For these behaviours the Mann-Whitney test was performed: no significant effects of 'time' or 'pretreatment' were observed. N.B. 'Time in light', significant effect of 'pretreatment' revealed by two-way ANOVA was not observed after Mann-Whitney test with 'pretreatment' as the main factor (P=0.7) n=6-14 per treatment group (saline AM, DSP-4 AM, saline PM, DSP-4 PM). See Figure 5.5 for graphical representation of these behaviours.

5.4.6. Experiment 3: Effect of *p*CPA pretreatment on behaviour of rats receiving acute fluoxetine

Locomotor 'activity in the dark' compartment was greater in *p*CPA pretreated animals (Figure 5.6). Two-way ANOVA revealed a main effect of 'pretreatment', ($F_{1,53}$=13.2, P=0.001) but no effect of 'time' and no 'time' x 'pretreatment' interaction. Data for 'activity in the dark' were also analyzed using Mann-Whitney test, since Levene's test was significant. There was an effect of 'pretreatment', (P<0.001), but not of 'time',

In saline pretreated animals, the number of 'grooms in the dark' compartment was greater in the morning than in the afternoon (Figure 5.6). However, *p*CPA pretreatment reversed this trend: *p*CPA pretreated animals made more grooms in the dark in the afternoon than morning. Thus, two-way ANOVA revealed a 'time' x 'pretreatment' interaction, ($F_{1,53}$=6.4, P=0.02), but no effect of 'time', or 'pretreatment'. Analysis of $\log_{10}$ transformed data for number of 'grooms in the dark' was performed, as Levene's test was significant and initial inspection of the data suggested there was a 'time' x 'pretreatment' interaction, which cannot be tested using Mann-Whitney analysis. Two-way ANOVA of 'log grooms in the dark' revealed a 'time' x 'pretreatment' interaction, ($F_{1,21}$=15.8, P=0.001), but no effect of 'time' or 'pretreatment'. One-way ANOVA of 'log grooms in the dark' revealed an effect of 'treatment group', ($F_{3,21}$=5.6, P=0.007). Post-hoc LSD analysis revealed differences between treatment groups (see Figure 5.6).

Neither one-way nor two-way ANOVA revealed any effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interactions for any other behaviours, (Figure 5.7, Table 5.6).



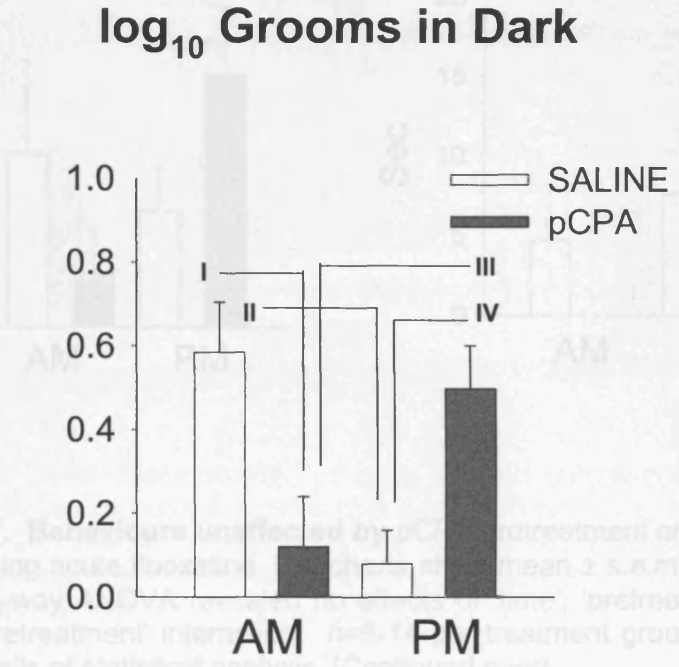


Figure 5.6. Effect of *p*CPA pretreatment and time of day on locomotor 'activity in the dark compartment' and log_{10} transformed number of 'grooms in the dark' compartment of animals receiving acute fluoxetine. Bar charts show mean + s.e.m. n= 13/14 per treatment group. Activity was greater in *p*CPA pretreated animals, (main effect of 'pretreatment' $P<0.001$). Post-hoc LSD test: **I**, $P=0.02$; **II**, $P=0.006$; **III**, $P=0.02$; **IV**, $P=0.006$. See section 5.4.6.

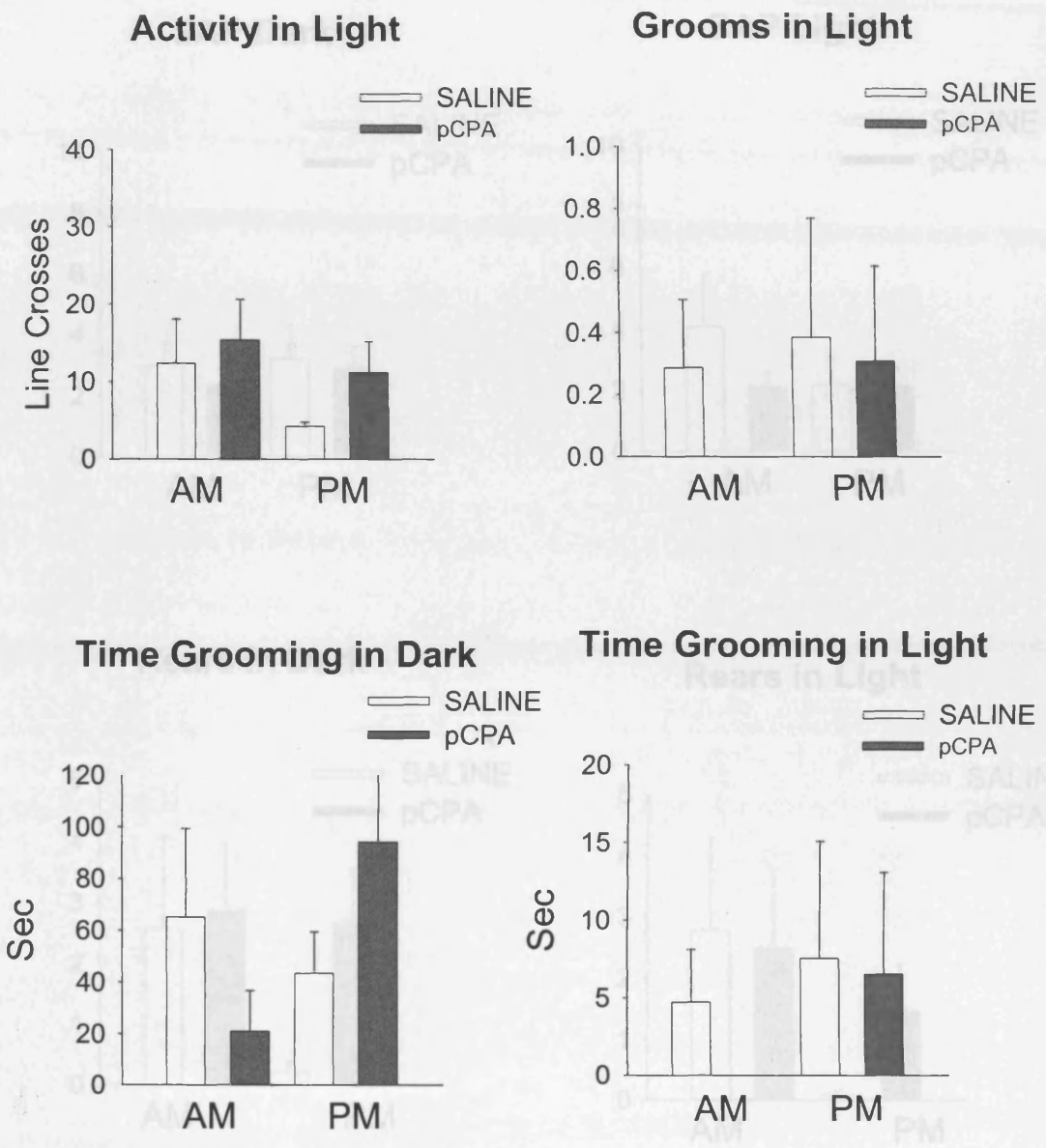


Figure 5.7. Behaviours unaffected by *p*CPA pretreatment or time of day in rats receiving acute fluoxetine. Bar charts show mean ± s.e.m of behaviours where two-way ANOVA revealed no effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interaction. n=5-14 per treatment group. See Table 5.5 for details of statistical analysis. [Continued over]

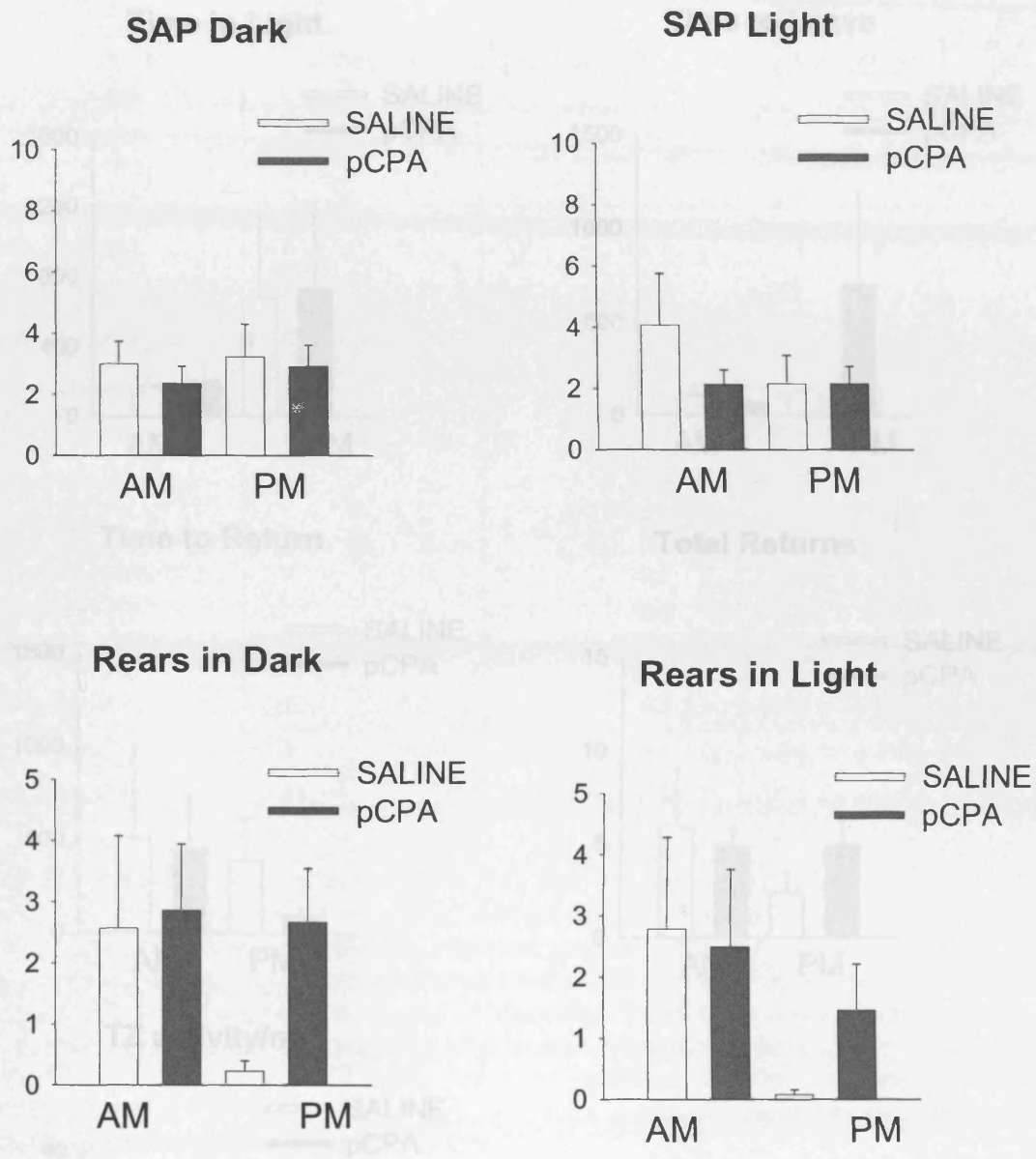


Figure 5.7. Behaviours unaffected by *p*CPA pretreatment or time of day in rats receiving acute fluoxetine. Bar charts show mean ± s.e.m of behaviours where two-way ANOVA revealed no effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interaction. n=5-14 per treatment group. See Table 5.5 for details of statistical analysis. [Continued over]

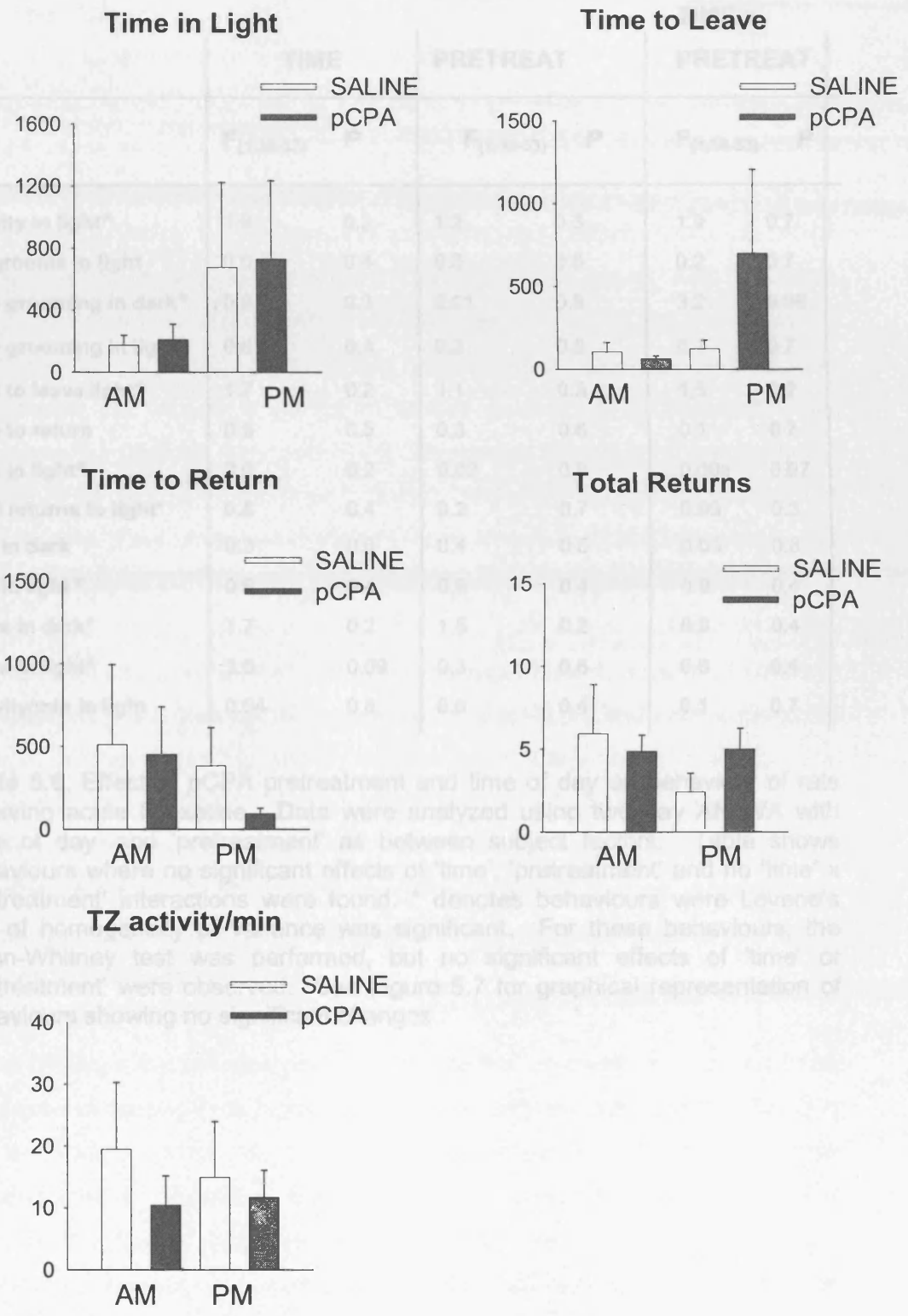


Figure 5.7. Behaviours unaffected by *p*CPA pretreatment or time of day in rats receiving acute fluoxetine. Bar charts show mean ± s.e.m of behaviours where two-way ANOVA revealed no effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interaction. n=5-14 per treatment group. See Table 5.5 for details of statistical analysis

	TIME		PRETREAT		TIME x PRETREAT	
	$F_{(1,38-53)}$	*P*	$F_{(1,38-53)}$	*P*	$F_{(1,38-53)}$	*P*
Activity in light*	1.9	0.2	1.2	0.3	1.9	0.7
No. grooms in light	0.6	0.4	0.5	0.5	0.2	0.7
Time grooming in dark*	0.9	0.3	0.01	0.9	3.2	0.08
Time grooming in light*	0.8	0.4	0.3	0.6	0.1	0.7
Time to leave light*	1.7	0.2	1.1	0.3	1.5	0.2
Time to return	0.5	0.5	0.3	0.6	0.1	0.7
Time in light*	2.0	0.2	0.02	0.9	0.001	0.97
Total returns to light*	0.8	0.4	0.2	0.7	0.99	0.3
SAP in dark	0.3	0.6	0.4	0.5	0.05	0.8
SAP in light *	0.8	0.4	0.9	0.4	0.9	0.4
Rears in dark*	1.7	0.2	1.5	0.2	0.9	0.4
Rears in light*	3.0	0.09	0.3	0.6	0.6	0.4
Activity/min in light	0.04	0.8	0.6	0.4	0.1	0.7

Table 5.6. Effect of *p*CPA pretreatment and time of day on behaviour of rats receiving acute fluoxetine. Data were analyzed using two-way ANOVA with 'time of day' and 'pretreatment' as between subject factors. Table shows behaviours where no significant effects of 'time', 'pretreatment' and no 'time' x 'pretreatment' interactions were found. * denotes behaviours were Levene's test of homogeneity of variance was significant. For these behaviours, the Mann-Whitney test was performed, but no significant effects of 'time' or 'pretreatment' were observed. See Figure 5.7 for graphical representation of behaviours showing no significant changes.

5.5. Discussion

The aim of experiments in this chapter was to establish whether changes in NA transmission could contribute to the behavioural effects of fluoxetine. The responses of rats to spatial novelty and bright light were measured to determine such effects, since these are paralleled by changes in central NA transmission (Dalley & Sanford, 1995; Mason *et al*, 1998; McQuade *et al*, 1999). Also, preliminary work from this laboratory shows that fluoxetine modulates locomotor activity in this test (Krzyzanowska *et al*, 2001). However, behaviour in tests involving exposure to novelty is also modulated by 5-HT. For this reason, DSP-4 and *p*CPA pretreatments were used, in combination with fluoxetine, to identify NA and 5-HT dependent behaviours, respectively. Finally, since circadian period can influence behaviour, as well as the response to drugs (*e.g.* Valentinuzzi *et al*, 2000; Lu and Nagayama, 1996; Krzyzanowska *et al*, 2001) the effect of time of day of testing was investigated throughout.

Pretreatment with both DSP-4 and *p*CPA produced robust, selective reductions of tissue content of their target amines. Thus, DSP-4 pretreatment reduced NA content in Experiments 1 and 2, both in the frontal cortex (-54/-60%) and hypothalamus, (-39/-42%) with no effects on 5-HT or DA. In Experiment 3, *p*CPA pretreatment decreased 5-HT content in the frontal cortex (-80%) and hypothalamus (-72%), with no effects on the other monoamines. However, the (possibly artifactual) variability of basal monoamine tissue concentrations was present, as in Chapters 3 and 4.

Since previous studies demonstrate that DSP-4 pretreatment can modulate behaviour of rodents (see below), it was necessary to determine the effects of this pretreatment alone, so effects of fluoxetine could be discriminated from those of DSP-4. Therefore, Experiment 1 compared the behaviour of DSP-4 and saline pretreated rats, all of which received acute vehicle immediately prior to testing. Neither DSP-4 nor time of day had any apparent effects on behaviour in this test.

That such a perturbation (see above) of central NA transmission had no effect on behaviour is remarkable. Several studies report reduced exploration of DSP-4 pretreated rats of arenas containing novel objects (Harro *et al*, 1995)

and in the open field (Harro *et al*, 2000). In line with the present results, other studies report no effect of DSP-4 on the locomotor response to novelty, (Lapiz *et al*, 2000; Haidkind *et al*, 2003; Stone *et al*, 2004). The lack of effect of DSP-4 on behaviour was not due to the absence of a NA lesion, since marked reductions in NA tissue content were found both here and in the studies above. Other differences such as the test apparatus and procedure, as well as the strain of rats used, could underlie these different results.

Whatever the reason for these differences, DSP-4 alone reduced NA content in the frontal cortex (-54%) and hypothalamus (-39%), but had no effect on behaviour. It could be that adaptive changes occur as a result of the lesion (*e.g.* upregulation of NA release, loss reuptake sites, upregulation of adrenergic receptors) that restore NA function to an extent where effects on behaviour are minimal. In support of this, results from the previous chapters, as well as other studies (*e.g.* Hughes & Stanford, 1996, 1998) confirm that the extracellular concentration of NA is not reduced under these circumstances.

Having established that neither DSP-4 nor time of testing alone influence behaviour, a further batch of rats was pretreated with DSP-4 or saline and all received acute, systemic fluoxetine prior to testing (Experiment 2). In this experiment, behaviours were modulated both by DSP-4 and time of day. Thus, behavioural effects of either DSP-4 or time of day were seen only in animals given acute fluoxetine immediately prior to testing. Therefore, it is reasonable to assume that these changes are due to the effects of fluoxetine.

The reduction in locomotor activity in the light compartment of DSP-4 pretreated rats is likely to be a NA-related effect of fluoxetine. For example, the difference in locomotor activity in the light compartment between saline and DSP-4 pretreated rats occurred only when all rats were administered fluoxetine (*i.e.* Experiment 2). The previous chapter described the finding that, when given systemically, fluoxetine decreased NA efflux in DSP-4 pretreated rats. The reduction is found both in the frontal cortex and hypothalamus (Chapter 3). It seems that the reduction in NA efflux in the forebrain is paralleled by a reduction in locomotor activity in the light compartment in DSP-4 pretreated rats.

Previous studies have reported parallel changes in locomotor activity in novel environments with changes in central NA transmission. Thus, the α_2-adrenoceptor antagonist, idazoxan, increases locomotor activity of rats in a novel, open field, (Haller *et al*, 1997). This effect is blocked by either α_1 or β adrenoceptor antagonists, (prazosin and propranolol respectively), suggesting a postsynaptic, NA-mediated behavioural change. Also, yohimbine and FG7142, both modulate the incremental increase in NA efflux which occurs when rats are exposed to a novel enclosure (yohimbine prevented it, FG7142 enhanced it; Mason *et al*, 1998). These drugs produced parallel changes in locomotor activity in novel arena (*i.e.* yohimbine enhanced locomotor activity in the novel enclosure, while FG7142 reduced it). The α_1-adrenoceptor agonist, phenylephrine and the antagonist, terazozin have permissive and inhibitory effects respectively on locomotor behaviour in a novel environment when infused to the LC of rats, (Stone *et al*, 2004). Taken together, these studies and the results presented here suggest that locomotor activity of rodents during exploration is sensitive to changes in central NA transmission. However, as with all studies that report parallel behavioural and neurochemical changes, a causative relationship cannot be certain.

Nevertheless, the finding that fluoxetine modulates behaviour in rats with a partial lesion of NA axon terminals supports the hypothesis that this drug is not entirely selective for effects on 5-HT transmission, *in vivo*. It is important to note that, despite reducing tissue content of 5-HT by 80% in the frontal cortex and 72% in the hypothalamus, *p*CPA pretreatment did not affect locomotor activity in the light compartment. This supports the idea that the reduction in this behaviour when DSP-4 pretreated rats are administered fluoxetine is a NA-related, as opposed to a 5-HT-related effect.

Fluoxetine administration also revealed effects that were dependent on the time of day. Thus, locomotor activity, rearing, the number of stretch attend postures in the dark compartment as well as the number of returns made to the light compartment, were all reduced during PM relative to AM testing, but only in animals receiving acute fluoxetine (*i.e.* Experiment 2). Analysis of covariance

demonstrated that the majority of these effects could be explained by the decrease in locomotor activity in the dark compartment in the afternoon.

The decrease in locomotor activity in the dark compartment during the afternoon is likely to be due to fluoxetine's effects on 5-HT transmission, for several reasons. Firstly, this effect occurred irrespective of DSP-4 pretreatment, so is unlikely to be mediated by fluoxetine acting on NA neurones. Secondly, *p*CPA pretreated rats exhibited increased locomotor activity in the dark compartment (Experiment 3), demonstrating that this behaviour is enhanced by a marked reduction in 5-HT transmission. Moreover, this behaviour was reduced when fluoxetine was administered to rats with intact 5-HT transmission (Experiment 2). Thus, drugs that have opposing effects on 5-HT transmission *in vivo* (*p*CPA and fluoxetine) have opposing effects locomotor activity in the dark compartment.

The fact that the locomotor activity in the dark compartment was reduced by fluoxetine during PM relative to AM testing, is also consistent with the known circadian variation of behaviours modulated by 5-HT. For example, the behavioural effects of postsynaptic 5-HT_{1A} receptor activation (with 8-0HDPAT) exhibit marked circadian variation, with maximal responses during the mid-dark phase, and lowest responses during the mid-light phase (Lu & Nagayama, 1996). This is true also of the head-twitch response to by the non-selective 5-HT agonist 5MeODMT in mice (Moser & Redfern, 1985). 5-HT_{1A} and 5-HT_2 receptor binding, as well as 5-HT transmission (determined by *in vivo* voltammetry) in the rat frontal cortex and hypothalamus vary across different times of the light-dark cycle (Weiner *et al*, 1992). As a reuptake inhibitor, the effects of fluoxetine will depend on the rate of 5-HT release. Since circadian variation is seen in both presynaptic (*i.e.* release) and postsynaptic (receptor sensitivity) elements of 5-HT transmission, is it not surprising that some behavioural effects of fluoxetine are dependent on the time of day. In support of the present results, a preliminary study from this laboratory demonstrated circadian effects of fluoxetine in the light/dark exploration box. Thus, fluoxetine decreased locomotor activity in the morning, (with respect to its vehicle) but increased it during the afternoon (Krzyzanowska *et al*, 2001).

Central NA efflux, (Feenstra *et al*, 2000; Kalen *et al*, 1989) β-adrenoceptor number (Vacas *et al*, 2001) and plasma NA concentration (De Boer & Van der Gugten, 1987) exhibit circadian variation, also. This raises the possibility that the time-dependent effects of fluoxetine reported here are NA-related. However, since none of these behavioural changes were modulated by DSP-4 pretreatment, but abolished or reversed by *p*CPA, a 5-HT related mechanism is more likely.

5.6. Conclusion

Since locomotor activity in light compartment was:

a) reduced only when fluoxetine was administered
b) reduced in DSP-4 pretreated animals
c) unaffected by *p*CPA pretreatment

it is concluded that this the decrease in this behaviour is a NA-related effect of fluoxetine.

Since locomotor activity in the dark compartment was:

a) reduced only when fluoxetine was administered
b) unaffected by DSP-4
c) increased by *p*CPA

it is concluded that this the decrease in this behaviour is a 5-HT-related effect of fluoxetine.

Thus, the results are consistent with 5-HT- and NA-related effects that influence different components of the behavioural response to fluoxetine.

DSP-4 pretreatment alone did not modulate any behaviour in this situation. This could reflect the compensatory mechanisms that occur after a partial lesion of NA axon terminals (see Chapters 3 & 4).

This behavioural approach was used in mice in experiments described in Chapter 7 to determine whether the behavioural consequences of genetic ablation of the NK1 receptor are related to changes in NA transmission.

Chapter 6
Comparison of Noradrenaline Efflux in the Frontal Cortex of NK1 Receptor Wild Type And Knockout mice

6.1. Introduction

Despite both pre-clinical and clinical (albeit equivocal) evidence for antidepressant efficacy of NK1 receptor antagonists (see Chapter 1), unlike all other antidepressant drugs, these compounds do not target directly monoamine transporters, receptors or monoamine oxidase (Kramer *et al*, 1998). This led to the proposal that these agents act by a mechanism independent of monoamines.

For example, repeated administration of substance P antagonists does not down-regulate β-adrenoceptors, an effect common to many conventional antidepressant strategies, (Kramer *et al*, 1998). Treatment with reserpine causes ptosis and hypothermia in rodents. This effect is reversed by phenelzine or imipramine, suggesting a monoaminergic-mediated effect. However, the NK1 receptor antagonist L-760735 does not reverse the effects of reserpine, suggesting that antagonism of this receptor does not lead to changes in monoamine transmission typical of conventional antidepressants, (Kramer *et al*, 1998). 5-HTP treatment, in combination with pargyline, induces wet dog shake, forepaw treading, hind paw splaying and flat body posture in gerbils: the number of animals displaying these behaviours is increased by MAO inhibitors, but not by L-760735, (Kramer *et al*, 1998). Also, acute NK1 receptor blockade has no effect on 5-HT efflux in the frontal cortex or hippocampus of rats, (Milan *et al*, 2001). The mechanism of NK1 antagonists in relieving the symptoms of depression was presumed to be distinct from that of conventional antidepressants, therefore.

However, an association between substance P and NA-containing neurones has been demonstrated in the brain. Substance P-containing fibres form

axodendritic synapses with noradrenergic neurones of the LC (Pickel *et al*, 1979). Also, 100% of tyrosine hydroxylase labeled neurones in the LC display NK1 receptor co-immunoreactiviy, (Chen *et al*, 2000). Application of substance P *in vivo* (Guyenet & Aghajanian, 1977), or to *in vitro* slice preparations (Cheesman *et al*, 1983) increases LC cell firing in rats. In mice, also, LC noradrenergic neurones express NK1 receptors, (Santarelli *et al*, 2001). Therefore, there is anatomical and physiological evidence that substance P could modulate NA transmission.

Given the strong evidence for a role of NA in the treatment of depression, modulation of central noradrenergic transmission could contribute to the antidepressant efficacy of NK1 receptor antagonists. In support of this, studies using NK1 receptor antagonists in rodents demonstrate effects on NA transmission. Thus, the NK1 antagonist RP67580 increases the number of LC noradrenergic cells expressing c-Fos in rats undergoing restraint stress (Hahn & Bannon, 1998). The NK1 antagonist, GR205171, elevates NA efflux, but not 5-HT efflux, in the frontal cortex and dorsal hippocampus of freely-moving rats (Millan *et al*, 2001). This drug also increases the firing rate of LC noradrenergic cells in anaesthetized rats. Maubach *et al*, (2002) reported increased burst-firing of LC neurones from brain slices prepared from guinea pigs chronically treated with L-760735. However, the NK1 antagonists, WIN51708 and CP-96345, did not modify the firing-rate, but diminished the suppressant effect of the α_2-adrenergic agonist, clonidine on LC noradrenergic neuronal firing in anaesthetized rats, (Haddjeri & Blier, 2000). These studies demonstrate that NK1 receptor blockade has a permissive effect of NA transmission.

Experiments in this chapter use the NK1 receptor knockout mouse as a model for lifelong inhibition of the NK1 receptor, (see Chapter 1). These mice have been generated so as not to express functional NK1 receptors (see Chapter 1 & De Felipe *et al*, 1998). For the first time, *in vivo* microdialysis is used to examine NA efflux in these mice. If ablation of the NK1 receptor modulates NA transmission *in vivo*, this should influence NA efflux in NK1 knockout mice.

Receptor knockout mice were used in place of NK1 receptor antagonist drugs because compounds that have high affinity for rat or mouse NK1 receptors

have poor brain penetrance and short half lives (Rupniak *et al*, 1997). To overcome this, large doses are often used to demonstrate effects *in vivo*. This can produce non-specific effects, unrelated to NK1 receptor blockade. Also, NK1 receptor ablation represents as a model of 'lifelong' inhibition of this receptor and therefore, may be more representative of the adaptive changes which occur as a result of chronic antidepressant therapy.

If the antidepressant effects of NK1 antagonists involve an action at central NA neurones, genetic depletion of these receptors could lead to long-term changes in NA function, similar to those seen with conventional antidepressant therapy. Chronic treatment with antidepressants increases NA efflux in terminal regions and leads to desensitization of somatodendritic and terminal α_2-adrenoceptors, (Charney *et al*, 1981; Thomas *et al*, 1998; Mateo *et al*, 2001, see Invernizzi & Garattini, 2004). If loss of NK1 receptor function increases NA transmission in a similar way, this may also decrease α_2-adrenoceptor function. This was investigated, by comparing the effect of the selective α_2-adrenoceptor antagonist, atipamezole, on NA efflux in NK1 knockout and wild type mice.

An abnormality in NA efflux in NK1 receptor knockout mice could also occur because of differences in NA transport. An interaction between NK1 receptor ablation and NA transport is feasible, since the NA transporter is a target for many antidepressant drugs (Chapter 1). Infusion of the selective NA reuptake inhibitor, desipramine (DMI) increases NA efflux in the cingulate cortex, (Mateo *et al*, 1998), hippocampus, (Thomas & Holman, 1991), ventral tegmental area (Chen & Reith, 1994) and hypothalamus (Morris *et al*, 1994). NA transport was compared in NK1 receptor wild type and knockout mice using local infusion of DMI to the frontal cortex of both genotypes.

6.2. Aims

The aim of experiments in this chapter was to determine the effect of genetic ablation of the NK1 receptor on NA transmission, using *in vivo* microdialysis. If mice lacking the NK1 receptor have disrupted, or abnormal, NA function and, in particular, changes resembling those reported with conventional antidepressant therapy, this will support a role for the NA system in the putative therapeutic efficacy of NK1 receptor antagonist drugs.

Two experiments were performed:

- Experiment 1: Basal efflux of NA was measured in the frontal cortex of NK1 knockout and wild type mice. The effect of α_2-adrenoceptor blockade was then compared by systemic administration of atipamezole.

- Experiment 2: Basal efflux of NA was compared, again followed by systemic administration of atipamezole. After 80 min, the effect of NA uptake inhibition was compared by locally infusing DMI by reverse dialysis.

6.3. Methods

6.3.1 *In vivo* microdialysis

Adult, male NK1 receptor wild type (+/+) and knockout (-/-) mice (25-30g) were used. See Chapter 2 (section 2.3.2) for details of probe implantation.

Once NA efflux was stable, at least 3 basal samples were then taken before administration of any drugs. Atipamezole (4-(2-ethyl-2,3-dihydro-1H-inden-2-yl)-1H-imidazole) hydrochloride ("Antisedan", Pfizer, UK) was dissolved in 0.9% saline and administered by intraperitoneal injection at a dose of 3mg/kg in a volume of 10ml/kg. This dose of atipamezole was chosen on the basis of a previous experiment that reported modulation of rodent behaviour in novel environments (Kauppila *et al*, 1992). Atipamezole was given systemically so that both somatodendritic and terminal α_2-adrenoceptors would be inhibited, maximizing any effects on NA efflux. Local infusion of DMI (Sigma, UK) *via* the probe was achieved by switching the perfusion medium to Ringer's containing 5μM DMI. This drug was administered locally to avoid the inhibitory effects of increased NA efflux in the LC (Mateo *et al*, 1998). At the end of each experiment mice were killed by cervical dislocation. The probes were removed and inspected for any damage or leaks. Only data from experiments where the probes were removed intact were used in the final analysis.

6.3.2. Statistical analysis.

Data were analyzed using two-way analysis of variance with repeated measures. 'Time' was a within subjects factor and 'genotype' a between subjects factors. In addition to analysis of raw data, net changes in efflux were calculated by subtracting the mean basal efflux from all other points on the time course, for each case. The effect of DMI infusion was determined by comparing time 'bins' of three time points each. Basal efflux was compared with the last three successive time points during DMI infusion. The Greenhouse Geisser 'ε' correction was performed where Mauchley's test of sphericity was significant.

6.4. Results

6.4.1. Experiment 1: Effect of systemic atipamezole on NA efflux

6.4.1.1. Basal efflux of NA in NK1 knockout and NK1 wild type mice

Basal efflux of NA was approximately two-fold greater in NK1 knockout mice, (Figure 6.1A; T_{-40}-T_0, fmoles/20min; NK1knockout , 19.57 ± 1.0; NK1 wild type,10.0 ± 0.5; main effect 'genotype', $F_{1,21}$=5.1, P=0.04).

6.4.1.2. Effect of systemic atipamezole

Administration of atipamezole did not change NA efflux in either NK1 knockout or wild type mice (Figure 6.1A). When all time points were analyzed (T_{-40}-T_{80}), there was no effect of 'time', ($F_{3,69}$=0.9, P=0.4), and no 'genotype' x 'time' interaction. NA efflux remained higher in NK1 knockout mice during this period, ($F_{1,21}$=6.0, P=0.02) and for the remaining time points after administration of atipamezole (T_{20}-T_{80}, $F_{1,21}$=5.7, P=0.03).

6.4.1.3 . Analysis of net changes in efflux.

Analysis of net changes revealed a similar pattern, with no change in efflux in either NK1 knockout or wild type mice after administration of atipamezole, (Figure 6.1B; T_{-40}-T_{80}; no effect of 'time', no 'time x 'genotype' interaction).

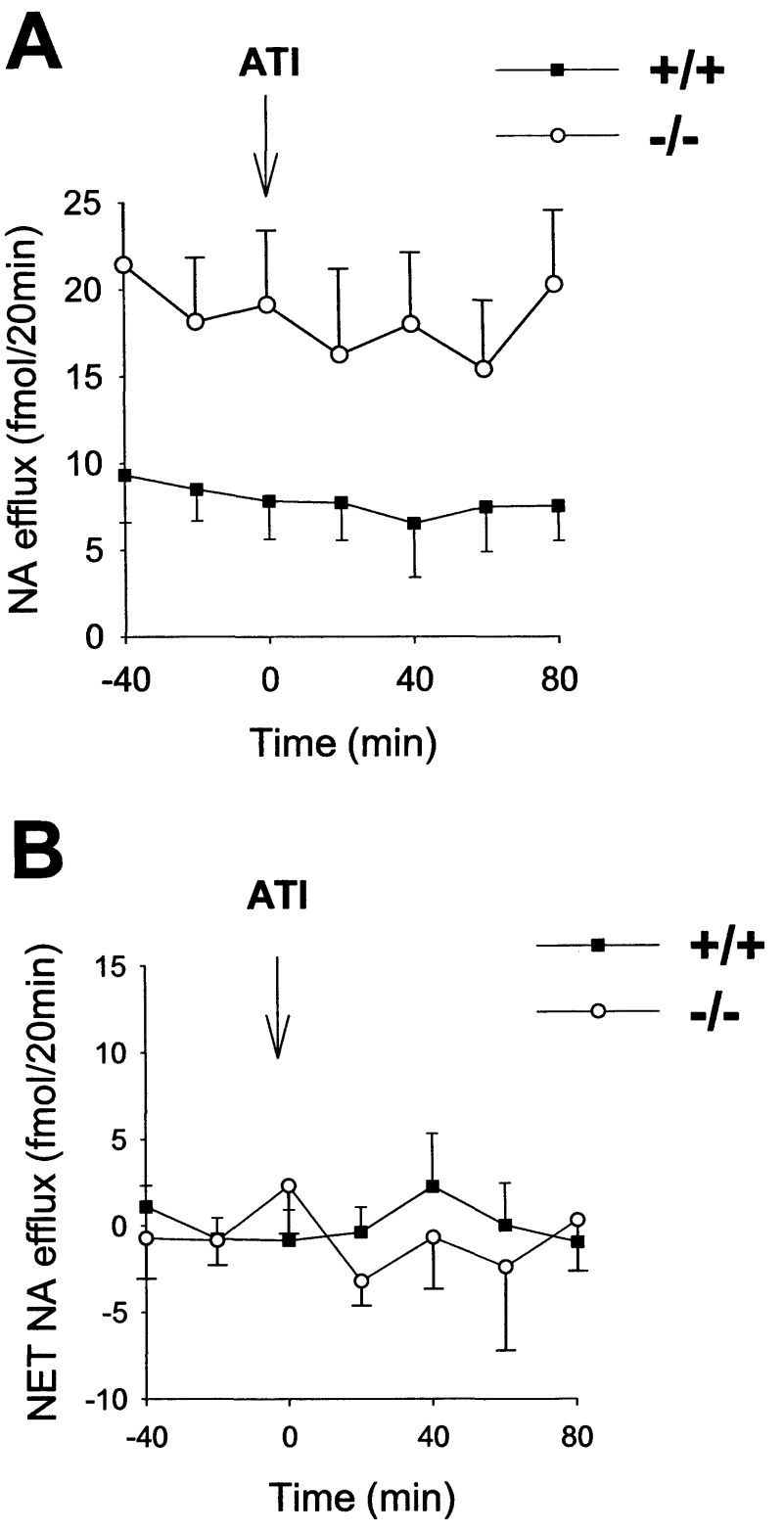


Figure 6.1. Effect of systemic atipamezole on NA efflux in the frontal cortex of NK1 wild type and knockout mice. Graphs show mean ± s.e.m. for A) raw data, B) net changes in NA efflux. Atipamezole was administered at Time 0 (arrow). n = 11/12 per data point. See section 6.4.1 for details of statistical analysis .

6.4.2. Experiment 2: Effect of systemic atipamezole followed by local infusion of DMI on NA efflux

6.4.2.1. Basal efflux of NA in NK1 knockout and NK1 wild type mice

Basal efflux of NA was 7.9 ± 0.5 in wild type mice and 15.7 ± 0.5 fmoles/20min in the knockouts (Figure 6.2A). On this occasion, the difference in basal efflux between knockout and wild type mice was not significant (T_{-40}-T_0, no effect of 'genotype', $F_{1,15}$=2.7, P=0.12). During the period T_{-40}-T_{80}, NA efflux was higher in knockout mice ($F_{1,15}$=6.0, P=0.02). Also, NA was greater in NK1 knockout mice when all time points were analyzed, (effect of 'genotype', $F_{1,15}$=5.3, P=0.04).

6.4.2.2. Effect of systemic atipamezole on NA efflux

Administration of atipamezole did not change NA efflux in either NK1 knockout or wild type mice. Thus, there was no effect of 'time', (T_{-40}-T_{80}; $F_{3,,69}$=0.9, P=0.4), and no 'genotype' x 'time' interaction, ($F_{3,69}$=0.3, P=0.8).

6.4.2.3. Effect of local infusion of DMI on NA efflux

Local infusion of 5μM DMI increased NA efflux in both knockout and wild type mice (Figure 6.2). Thus, analysis of time points T_{80}-T_{180} revealed a main effect of 'time', ($F_{5,75}$=3.9, P=0.03), a main effect of 'genotype', ($F_{1,15}$=6.0, P=0.03), but no 'time' x 'genotype' interaction, ($F_{5,75}$=0.3, P=0.9). Comparison of basal efflux (T_{-40}-T_0: bin 1) with efflux during infusion of DMI (T_{140}-T_{180}: bin 2) revealed a main effect of 'bin' ($F_{1,15}$=6.6, P=0.02), but no 'bin' x 'genotype' interaction. A main effect of 'genotype' was found ($F_{1,15}$=5.9, P=0.03), indicating that although there was no difference in the effects of DMI, NA efflux was greater NK knockout mice.

6.3.2.4. Analysis of net changes in NA efflux

Analysis of net changes gave a similar pattern. Injection of atipamezole did not change NA efflux in either genotype (T_{-40}-T_{80}; no effect of 'time', $F_{3,90}$=0.4, P=0.8; Figure 6.2B). Infusion of DMI increased NA efflux in both genotypes, (effect of 'time', $F_{4,64}$=3.0, P=0.02). Comparison of basal efflux with efflux during DMI infusion revealed a main effect of 'bin', ($F_{1,15}$=6.6, P=-0.02), but no 'bin' x 'genotype' interaction, ($F_{1,15}$=0.002, P=0.97).

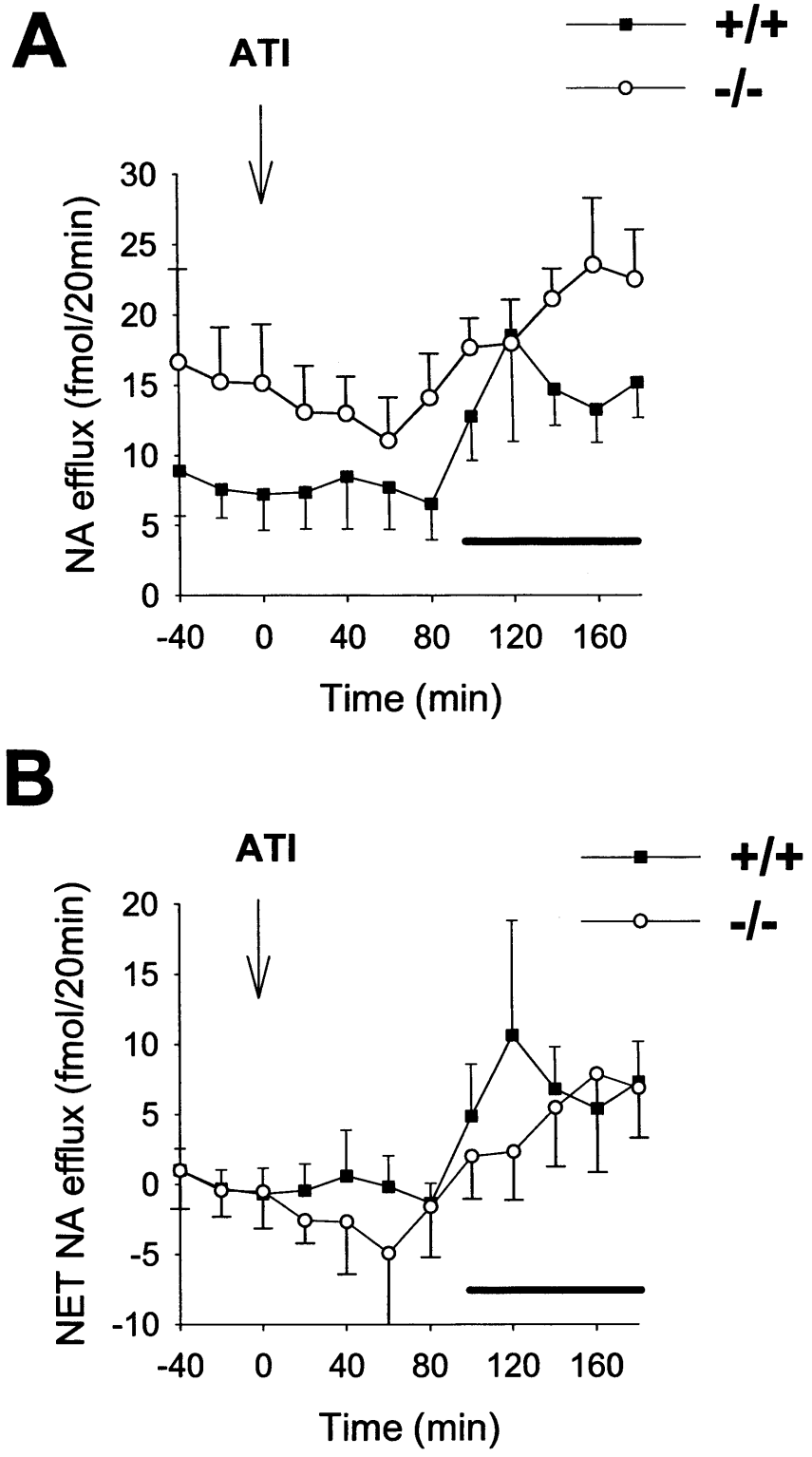


Figure 6.2. Effect of systemic atipamezole and local infusion of DMI on NA efflux in the frontal cortex of NK1 wild type and NK1 knockout mice. Graphs show mean ± s.e.m. for A) raw data, B) net changes. Atipamezole was administered at T_0 (arrow); DMI was infused during T_{100}-T_{180} (solid line). n = 8/9. See section 6.4.2 for details of statistical analysis.

6.5. Discussion

The aim of experiments described in this chapter was to determine the effect of NK1 receptor ablation on NA transmission *in vivo*. *In vivo* microdialysis was used to measure NA efflux in the frontal cortex of NK1 receptor knockout and wild type mice. Anaesthetized animals were used since these were the first microdialysis experiments performed in mice in this lab, before the technique of recovery surgery had been developed in this species. Knockout mice were used as a model of lifelong inhibition of the NK1 receptor and to avoid complication of any lack of selectivity of NK1 receptor antagonists.

In Experiment 1, there was a marked increase in basal NA efflux in mice lacking the NK1 receptor. The difference in basal efflux in the knockout mice failed to reach significance in Experiment 2. However, analysis of raw data over the entire time course revealed that NA efflux was greater in NK1 knockout mice, despite no differences in the NA response of these mice to either drug (see below). Greater basal NA efflux in NK1 receptor knockout mice has been confirmed in two subsequent studies from this lab (Fisher *et al*, 2004; Herpfer *et al,* 2005).

Elevated NA efflux is reported after chronic treatment with a variety of antidepressants (see Invernizzi & Garattini, 2004). Repeated administration of NA selective drugs, such as reboxetine, (Invernizzi *et al*, 2001; Sacchetti *et al*, 1999; Page & Lucki, 2002), and DMI (Seo *et al*, 1999; Mateo *et al*, 2001) elevates NA efflux in the frontal cortex and hippocampus of rats. Chronic treatment with the SSRIs, paroxetine (Hajos-Korcsok *et al*, 2000) and sertraline (Thomas *et al*, 1998) elevate basal NA efflux, also. Thus, the effect of NK1 receptor ablation on NA efflux is similar to the effects of chronic treatment with antidepressants. This supports a role for the NA system in the antidepressant efficacy of NK1 receptor antagonist.

Chronic treatment with antidepressants decreases the density and activity of α_2-adrenoceptor in the brain (Charney *et al*, 1981; Thomas *et al*, 1998; Invernizzi *et al*, 2001; Mateo *et al*, 2001; Subhash *et al*, 2003; see Invernizzi & Garatitini, 2004). This is thought to be an adaptive response to the prolonged activation of these receptors, due to increased release of NA (see above). If

NK1 receptor antagonists modulate NA function in similar way to conventional antidepressants, decreased responsiveness of α_2-adrenoceptors would be expected.

α_2-Adrenoceptors are inhibitory autoreceptors on the cell bodies and terminals of NA neurones (Cedarbaum & Aghajanian, 1977). Therefore, inhibition of these receptor reduces negative feedback on NA cell firing and NA efflux, (Svensson *et al*, 1980; Laitinen *et al*, 1995; Wortley *et al*, 1999; Gobert *et al*, 2004; Fernandez-Pastor *et al*, 2005). However, systemic administration of atipamezole had no effect on NA efflux in NK1 knockout or wild type mice. A lack of effect of α_2-adrenoceptor antagonists on NA efflux (Géranton *et al*, 2003), as well as LC cell firing (Mateo *et al*, 1998) been reported previously. This is thought to be due to low tonic activation of these receptors, which are activated only during periods of high NA release-rate (Callado & Stamford, 2000).

With this in mind, it is interesting that atipamezole was inactive in both genotypes. The lack of effect of atipamezole in wild type mice could be due to the low extracellular concentration of NA in these animals. However, in the knockout mice, which have two-fold greater basal NA efflux, atipamezole had no effect, also. It could be that despite elevated extracellular NA concentration, α_2-adrenoceptors are not tonically active NK1 receptor knockout mice, either. Alternatively, α_2-adrenoceptors could be down-regulated in the knockout mice, due to the elevated NA efflux. This was suggested in a subsequent study by Herpfer *et al*, (2005), who reported a lack of effect of local infusion of the α_2-adrenoceptor antagonist, RX821002, on NA efflux in NK1 receptor knockout mice.

Down-regulation of α_2-adrenoceptors is seen after chronic treatment with antidepressants, (see above). Froger *et al*, (2001) and Santarelli *et al*, (2001) report down-regulated 5-HT_{1A} receptors in NK1 receptor knockout mice, indicating that ablation of this receptor leads to long-term adaptations that are seen with conventional antidepressants (Blier & Montigny, 1980; Invernizzi *et al*, 1996). However, given that atipamezole was inactive in NK1 receptor wild type mice also, the present evidence suggests that that case for disrupted

α_2-adrenoceptor function is suggestive, but not certain. Experiments in Chapter 7 explore this further, by comparing the effect of two α_2-adrenoceptor antagonists on behaviour of NK1 receptor knockout and wild type mice.

The elevated basal NA efflux the NK1 knockout mice could also be due to impaired NA reuptake. However, the results here suggest that this process is functional in NK1 knockout mice. Local infusion of the selective NA reuptake inhibitor, DMI, increased NA efflux in both genotypes. If the increase in basal NA efflux were due to a reduced rate of NA clearance in knockout mice, a diminished response to DMI would be expected. A subsequent study, using systemic administration of DMI, confirmed that there is no difference in the effects of this uptake inhibitor on NA efflux in NK1 receptor knockout and wild type mice, (Herpfer *et al*, 2005). The finding that DMI was equally effective in both genotypes suggests that reduced NA clearance is unlikely to be responsible for the increase in basal efflux in the knockouts. Therefore, the most likely explanation for an increase in NA efflux in NK1 knockout mice is an increased rate of release.

An increased rate of NA release in NK1 receptor knockout mice appears paradoxical, given that activation of NK1 receptors in the LC increases the firing rate of noradrenergic neurones (Guyenet & Anghajanian, 1977; Cheeseman *et al*, 1983; Shen & North, 1992). NK1 receptor activation increases cation conductance and decreases rectifying K^+ current, (Shen & North, 1992). Therefore, the elevated NA efflux in NK1 receptor knockout mice seen here cannot be explained by a loss of receptors located on LC neurones. In support of the present findings, previous *in vivo* studies demonstrate that NK1 receptor antagonists elevate NA transmission. For example, systemic administration of the NK1 receptor antagonist, GR205171, increases NA efflux in the frontal cortex and dorsal hippocampus of freely moving rats, (Millan *et al*, 2001). Moreover, GR205171 increases the firing-rate of noradrenergic cells in the LC of anaesthetized rats. The effect of NK1 ablation could be due to the loss of a population of receptors located on inhibitory afferents to the LC. The location of NK1 receptors that inhibit NA transmission indirectly is as yet unknown, but GABAergic afferents from the nucleus prepositus hypoglossi are

a possibility (Ennis & Aston-Jones, 1989). GABAergic neurones expressing NK1 receptors have been identified in the CNS (Bailey *et al*, 2004).

6.6. Conclusion

In conclusion, genetic ablation of the NK1 receptor leads to an increase in NA transmission as seen following chronic treatment with conventional antidepressants. The mechanism behind this increase in extracellular NA concentration in NK1 receptor knockout mice is unknown, but is most likely due to an increase in the rate of release of transmitter. The lack of effect of atipamezole in NK1 knockout mice, despite elevated NA efflux, suggests a possible impairment of α_2-adrenoceptor function. Whether this is due to down-regulation of these receptors as a consequence of increased extracellular NA concentration is, as yet, uncertain. Nevertheless, experiments presented here support a role for the noradrenergic system in the antidepressant efficacy of NK1 receptor antagonists.

Experiments in the following chapter compare behavioural responses of NK1 knockout and wild type mice in the light/dark exploration box, a test known to elicit changes in central NA transmission (see Chapter 5). The possibility of impaired α_2-adrenoceptor function was explored further.

Chapter 7

Comparison of Behaviour of NK1 Receptor Wild Type and Knockout Mice: Effect of the α_2 -Adrenoceptor Antagonists, Atipamezole and Yohimbine

7.1. Introduction

Experiments described in the previous chapter demonstrate elevated NA efflux in NK1 knockout mice compared with spontaneous efflux in wild type mice. This is the first demonstration that genetic ablation of this putative, novel antidepressant target leads to changes in central NA transmission that resemble those seen after chronic treatment with established antidepressants (Invernizzi *et al*, 2001; Sacchetti *et al*, 1999; Page & Lucki, 2002; Seo *et al* 1999; Mateo *et al*, 2001; Hajos-Korcsok *et al*, 2000; Thomas *et al*, 1998; see Invernizzi & Garattini, 2004).

NK1 receptor antagonists (Kramer *et al*, 1998; Papp *et al*, 2000) and NK1 receptor ablation (Rupniak *et al*, 2001; Santarelli *et al*, 2001) produce changes in behaviour that are seen with established antidepressants/anxiolytic drugs (see Chapter 1). However, the neurochemical differences underlying these effects are unknown. The aim of experiments here was to determine whether any of the behavioural differences of NK1 receptor knockout mice could be attributed to augmented NA transmission.

The light/dark exploration box was used since marked increases in NA transmission are seen when rodents are confined to this test environment (Dalley & Stanford, 1995; McQuade *et al*, 1999). Also, behaviour in novel environments is modified by drugs that target NA neurones. For example, the α_2-adrenoceptor antagonist, idazoxan, increases exploratory behaviour of rats in the open field, (Siviy *et al*, 1990; Haller *et al*, 1997). α_{2A}-Adrenoceptor knockout mice make fewer rears in the open field than wild type mice and spend more time in the dark compartment in the light/dark test (Schramm *et al*,

2001). α_2-Adrenoceptor antagonists also increase locomotor behaviour in the light/dark test (Haapalinna *et al*, 1997). Therefore, the increase in NA efflux in NK1 receptor knockout mice should affect these behaviours

The hypothesis to be tested is that at least some of the behavioural differences of NK1 receptor knockout mice are due to the elevated NA transmission seen in these mice (Chapter 6). If this is the case, then drugs that augment NA transmission (*e.g.* α_2-adrenoceptor antagonists) will produced effects that resemble those of NK1 receptor ablation. Thus, the effects of the α_2-adrenoceptor antagonists, atipamezole and yohimbine, were determined in both strains of mice. Also, if NK1 receptor ablation leads to down-regulation of α_2-adrenoceptors (see Chapter 6), it would be predicted that the behavioural effects of atipamezole and yohimbine are diminished in NK1 receptor knockout mice.

7.2. Aims

- To identify behaviours in the light/dark exploration box that are sensitive to changes in NA transmission and which are modulated by the α_2-adrenoceptors antagonists, atipamezole and yohimbine.

- To determine whether NK1 receptor ablation produces behavioural effects that are consistent with elevated NA transmission, to support the microdialysis evidence of Chapter 6.

- To determine whether NK1 receptor ablation leads to a deficit in α_2-adrenceptor function, by comparing the effects of both α_2-adrenoceptor antagonists in NK1 receptor knockout and wild type mice.

7.3. Methods

7.3.1 Light/dark exploration box

Adult, male NK1 wild type and NK1 knockout mice were used. See Chapter 2 (2.7.2) for a description of the protocol of behaviour testing and scoring of behaviour. Atipamezole, (3mg/kg), yohimbine, (2.5mg/kg) or 0.9% saline were administered 30 min prior to testing. The doses were chosen on the basis of previous experiments that report modulation of rodent behaviour in novel environments by these drugs (Mason *et al*, 1998; Kauppila *et al*, 1992). At the end of each experiment the mice were removed and killed by CO_2 overdose and cervical dislocation. Atipamezole and yohimbine were dissolved in 0.9% saline. All injections were given in a volume of 10mg/kg i.p.

7.3.2. Statistical analysis

Data were analyzed routinely using two-way ANOVA with 'genotype' (NK1 knockout vs NK1 wild type) and 'drug treatment' (saline vs atipamezole or saline vs yohimbine) as 'between subjects' factors. Where a significant effect was revealed by two-way ANOVA data were also analyzed using one-way ANOVA with 'treatment group' (n=4) as the 'between subjects' variable and post-hoc LSD test performed. Levene's test of homogeneity of the variances was performed (a condition required for use of ANOVA). Where this was significant, non-parametric analysis was performed using the Mann-Whitney test with either 'drug treatment' or 'genotype' as grouping variables. Where an interaction was suspected, but Levene's test was significant, data were Log_{10} or square-root transformed and analyzed by two-way analysis of variance.

To explore the possibility that any behavioural effects were due to differences in locomotor activity, analysis of covariance (ANCOVA) was performed routinely, including either locomotor activity in the light or dark zone as a co-variate in the analysis. This was only performed for behaviours where Levene's test was not significant, a prerequisite for ANCOVA.

7.4. Results

7.4.1. Effects of atipamezole on NK1 knockout and wild type mice in the light/dark box

Behavioural effects of NK1 receptor ablation

NK1 receptor knockout mice displayed greater *locomotor activity* in the light compartment (Figure 7.1). Two-way ANOVA revealed a main effect of 'genotype', ($F_{1,36}$=5.8, P=0.02). One-way ANOVA revealed an effect of 'treatment group', ($F_{3,39}$=3.3, P=0.03). Multiple comparisons using the LSD test revealed that spontaneous locomotor activity in the light compartment of knockout mice was greater than wild types. Also, atipamezole increased locomotor activity in wild type mice but had no effect in the knockouts.

NK1 receptor knockout mice made fewer *rears in the dark* compartment, (Figure 7.2). Two-way ANOVA revealed a main effect of 'genotype', ($F_{1,36}$=7.5, P=0.01), but no effect of 'drug treatment' and no 'drug' x 'genotype' interaction. ANCOVA revealed a main effect of 'activity in the dark' ($F_{1,35}$=22, P<0.001) with residual variance due to 'genotype' ($F_{1,35}$=4.7, P=0.04). Mann-Whitney analysis was performed since Levene's test of error variance was significant for this behaviour. This also revealed an effect of 'genotype', (P=0.01).

Behavioural effects of atipamezole

Atipamezole increased the total *number of returns* to the light compartment both in NK1 receptor knockout and wild type mice, (Figure 7.3). Two-way ANOVA revealed a main effect of 'drug treatment', ($F_{1,36}$=7.9, P=0.01), but no effect of 'genotype' and no 'drug treatment' x 'genotype' interaction. ANCOVA revealed a main effect of 'activity in the dark' ($F_{1,35}$=6.4, P=0.02) with residual variance due to 'drug treatment' ($F_{1,35}$=7.6, P=0.01). Mann- Whitney analysis was performed as Levene's test was significant for this behaviour. This also revealed a main effect of 'drug treatment', (P=0.005).

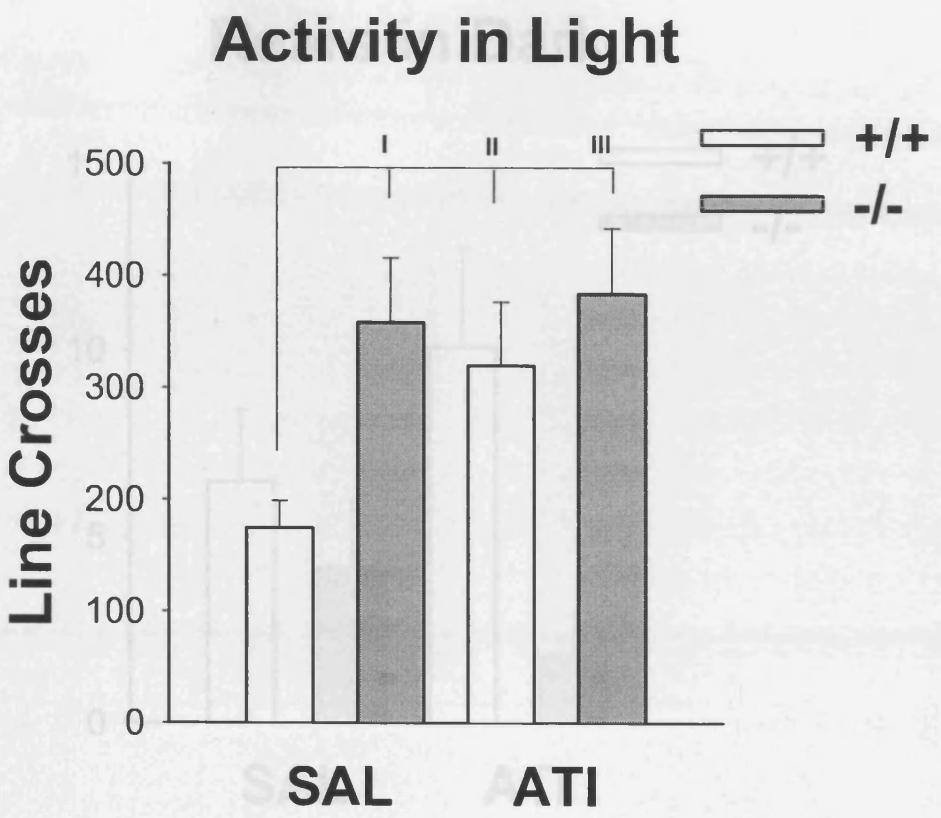


Figure 7.1. NK1 receptor knockout mice displayed increased locomotor activity in the light compartment of the exploration box. Chart shows mean line crosses ± s.e.m., n=10 each group. Post-hoc LSD test: **I**: *P*=0.02; **II**: *P*=0.05; **III**: *P*=0.007 *c.f.* NK1 wild type mice given saline. See section 7.4.1 for details of statistical analysis.

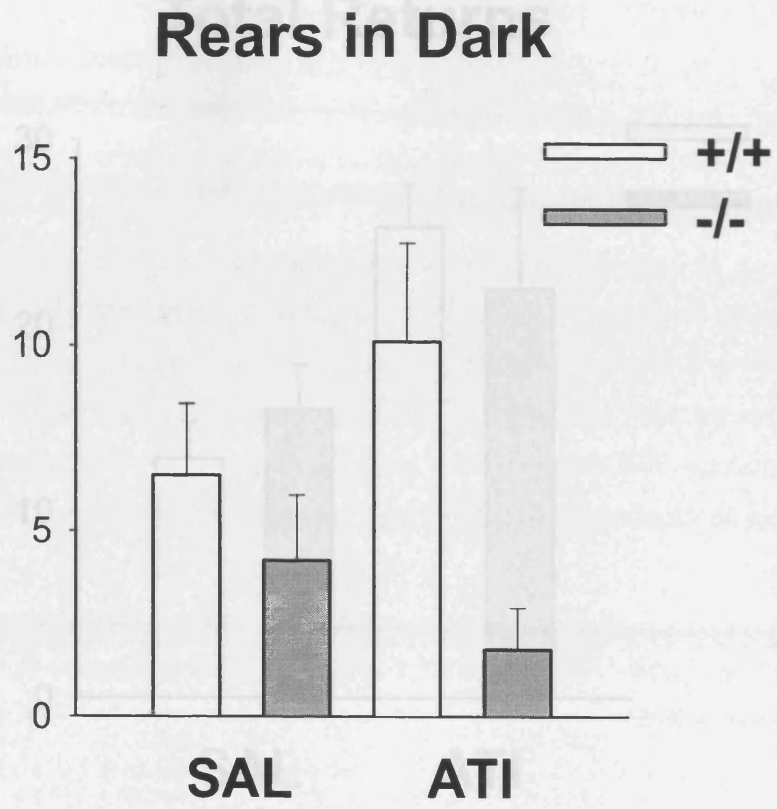


Figure 7.2. NK1 receptor knockout mice made fewer rears in the dark compartment than wild type mice. Chart shows mean number of rears ± s.e.m. (n=10). See section 7.4.1 for details of statistical analysis.

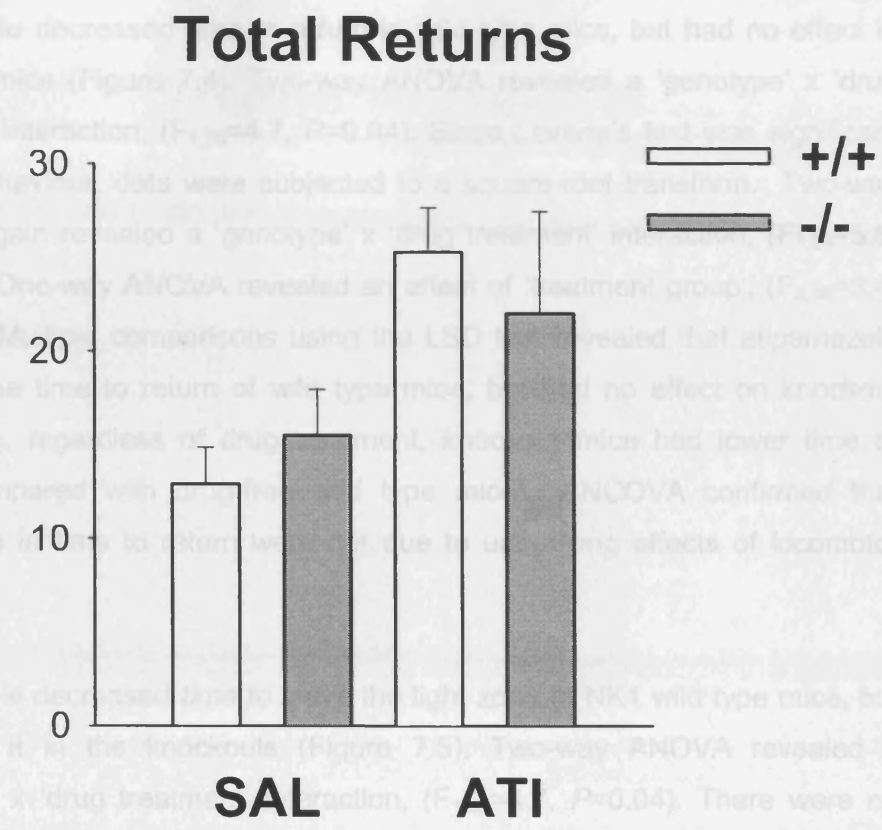


Figure 7.3. Atipamezole increased the total number of returns to the light compartment made by NK1 receptor knockout and wild type mice. Chart shows mean ± s.e.m., (n=10). See section 7.4.1 for details of statistical analysis.

Behaviours showing a drug x genotype interaction

Atipamezole decreased *time to return* in wild type mice, but had no effect in knockout mice (Figure 7.4). Two-way ANOVA revealed a 'genotype' x 'drug treatment' interaction, ($F_{1,35}$=4.7, P=0.04). Since Levene's test was significant for this behaviour, data were subjected to a square-root transform. Two-way ANOVA again revealed a 'genotype' x 'drug treatment' interaction, ($F_{1,35}$=5.6, P=0.02). One-way ANOVA revealed an effect of 'treatment group', ($F_{3,38}$=3.4, P=0.03). Multiple comparisons using the LSD test revealed that atipamezole reduced the time to return of wild type mice, but had no effect on knockout mice. Also, regardless of drug treatment, knockout mice had lower time to return compared with drug-free wild type mice. ANCOVA confirmed that differences in time to return were not due to underlying effects of locomotor activity.

Atipamezole decreased *time to leave* the light zone of NK1 wild type mice, but increased it in the knockouts (Figure 7.5). Two-way ANOVA revealed a 'genotype' x 'drug treatment' interaction, ($F_{1,35}$=4.7, P=0.04). There were no effects of 'drug treatment' or 'genotype'. One way ANOVA revealed no effect of 'treatment group'. Differences in time to leave were not due to underlying effect on locomotor activity, demonstrated by ANCOVA.

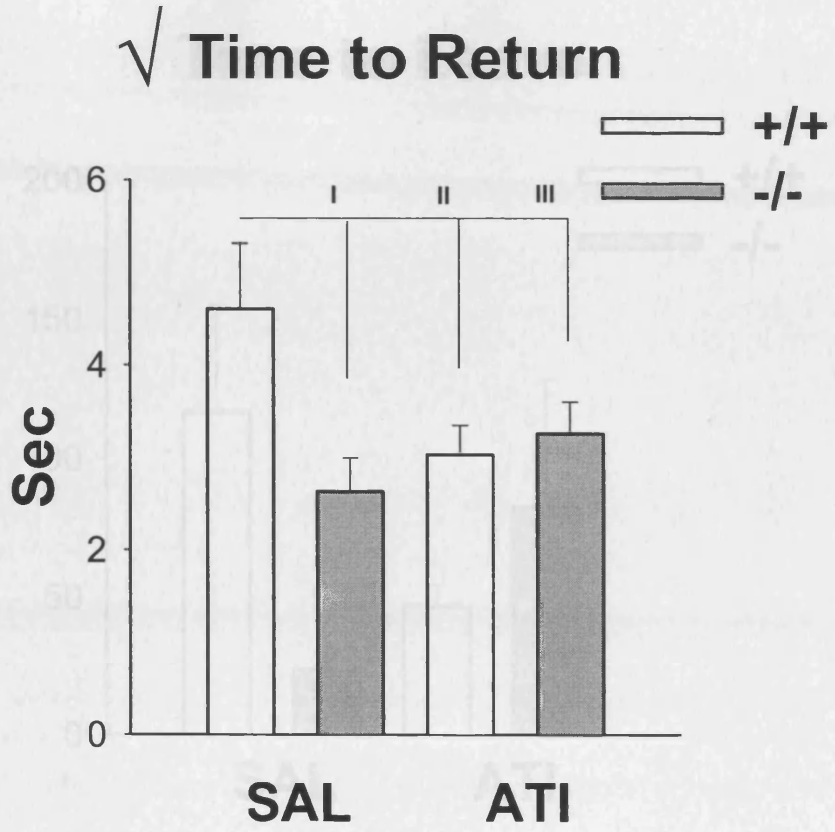


Figure 7.4. Effect of atipamezole on time to return to light (√-transformed data), by NK1 knockout and wild type mice. Chart shows mean ± s.e.m., (n=10). Post-hoc LSD test: **I**: *P*=0.005; **II**: *P*=0.02; **III**: *P*=0.05 *c.f.* wild type mice treated with saline. See section 7.4.1 for details of statistical analysis.

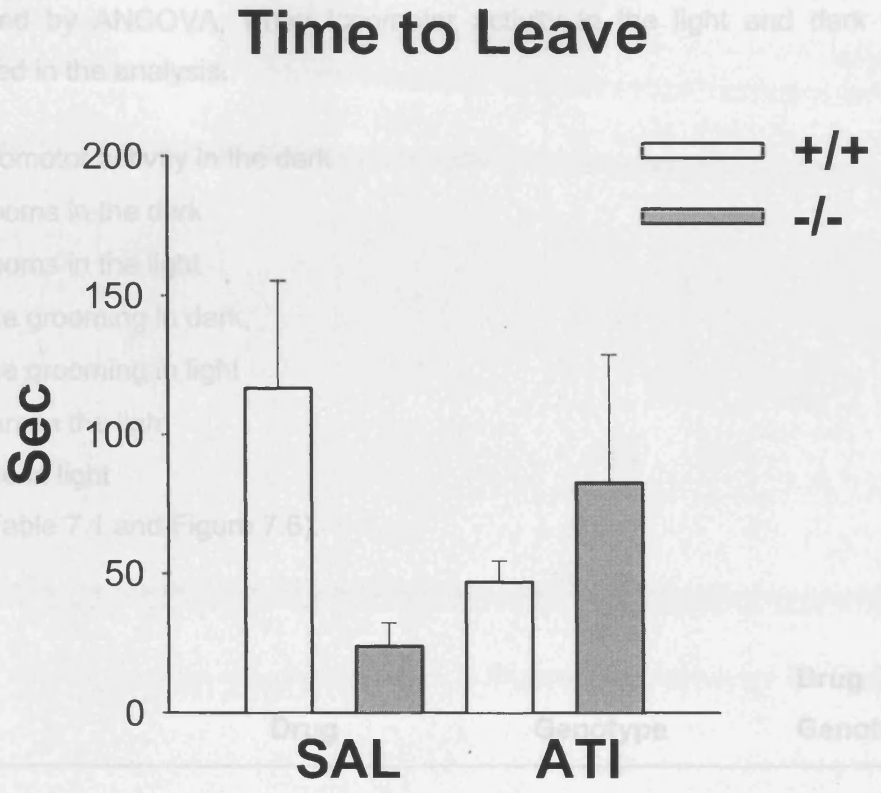


Figure 7.5. Effect of atipamezole on time to leave light compartment by NK1 knockout and wild type mice. Chart shows mean ± s.e.m., (n=10). See section 7.4.1 for details of statistical analysis.

The following behaviours were unaffected by either genotype or atipamezole, as revealed by two-way or one-way ANOVA. Likewise, no effects were revealed by ANCOVA, when locomotor activity in the light and dark were included in the analysis.

- locomotor activity in the dark
- grooms in the dark
- grooms in the light
- time grooming in dark,
- time grooming in light
- rears in the light
- time in light

(see Table 7.1 and Figure 7.6).

	Drug		**Genotype**		**Drug X Genotype**	
	$F_{1,36}$	*P*	$F_{1,36}$	*P*	$F_{1,36}$	*P*
Activity in dark	0.3	0.6	2.4	0.1	1.7	0.2
Grooms in dark*	1.3	0.3	0.001	0.9	0.5	0.5
Grooms in light*	0.1	0.7	0.1	0.8	0.1	0.7
Time grooming in dark	0.3	0.6	0.03	0.9	0.6	0.4
Time grooming in light	0.2	0.6	0.2	0.7	0.9	0.3
Rears in light	1.6	0.2	0.9	0.3	0.8	0.4
Time in light	0.4	0.5	3.1	0.09	0.3	0.9

Table 7.1. Behaviours in the light/dark exploration unaffected by atipamezole on NK1 receptor ablation. n=10. Table shows results of two-way ANOVA. * denotes behaviours where Levene's test of error variance was significant. For these behaviours, Mann-Whitney test was performed. No effects of 'drug' or 'genotype' were seen.

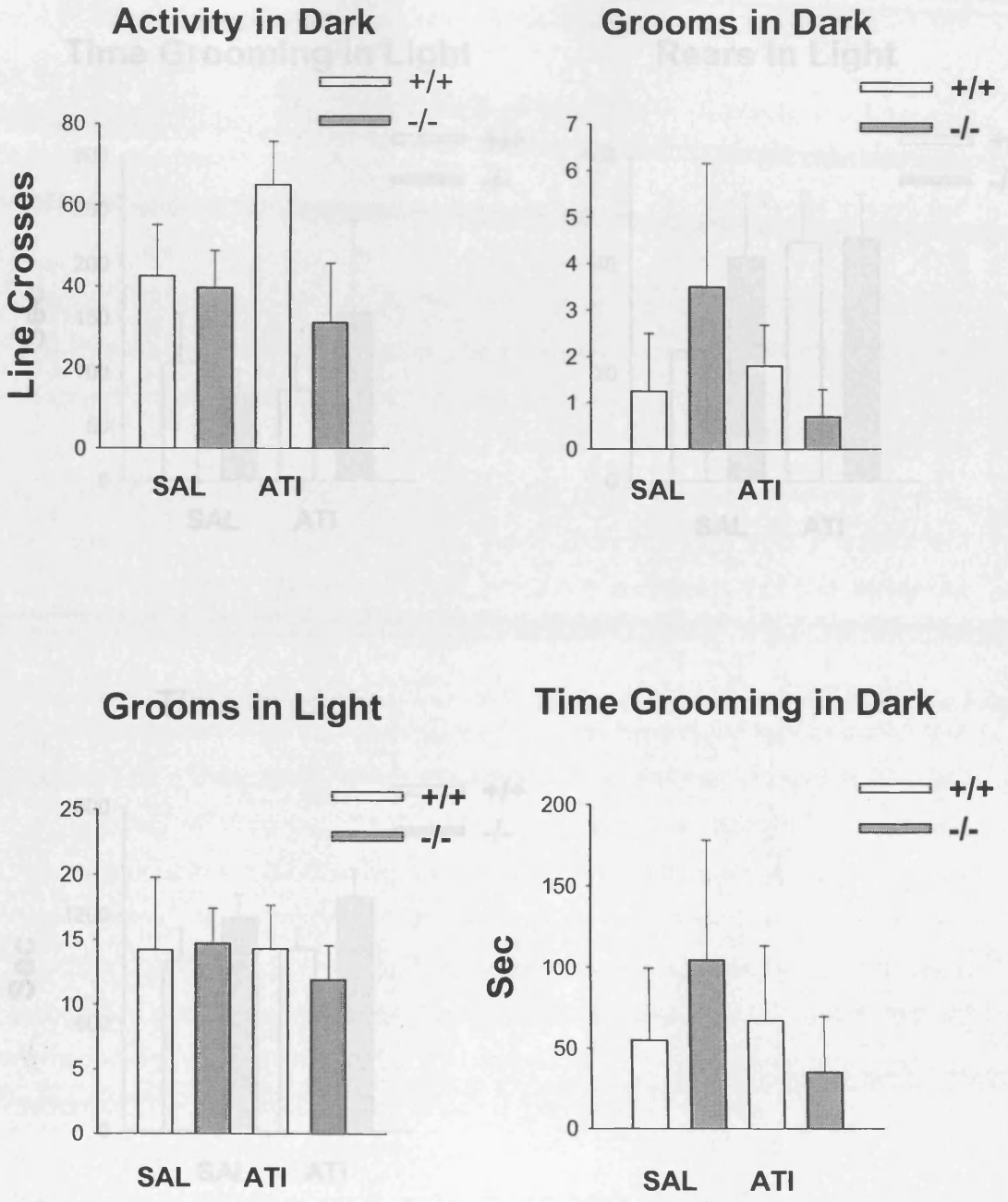


Figure 7.6. Behaviours of mice in the light/dark exploration box that were unaffected by either NK1 receptor ablation or atipamezole. N=10. See Table 7.1 for statistical analysis [continued over].

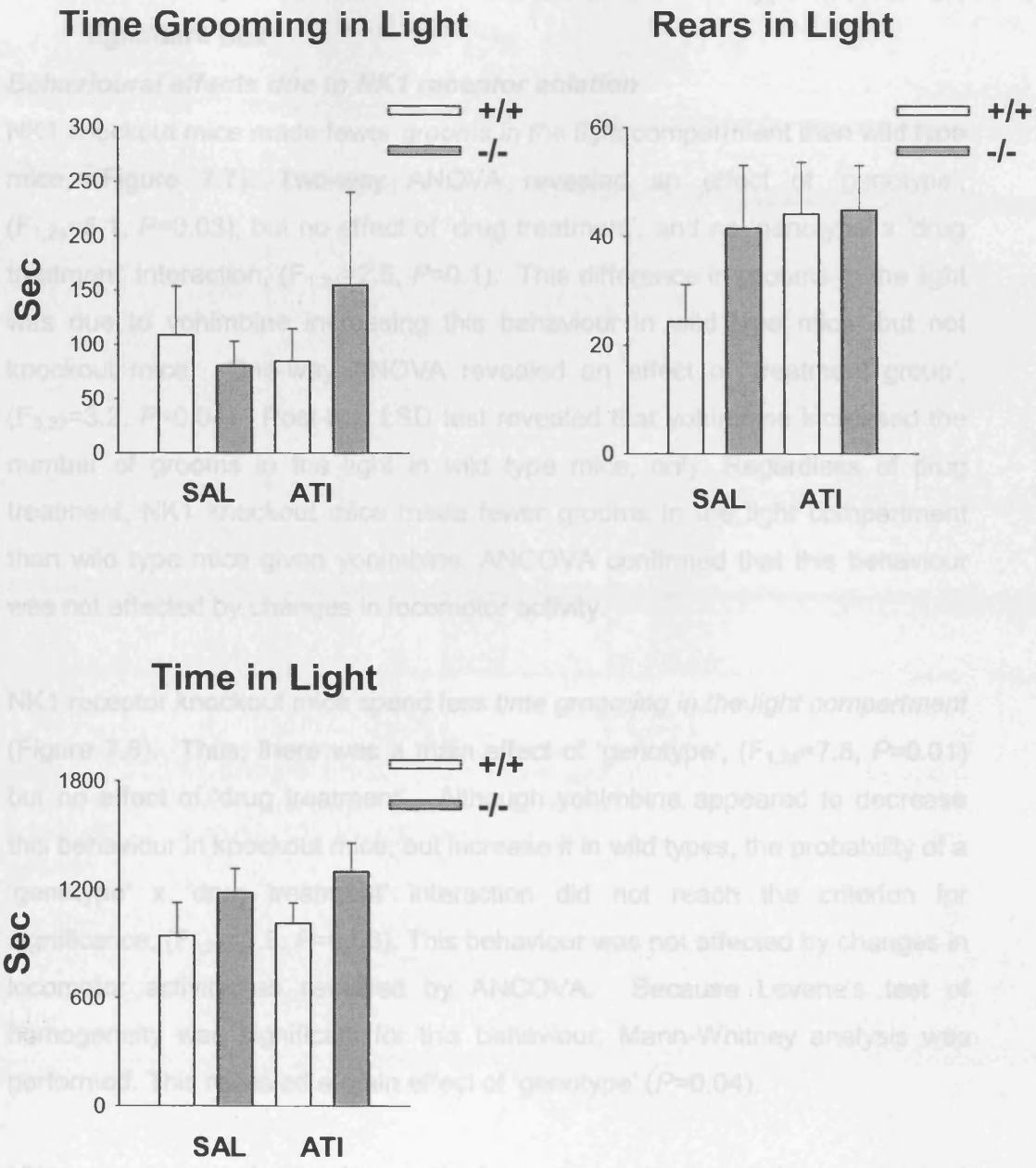


Figure 7.6 [continued]. Behaviours of mice in the light/dark exploration box that were unaffected by either NK1 receptor ablation or atipamezole. N=10. See Table 7.1 for statistical analysis

7.4.2. Effect of yohimbine on NK1 knockout and wild type mice in the light/dark box

Behavioural effects due to NK1 receptor ablation

NK1 knockout mice made fewer *grooms in the light* compartment than wild type mice, (Figure 7.7). Two-way ANOVA revealed an effect of 'genotype', ($F_{1,24}$=5.1, P=0.03), but no effect of 'drug treatment', and no 'genotype' x 'drug treatment' interaction, ($F_{1,24}$=2.5, P=0.1). This difference in grooms in the light was due to yohimbine increasing this behaviour in wild type mice, but not knockout mice. One-way ANOVA revealed an effect of 'treatment group', ($F_{3,27}$=3.2, P=0.04). Post-hoc LSD test revealed that yohimbine increased the number of grooms in the light in wild type mice, only. Regardless of drug treatment, NK1 knockout mice made fewer grooms in the light compartment than wild type mice given yohimbine. ANCOVA confirmed that this behaviour was not affected by changes in locomotor activity.

NK1 receptor knockout mice spend less *time grooming in the light compartment* (Figure 7.8). Thus, there was a main effect of 'genotype', ($F_{1,24}$=7.8, P=0.01) but no effect of 'drug treatment'. Although yohimbine appeared to decrease this behaviour in knockout mice, but increase it in wild types, the probability of a 'genotype' x 'drug treatment' interaction did not reach the criterion for significance, ($F_{1,24}$=3.8, P=0.06). This behaviour was not affected by changes in locomotor activity, as revealed by ANCOVA. Because Levene's test of homogeneity was significant for this behaviour, Mann-Whitney analysis was performed. This revealed a main effect of 'genotype' (P=0.04).

NK1 receptor knockout mice made fewer *rears in the dark compartment*, (Figure 7.9). Two-way ANOVA revealed an effect of 'genotype', ($F_{1,23}$=5.1, P=0.03), but no effect of 'drug treatment' and no 'genotype' x 'drug treatment' interaction, ($F_{1,23}$=0.6, P=0.4). Mann-Whitney analysis was performed due to significance of Levene's test. This gave the same results as ANOVA, with an effect of 'genotype', (P=0.005), but no effect of 'drug treatment'. The genotype difference in rearing in the dark could have been due to underlying differences in locomotor activity. Thus, ANCOVA revealed a main effect of 'activity in dark' ($F_{1,22}$=7.6, P=0.01) with no residual variance due to 'genotype'.

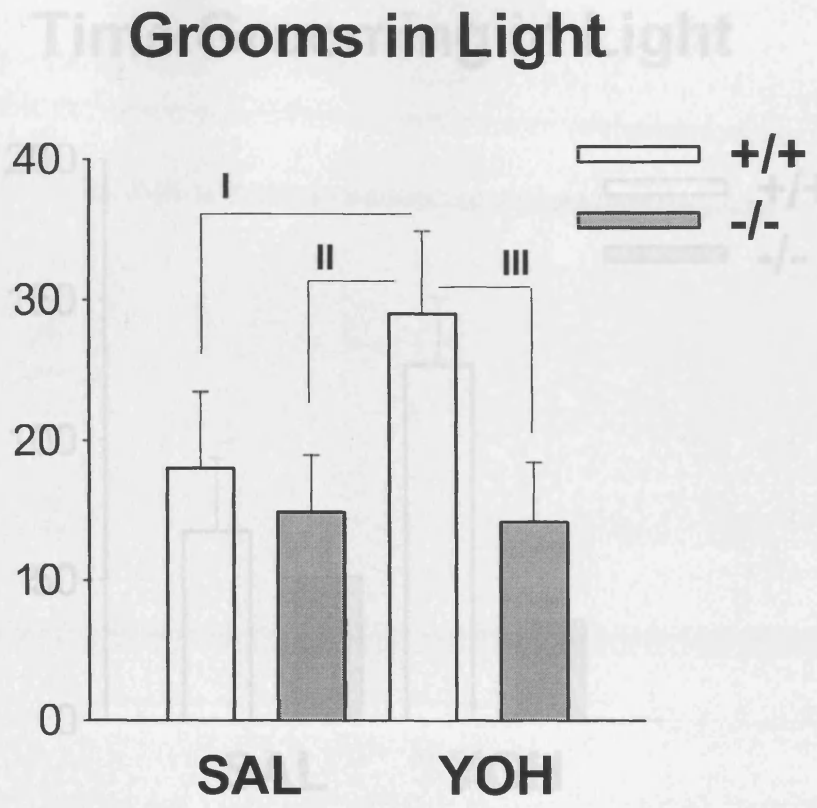


Figure 7.7. NK1 knockout mice made fewer grooms in the light compartment than wild type mice. Bar chart shows mean ± s.e.m., (n=7). Post-hoc LSD test: **I**: *P*=0.05; **II**: *P*=0.02; **III**: *P*=0.01. See section 7.4.2 for details of statistical analysis.

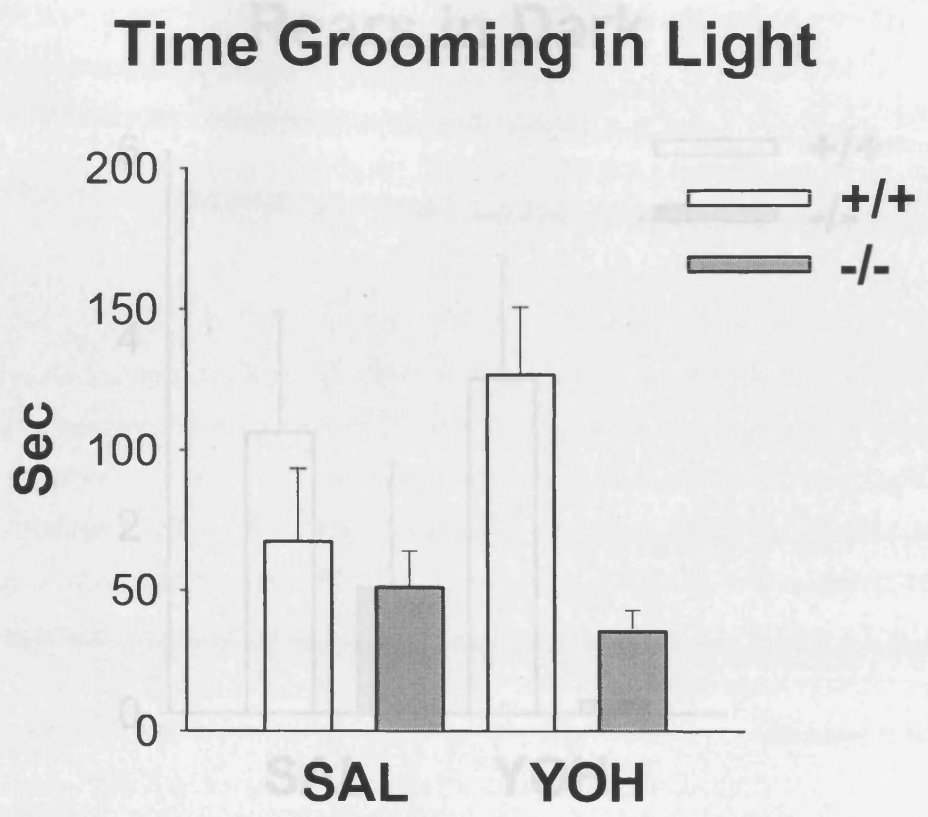


Figure 7.8. NK1 receptor knockout mice spent less time grooming in the light compartment than wild type mice. Plots show mean ± s.e.m., (n=7). See section 7.4.2 for details of statistical analysis.

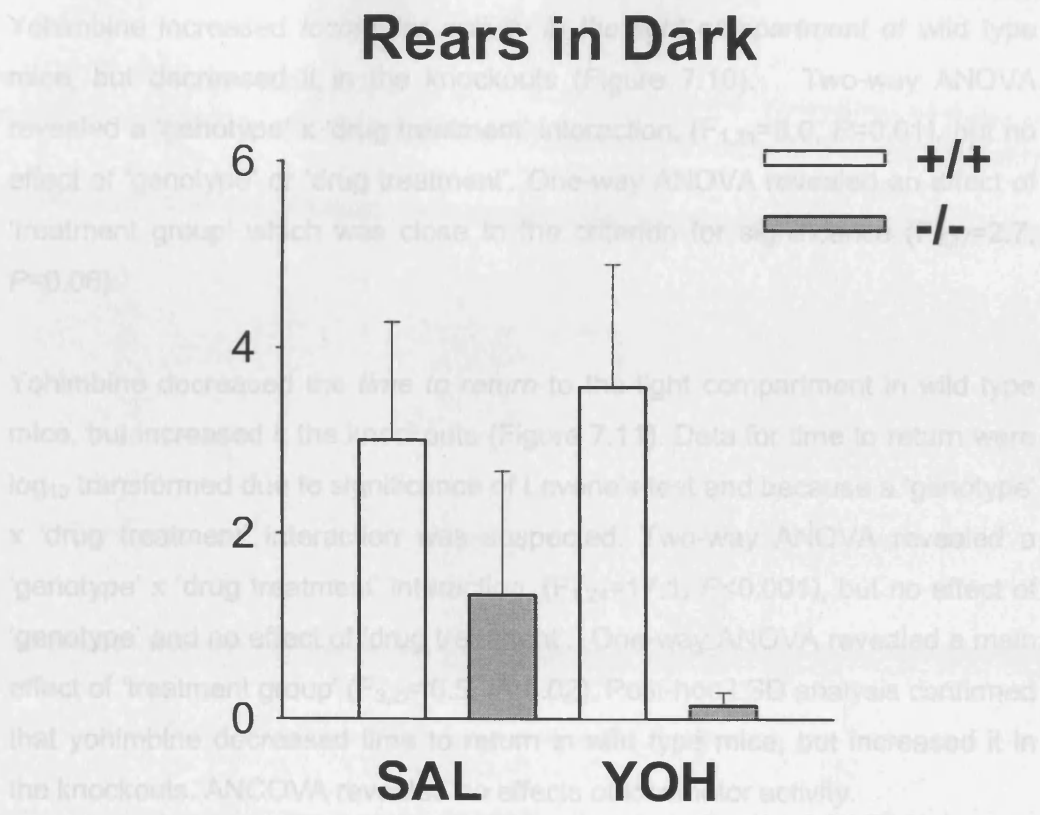


Figure 7.9. NK1 receptor ablation reduced the total number of rears in the dark compartment. Bar chart shows mean ± s.e.m., (n=7). See section 7.4.2 for details of statistical analysis.

Behaviours showing a drug x genotype interaction

Yohimbine increased *locomotor activity in the light compartment* of wild type mice, but decreased it in the knockouts (Figure 7.10). Two-way ANOVA revealed a 'genotype' x 'drug treatment' interaction, ($F_{1,24}$=8.0, P=0.01), but no effect of 'genotype' or 'drug treatment'. One-way ANOVA revealed an effect of 'treatment group' which was close to the criterion for significance ($F_{3,27}$=2.7, P=0.06).

Yohimbine decreased the *time to return* to the light compartment in wild type mice, but increased it the knockouts (Figure 7.11). Data for time to return were log_{10} transformed due to significance of Levene's test and because a 'genotype' x 'drug treatment' interaction was suspected. Two-way ANOVA revealed a 'genotype' x 'drug treatment' interaction, ($F_{1,24}$=17.1, P<0.001), but no effect of 'genotype' and no effect of 'drug treatment'. One-way ANOVA revealed a main effect of 'treatment group' ($F_{3,27}$=6.5, P=0.02). Post-hoc LSD analysis confirmed that yohimbine decreased time to return in wild type mice, but increased it in the knockouts. ANCOVA revealed no effects of locomotor activity.

Yohimbine increased the number of *rears in light compartment* in NK1 wild type mice, but decreased this behaviour in the knockouts (Figure 7.12). Data for rears in the light were log_{10} transformed due to significance of Levene's test and because a 'genotype' x 'drug treatment' interaction was suspected. Two-way ANOVA revealed a 'genotype' x 'drug treatment' interaction, ($F_{1,24}$=6.4, P=0.02) but no effects of 'genotype', or 'drug treatment'. ANCOVA revealed an effect of locomotor activity in the light compartment, ($F_{1,28}$=43, P<0.001), with no residual variance due to a 'genotype' x 'drug' interaction. Despite interaction between genotype and drug effects, there were no differences between any of the treatment groups, as demonstrated by one-way ANOVA.

Behavioural effects of Yohimbine

No behaviours were influenced by a main effect of yohimbine 'drug treatment' alone, free from effects of 'genotype', or a 'drug treatment' x 'genotype' interaction, as revealed by two-way ANOVA.

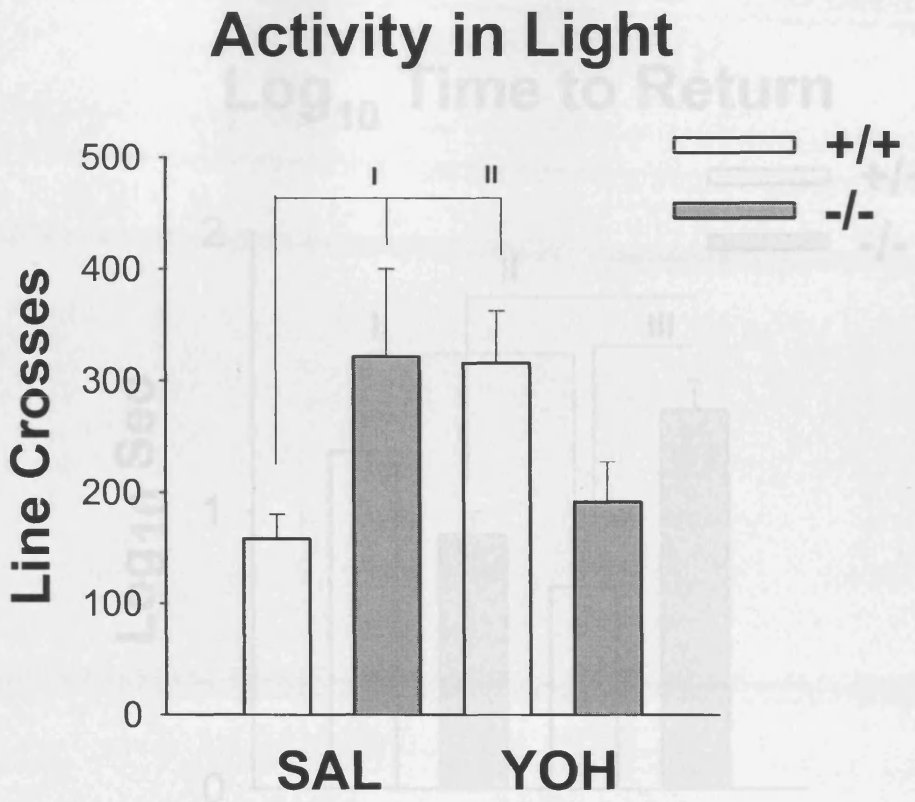


Figure 7.10. Yohimbine increased and decreased locomotor activity in the light compartment of NK1 receptor wild type and knockout mice, respectively. Chart shows mean ± s.e.m., (n=7). Post-hoc LSD test: **I**: *P*=0.03; **II**: *P*=0.04 *c.f.* NK1 wild type mice given saline. See section 7.4.2. for details of statistical analysis.

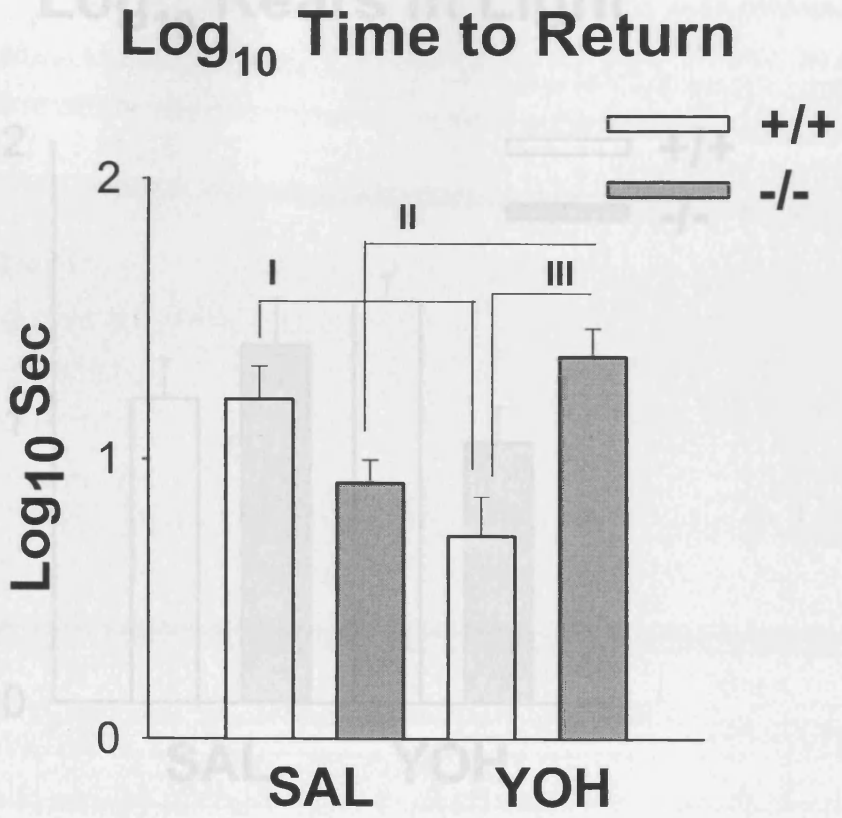


Figure 7.11. Yohimbine decreased log_{10} time to return in wild type mice, but increase it the knockouts. Chart shows mean ± s.e.m., log_{10} transformed data, (n=7). **I**: *P*=0.006, **II**: *P*=0.001, **III**: *P*=0.01. See section 7.4.2 for details of statistical analysis.

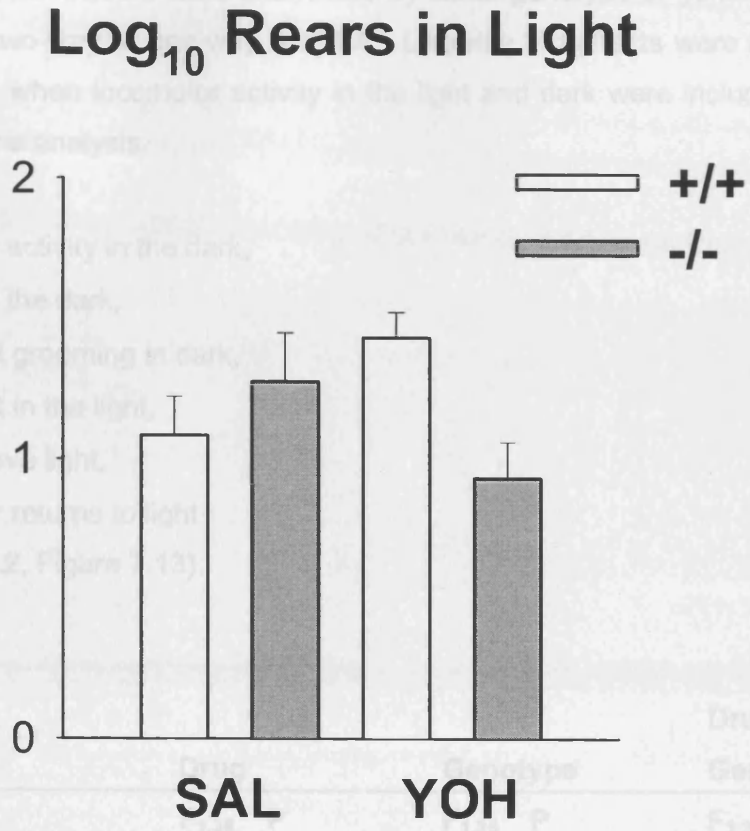


Figure 7.12. Yohimbine increased and decreased the number of rears in the light compartment of NK1 receptor wild type and knockout mice, respectively Chart shows mean ± s.e.m., log10 transformed data, (n=7). See section 7.4.2. for details of statistical analysis.

The following behaviours were unaffected by either genotype or yohimbine, as revealed by two-way or one-way ANOVA. Likewise, no effects were revealed by ANCOVA, when locomotor activity in the light and dark were included as a covariate in the analysis.

- locomotor activity in the dark,
- grooms in the dark,
- time spent grooming in dark,
- time spent in the light,
- time to leave light,
- number or returns to light

(see Table 7.2, Figure 7.13).

	Drug		Genotype		Drug x Genotype	
	$F_{1,36}$	P	$F_{1,36}$	P	$F_{1,36}$	P
Activity in dark	1.7	0.2	1.7	0.2	0.0	0.9
Grooms in dark*	1.9	0.2	4.0	0.06	1.9	0.2
Time grooming in dark	1.7	0.2	2.8	0.1	1.7	0.2
Time in light	0.1	0.7	0.005	0.9	0.08	0.8
Time to leave	1.2	0.3	0.06	0.8	0.9	0.3
Number of returns	0.1	0.7	0.0	1.0	0.2	0.6

Table 7.2. Behaviours in the light/dark exploration box unaffected by yohimbine or NK1 receptor ablation n=7. Table shows results of two-way ANOVA. * denotes behaviour where Levene's test of error variance was significant. For these behaviours, Mann-Whitney test was performed. No effects of 'drug' or 'genotype' were found.

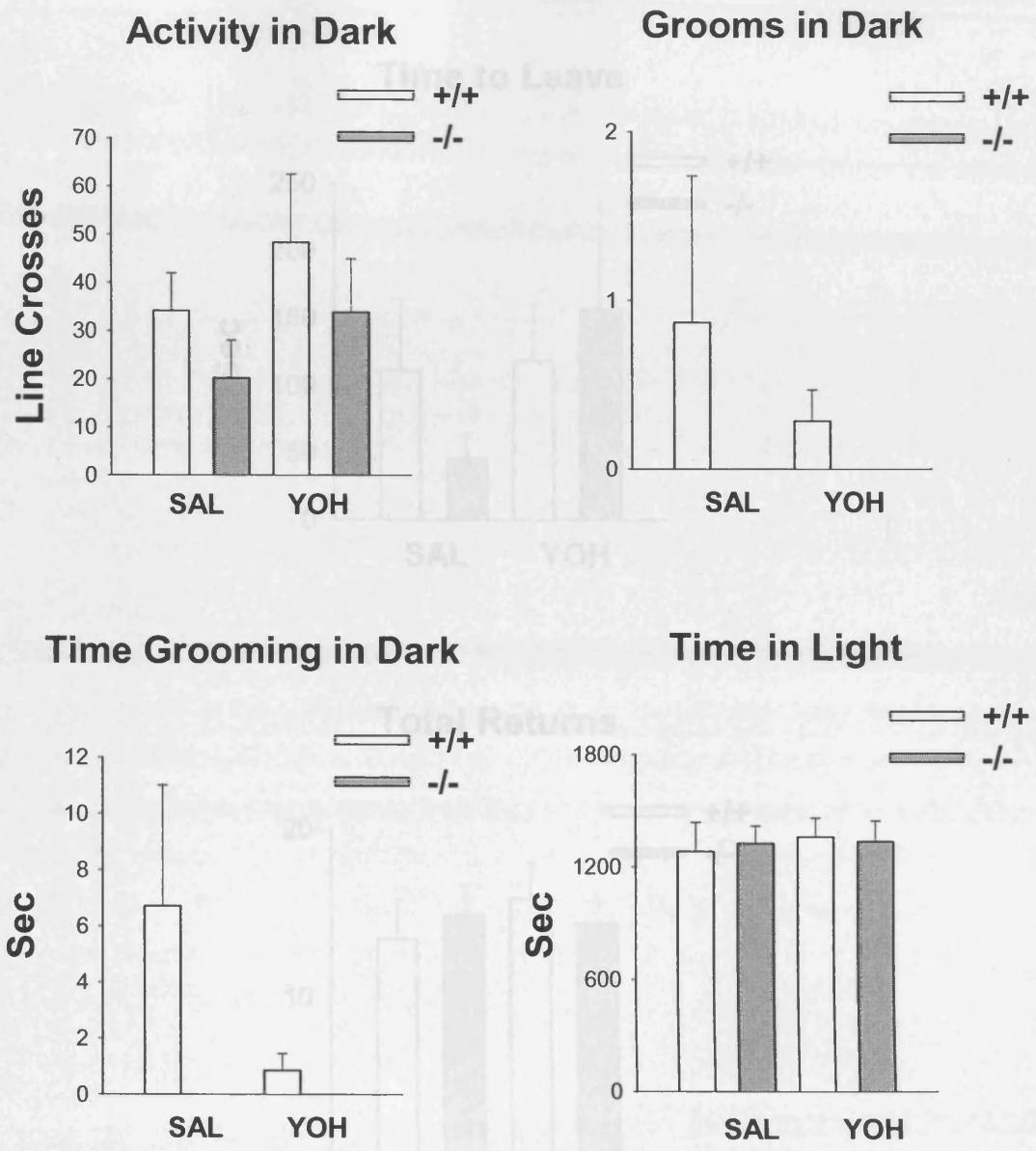


Figure 7.13. Behaviours in the light/dark exploration box that were unaffected by either yohimbine or NK1 receptor ablation. Charts show mean ± s.e.m., (n=7) [continued over]. See Table 7.2 for details of statistical analysis.

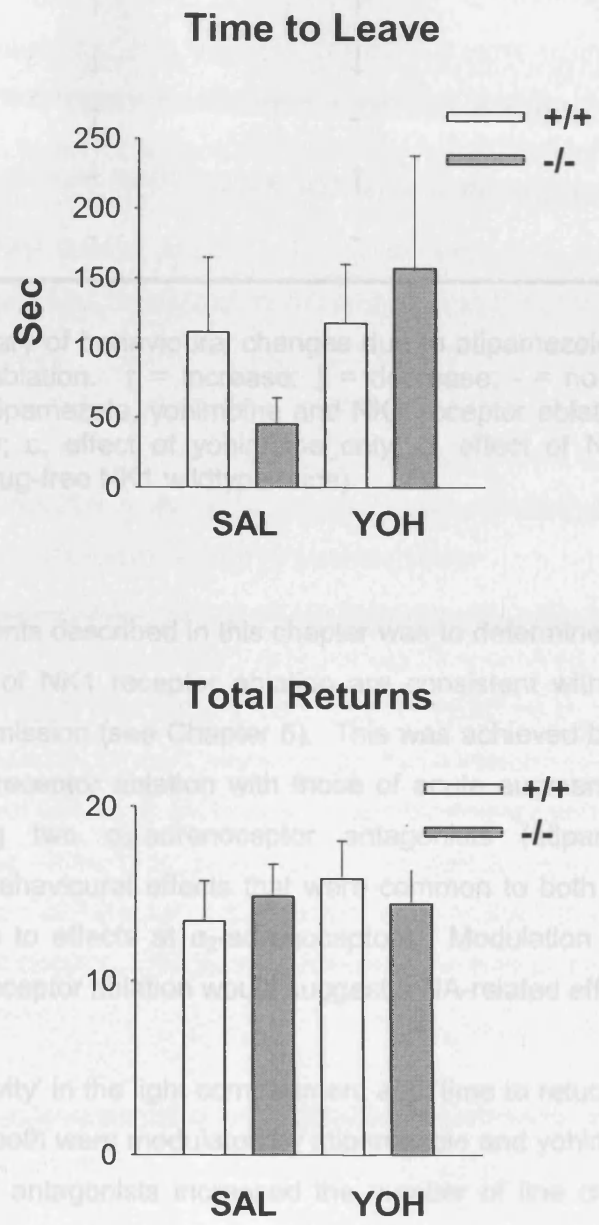


Figure 7.13. [continued] Behaviours in the light/dark exploration box that were unaffected by either yohimbine or NK1 receptor ablation. Charts show mean ± s.e.m., (n=7). See Table 7.2 for details of statistical analysis.

	ATI	YOH	NK1-/-
Activity in light[a]	↑	↑	↑
Time to return[a]	↓	↓	↓
Number of returns[b]	↑	-	-
Grooming in light[c]	-	↑	-
Rears in dark[d]	-	-	↓

Table 7.3. Summary of behavioural changes due to atipamezole, yohimbine and NK1 receptor ablation. ↑ = increase; ↓ = decrease; - = no change. a, effects shared by atipamezole, yohimbine and NK1 receptor ablation; b, effect of atipamezole only; c, effect of yohimbine only; d, effect of NK1 receptor ablation only, (*c.f.* drug-free NK1 wildtype mice).

7.5. Discussion

The aim of experiments described in this chapter was to determine whether the behavioural effects of NK1 receptor ablation are consistent with augmented noradrenergic transmission (see Chapter 6). This was achieved by comparing the effects of NK1 receptor ablation with those of acute augmentation of NA transmission, using two α_2-adrenoceptor antagonists (atipamezole and yohimbine). Only behavioural effects that were common to both these drugs are likely to be due to effects at α_2-adrenoceptors. Modulation of any such behaviour by NK1 receptor ablation would suggest a NA-related effect.

Both 'locomotor activity' in the light compartment and 'time to return' to the light fitted these criteria: both were modulated by atipamezole and yohimbine. Thus, the α_2-adrenoceptor antagonists increased the number of line crosses in the light compartment and decreased the time to return to the light compartment for the first time. Subsequent experiments from this laboratory demonstrate that both these effects are produced by a third α_2-adrenoceptor antagonist, RX821002 (Fisher *et al*, 2004). Increased locomotor activity in a novel compartment in response to α_2-adrenoceptor blockade has been demonstrated previously (Siviy *et al*, 1990; Haller *et al*, 1997; Haapalinna *et al*, 1997).

The ability of α_2-adrenoceptor antagonists to stimulate locomotor activity is probably due to elevated central NA transmission (due to blockade of

presynaptic autoreceptors). This augmented NA transmission activates postsynaptic adrenoceptors (*e.g.* α_1/β) which has a permissive role on locomotor activity, generally. For example, α_1-adrenoceptor agonists, such as phenylephrine and methoxamine, stimulate locomotor activity in mice (Heal, 1984). Elevated locomotor activity in the open field, induced by idazoxan, is prevented by the α_1-adrenoceptor antagonist, prazosin, or the β-adrenoceptor antagonist, propranolol (Haller *et al*, 1997). Moreover, i.c.v. infusion of the α_1-adrenoceptor antagonist, terazosin, completely blocks locomotor activity of mice in a novel cage, (Stone *et al*, 1999). Locomotor stimulation by amphetamine or cocaine is also mediated partly by effects on α_1-adrenoceptors, (Snoddy & Tessel, 1985). Thus, atipamezole and yohimbine increase locomotor activity in the light compartment of wild type mice in a manner consistent with elevated central NA transmission.

With this in mind, it is striking that NK1 receptor ablation consistently produced the same effects on 'locomotor activity' and 'time to return' as α_2-adrenoceptor blockade (*i.e.* increased locomotor 'activity in light', decreased 'time to return'). The same behavioural effects of NK1 receptor ablation have been confirmed in a subsequent study from this laboratory (Herpfer *et al*, 2005). This demonstrates that loss of NK1 receptor function leads to the same behavioural changes as α_2-adrenoceptor antagonism, presumably due to augmented NA transmission (see above).

This finding is consistent with the microdialysis experiments of Chapter 6, which report elevated spontaneous efflux of NA in knockout mice. In support of the present findings, evidence for augmented NA transmission after pharmacological blockade of the NK1 receptor has also been reported (Millan *et al*, 2001; Maubach *et al* 2002). Thus, NK1 receptor ablation and α_2-adrenoceptor antagonism have the same effect on certain behaviours of mice in a novel environment. Along with the results of Chapter 6, this supports a role for central NA transmission in the effects of NK1 receptor antagonist drugs on behaviour.

It is interesting that yohimbine and atipamezole *decreased* 'time to return'. 'Time to return' is also reduced by benzodiazepines (Chaouloff *et al*, 1997), an

effect attributed to the anti-conflict properties of these drugs that disinhibit exploration of the novel environment and so decrease the latency to return to there after leaving for the first time. Such an effect of α_2-adrenoceptor antagonists is surprising since yohimbine induces anxiety in healthy human subjects (Mattila *et al*, 1989) and panic attacks in panic disorder patients (Charney *et al*, 1987), an effect attributed to α_2-adrenoceptor blockade (Charney *et al*, 1992). However, the effects of benzodiazepines were not tested in the present study, so it is not valid to describe a reduction in 'time to return' as an anti-conflict property of atipamezole and yohimbine. Also, it is unlikely that the α_2-adrenoceptor antagonists reduced time to return by making mice more active (see above), since analysis of covariance did not reveal an effect of locomotor activity on 'time to return'. It is not clear how α_2-adrenoceptor antagonists reduce 'time to return'. However, as with 'activity in light', this effect was also produced by NK1 receptor ablation, consistent with an effect of this genetic ablation on NA transmission.

An interesting finding was that effects of atipamezole and yohimbine in wild type mice (*i.e.* increased locomotor 'activity in light', reduced 'time to return') were not seen in NK1 knockout mice. It is possible that floor and ceiling effects prevented any further effect of the antagonists in the knockout mice. Alternatively, there could be a deficit in α_2-adrenoceptor-mediated responses as a result of the knockout. There is some support for the latter possibility from the microdialysis data of Chapter 6: NA efflux was unchanged by atipamezole in the knockout mice, despite elevated spontaneous efflux. Together, these findings are consistent with impaired α_2-adrenoceptor function in NK1 receptor knockout mice. However, alternative explanations remain (*i.e.* a lack of tonic activity at α_2-adrenoceptors, floor and ceiling effects on behaviour).

NK1 receptor ablation also decreased rearing in the dark compartment. It is unlikely that this is a NA-related effect, since neither of the α_2-adrenoceptor antagonists affected rearing. This illustrates the sensitivity and versatility of the light/dark exploration box when used in this way. In addition to NA-related behavioural changes, effects at unidentified targets were seen. However, without further testing the effects of other reference compounds (*e.g.* 5-HT

receptor ligands) the mechanism behind the decreased rearing in the dark compartment in knockout mice cannot be identified.

For certain behaviours (number of returns, grooming in light), the effect of yohimbine and atipamezole were not the same. For example, yohimbine, but not atipamezole, increased grooming in the light compartment in wild type mice. Conversely, atipamezole, but not yohimbine, increased the number of returns to the light zone. These disparate effects likely reflect differences in the pharmacology of these drugs. The α_2:α_1 selectivity ratio of yohimbine is 200-fold lower than that of atipamezole (Virtanen *et al*, 1989). Yohimbine also has high affinity for other receptors, *e.g.* D_2 dopaminergic, (Scatton *et al*, 1980, Van Oene *et al*, 1984) nicotinic acetylcholine, (Abelson & Höglund, 2004) and 5-HT_{1A} receptors, (Winter & Rabin, 1992) as well as Na^+ channels and VR1 vanilloid receptors, (Dessaint *et al*, 2004). Atipamezole on the other hand, is a relatively specific α_2-adrenoceptor ligand, (Virtanen *et al*, 1989) although binding to imadazoline receptors, (Romer *et al*, 2003), as well as unidentified binding sites (Sjoholm *et al*, 1999) have been reported. Also, despite having similar affinities for the α_{2A}, α_{2B} and α_{2C} subtypes of the α_2-adrenoceptor, atipamezole has approximately 100-fold greater affinity for the α_{2D} receptor (*i.e.* the rodent isoform of the presynaptic α_2-adrenoceptor), compared with yohimbine, (Schwartz & Clark, 1998). As a result of these differences in drug selectivity, only behaviour effects that were common to both antagonists (increased activity in light, decreased time to return) were considered to be NA-related. The finding that both drugs had behavioural effects that were likely due to other actions demonstrates the importance of proper validation in this test (in this case, for effects on NA transmission) as well as the breadth of effects that can be detected.

It is interesting that yohimbine increased grooming in the light compartment in wild type mice, but not knockouts. This lack of effect in the knockout mice suggests a deficit in the site through which yohimbine produces this change in wild type mice. This is unlikely to be the α_2-adrenoceptor, since the effect was not shared by atipamezole (see above). Yohimbine has agonist actions at 5-HT_{1A} receptors (K_d 74nM) an effect not shared by atipamezole (K_d, 13μM; Winter & Rabin, 1992) but these are desensitized in NK1 receptor knockout

mice, (Santarelli *et al*, 2001, Froger *et al*, 2001). It is possible that the increased rearing induced by yohimbine is due to activation of 5-HT_{1A} receptors, because it is not seen in NK1 knockout mice and is not caused by atipamezole. Previous work has demonstrated an involvement of central 5-HT in grooming behaviour (Elbin & Randall, 1977; del Angel-Meza *et al*, 1996), but 5-HT_{1A} receptor activation is associated with a *reduction* in grooming (Higgins *et al*, 1991; Lazosky *et al*, 1991). Further studies using selective 5-HT receptor ligands would confirm whether grooming in the light/dark test is sensitive to changes in 5-HT transmission.

7.6. Conclusion

NK1 receptor ablation changes behaviour. Specifically, NK1 receptor knockout mice showed increased locomotor activity in the light compartment and decreased time to return to the light zone. The same behavioural effects were induced by two α_2-adrenoceptor antagonists, consistent with elevated NA transmission. Therefore, the results presented here support the microdialysis conclusions predicted from experiments of Chapter 6, which demonstrated increased NA efflux in NK1 knockout mice. Moreover, the behavioural effects of α_2-adrenoceptor antagonists are absent in NK1 receptor knockout mice, consistent with a deficit in α_2-adrenoceptor function in these mice. This work supports a role for central NA neurones in the action of NK1 receptor antagonist drugs.

Chapter 8: General Discussion

Along with 5-HT, NA is central to the mechanism of action of most, if not all antidepressant drugs. Whether effects on NA contribute to the action of SSRIs is less well established. Also, the mode of action of the putative antidepressant/anxiolytic NK1 receptor antagonists is not at all clear, although effects on NA transmission are possible. The aim of experiments described in this thesis was to determine the effects of acute fluoxetine and NK1 receptor ablation on central NA transmission and establish possible mechanisms contributing to these effects.

8.1. Summary of findings

When administered systemically, fluoxetine increased NA efflux in both the frontal cortex and hypothalamus of freely moving rats (Chapter 3). In the frontal cortex, this increase was sustained over a 4 h period, but in the hypothalamus, the increase lasted only 100 min. This difference in the duration of elevated NA efflux is similar to that reported previously using the mixed NA/5-HT reuptake inhibitor, sibutramine, and could reflect the greater inhibitory control of NA release by α_2-adrenoceptors in the hypothalamus (Wortley *et al*, 1999).

In subsequent experiments systemic fluoxetine had no apparent effect on NA efflux in otherwise untreated rats. Comparison of relevant microdialysis studies reveals that fluoxetine's effects on NA efflux are inconsistent: both increased efflux and no effect have been reported. However, this is the first time this inconsistency has been confirmed in two brain regions within a single study.

Fluoxetine's actions on NA efflux could depend on brain region, since the limited number of studies available report effects in the frontal cortex more consistently than subcortical regions. Whether this is due to inter-region variability in fluoxetine's effects, or simply the greater number of studies performed in the frontal cortex, is unknown. The present experiments demonstrate that fluoxetine has a variable effect both in the frontal cortex and hypothalamus. This demonstrates that, with respect to these two areas at

least, brain region is not the sole determinant of whether or not fluoxetine elevates NA efflux.

In both brain regions, systemic fluoxetine decreased NA efflux in rats that had a partial lesion of noradrenergic axon terminals (using DSP-4). The site of action of this decrease could not be determined, since fluoxetine was administered systemically. However, it was unlikely to be noradrenergic axon terminals, since these are partially lesioned by DSP-4. This suggests that fluoxetine decreases NA efflux by an effect upstream of axon terminals. This could be *via* increased extracellular concentration of NA and activation α_2-adrenoceptors at the somatodendritic level. When using microdialysis, the effects of fluoxetine at cell bodies could not be distinguished from those at axon terminals. Therefore, subsequent experiments used 'reverse dialysis' to determine the effects of fluoxetine infused to the frontal cortex and hypothalamus terminal field.

When infused locally, fluoxetine increased NA efflux in both brain regions in a more consistent way than when given systemically (Chapter 4). Thus, the route of administration is critical in determining fluoxetine's effects on NA efflux. This pattern of effects (*i.e.* permissive effect at axon terminals, inhibitory effect at a site upstream of the terminals) is entirely consistent with the known regulatory mechanism of central NA transmission (*i.e.* activation of somatodendritic α_2-adrenoceptors inhibits noradrenergic cell firing and release of NA; Mateo *et al*, 2000).

Any effect of fluoxetine on NA efflux could be mediated by the drug elevating the concentration of 5-HT in the extracellular space (and subsequent activation of excitatory heteroceptors on noradrenergic axon terminals). However, in rats pretreated with *p*CPA, (which depleted tissue stores of 5-HT) fluoxetine increased NA efflux in both brain regions at the highest concentration tested (50μM). This 5-HT *independent* effect occurred at a concentration of fluoxetine that is within its range to inhibit NA reuptake. This, along with the 5-HT independent nature of this response, suggests a direct effect of fluoxetine on noradrenergic neurones *in vivo*.

In the frontal cortex, but not the hypothalamus, fluoxetine also had a 5-HT-*dependent* effect on NA efflux. This was abolished by *p*CPA and was seen with a ten-fold lower concentration of fluoxetine than that required to modulate NA efflux directly (see above). Thus, fluoxetine increases NA efflux in the frontal cortex by two distinct mechanisms: by direct inhibition of NA reuptake and by a higher affinity, 5-HT-dependent mechansim. This 5-HT dependent mechanism exhibits regional selectivity, since it was present in the frontal cortex, but not the hypothalamus.

The 5-HT dependent effect of fluoxetine is not due to increased extracellular concentration of 5-HT alone. Thus, citalopram, (a more potent inhibitor of the 5-HT transporter than fluoxetine) had no effect on NA efflux even at the highest concentration tested. This suggests that an additional property of fluoxetine, not shared by citalopram, (and so not obviously related to inhibition of 5-HT uptake), contributes to its effects on NA in the frontal cortex. Thus, at low concentrations, fluoxetine has effects on NA efflux that are not fully explained by inhibition of either NA or 5-HT reuptake.

It is known that both NA and 5-HT transmission modulate the behaviour of rodents in the light/dark exploration box. Effects of fluoxetine were seen in this test that were consistent with an action on NA transmission, while other effects were suggestive of an action on 5-HT transmission (Chapter 5). Thus, although a DSP-4-induced lesion of NA axon terminals had no effect on behaviour, when fluoxetine was administered to DSP-4 pretreated rats, locomotor activity in the light compartment was *decreased*. This combination of DSP-4 and fluoxetine also *decreased* NA efflux in the frontal cortex and hypothalamus (Chapter 3), demonstrating a parallel change in NA efflux and locomotor activity in the light compartment of this test. Parallel changes in NA transmission and locomotor activity have been described before, (Mason & Fibiger, 1977; Mason *et al*, 1998; Stone *et al*, 1999) suggesting that the decrease in locomotor activity seen in DSP-4 pretreated rats given fluoxetine could be due to the diminished NA efflux resulting from this treatment (Chapter 3).

Since locomotor activity in the light compartment was unaffected by *p*CPA pretreatment, the reduction in this behaviour seen with fluoxetine is unlikely to be related to effect on 5-HT. Time of day-dependent effects were seen with fluoxetine (*e.g.* decreased locomotor activity in the dark compartment) that were abolished or reversed by *p*CPA pretreatment and therefore, consistent with effects on 5-HT-mediated regulation of circadian rhythms.

To investigate the effect of NK1 receptor ablation on NA transmission, NK1 receptor knockout mice were used as a model of life long inhibition of this receptor. Compared to wild type mice, knockout mice had abnormal NA transmission: spontaneous NA efflux was approximately two-fold greater in the knockouts (Chapter 6). Subsequent studies from this laboratory have confirmed this elevated NA efflux in NK1 receptor knockout mice (Fisher *et al*, 2004; Herpfer *et al*, 2005). This increase was not due to decreased NA reuptake, since the reuptake inhibitor, desipramine, was equally effective at increasing NA efflux in both groups of mice, when infused to the frontal cortex. NA efflux was unaffected by the α_2-adrenoceptor antagonist, atipamezole, in either group of mice. This was surprising in the knockout mice, since the increased extracellular concentration of NA efflux would be expected to activate α_2-adrenoceptors and diminish noradrenergic cell firing and release rate. That atipamezole had no effect on NA efflux in knockout mice could be due to a lack of tonic activation of α_2-adrenoceptor or some deficit in their function. Behavioural experiments explored this further by testing the response of NK1 receptor knockout and wild type mice to α_2-adrenoceptor antagonist drugs.

The light/dark exploration box revealed NA-related behaviours in mice. Thus, two α_2-adrenoceptor antagonists (atipamezole and yohimbine) increased locomotor activity in the light compartment and reduced the time to return, demonstrating that these behaviours are sensitive to drugs that modulate NA transmission. Subsequent experiments from this laboratory confirmed these effects using a third α_2-adrenoceptor antagonist, RX821002 (Fisher *et al*, 2004). By demonstrating reproducible effects of α_2-adrenoceptor antagonists, behaviour in the light/dark exploration box was used to demonstrate NA-related effects of NK1 receptor ablation.

NK1 receptor ablation produced the same changes in behavioural that were common to both α_2-adrenoceptor antagonists (*i.e.* increased locomotor activity in the light compartment, reduced the time to return: Chapter 7). Subsequent experiments have confirmed both these behavioural effects of NK1 receptor ablation (Herpfer *et al*, 2005). Since the same changes were seen with both α_2-adrenoceptor antagonists, the behaviour of NK1 receptor knockout mice is consistent with elevated NA transmission. This is in agreement with elevated spontaneous NA efflux in the knockout mice (Chapter 6) and previous experiments reporting increased LC firing rate and NA efflux after administration of NK1 receptor antagonist (Maubach *et al*, 2002; Haddjeri & Blier, 2000).

Neither α_2-adrenoceptor antagonist modulated behaviour of NK1 receptor knockout mice. Although this could be due to a deficit in α_2-adrenoceptor function in the knockout mice, floor and ceiling effects on behaviour (time to return, locomotor activity in light) could have prevented the effects of these drugs being seen in knockout mice. Thus, although the microdialysis and behavioural experiments are suggestive of a deficit in α_2-adrenoceptor function in NK1 knockout mice, further experiments are required to establish this.

8.2. Implications of this work

8.2.1. Modulation of central noradrenergic transmission by fluoxetine

The experiments in freely-moving rats demonstrate that fluoxetine has an unpredictable effect on NA efflux in the frontal cortex and hypothalamus, when administered systemically. This is strikingly similar to the effects of SSRIs on 5-HT efflux, where both increased and no change in efflux have been reported after their acute, systemic administration. An important difference with studies of 5-HT efflux and those of NA efflux is that a lack of effect of acute, systemic SSRIs on 5-HT efflux is not interpreted as an lack of effect of the drug. On the contrary, this is explained by the influence of additional mechanisms (feedback inhibition of 5-HT release *via* activation of 5-HT_{1A} receptors). This additional level of scrutiny is rarely extended to the study of SSRI effects on NA efflux.

It is concluded that microdialysis studies that report no effect of acute, systemic SSRIs on NA efflux in forebrain regions should not be interpreted as a lack of

effect of these drugs. Just as has been the case for microdialysis studies of 5-HT, additional manipulations (*e.g.* inhibition of α_2-adrenoceptors) may be necessary to demonstrate acute effects of SSRIs on NA efflux *in vivo*. In support of this, the increase in NA efflux by systemic fluoxetine is augmented by α_2-adrenoceptor antagonism (Gobert *et al*, 1999). It could be argued that this treatment should be used routinely to determine the *in vivo* effects of acute, systemic administration of other SSRIs on NA efflux.

Inhibition of noradrenergic cell firing and release due to increased activation of inhibitory α_2-autoreceptors could explain how acute, systemic SSRIs have no effect on monoamine efflux, but not how their effects are inconsistent across experiments. How is it that sometimes fluoxetine elevates NA and 5-HT efflux and not others? How does activation of somatodendritic autoreceptors diminish the increase in NA and 5-HT efflux sometimes, but not others? Unlike releasing agents such as amphetamine or fenfluramine, reuptake inhibitors do not have intrinsic effects on synaptic transmission. Rather, they prolong ongoing synaptic transmission by reducing clearance of released transmitter. Therefore, SSRIs might only augment NA efflux if NA release rate is high. The effect of uptake inhibitors would depend on the neurochemical status of the animal at the time of administration. This could explain how the effects of antidepressants vary across individuals (*i.e.* patients that respond to antidepressant therapy compared with patients who do not).

Experiments here demonstrate that changes in locomotor activity in a light, novel environment are paralleled by changes in NA efflux in the frontal cortex and hypothalamus. Thus, fluoxetine administration to DSP-4 pretreated rats *decreased* NA efflux and *decreased* locomotor activity. In mice, interventions that increase NA transmission (α_2-adrenoceptor antagonists and NK1 receptor ablation) *increased* locomotor activity. Although a causative relationship cannot be certain, this bi-directional, cross-species association between NA efflux and locomotor activity in the light compartment of the exploration box is striking and supports the use of this behavioural parameter as a measure of NA transmission in rats and mice.

8.2.2. Impact of NK1 receptor ablation on central NA transmission

Life-long inhibition of the NK1 receptor leads to changes in central NA transmission similar to those reported after chronic treatment with conventional antidepressants of different classes. This demonstrates two points: firstly, this adds to the growing body of evidence that NK1 receptor antagonists are not a novel antidepressant strategy, acting independently from any effects on monoamine transmission, as originally proposed (Kramer *et al*, 1998). Secondly, that modulation of central NA transmission is a feature common to all antidepressant classes, regardless of the molecular drug target.

8.3 Future work

Future experiments could test the hypothesis that the effects of SSRIs on NA efflux are offset by activation of α_2-adrenoceptors. Co-administration of atipamezole with fluoxetine augments its effects on NA efflux in the frontal cortex (Gobert *et al*, 1997). If co-administration of other SSRIs with an α_2-adrenoceptor antagonist discloses effects on NA efflux not otherwise apparent, this would demonstrate a lack of selectivity of these drugs for 5-HT transmission *in vivo*. Given that this selectivity is the defining feature of SSRIs, demonstration that these drugs also modulate NA transmission *in vivo* (as is the case for fluoxetine), would challenge this definition. This would also highlight the difficulty when drawing conclusions about a drug's effects *in vivo*, based on data from experiments performed *in vitro*.

Experiments using DSP-4 and fluoxetine demonstrated a decrease in NA efflux in the frontal cortex and hypothalamus. The most simple explanation for this finding is that fluoxetine preferentially increases NA efflux at the somatodendritic region, thereby diminishing cell firing and NA release. This could be tested directly, by administering fluoxetine, by reverse dialysis, to the LC and LTA. If the above hypothesis is correct, a decrease in NA efflux will be observed in the frontal cortex and hypothalamus, respectively. This effect has been demonstrated by local infusion of the SSRI citalopram to the LC, (Mateo *et al*, 2000), but no other SSRI, thus far.

The ability of SSRIs to augment NA transmission depends on the neurochemical status of the animal. This hypothesis could be tested by

comparing the effects of systemic fluoxetine on NA efflux in the homecage with those in light compartment of the light/dark exploration box. Exposure to novel environments increase NA efflux, (Dalley & Stanford, 1995; McQuade *et al*, 1999). If the hypothesis that the effect of SSRIs on NA efflux depends on the ongoing activity of noradrenergic transmission, then fluoxetine should produce a more consistent effect on NA efflux, thereby demonstrating directly that the neurochemical effect of SSRIs depend on the ongoing activity of the transmitter systems they target.

The finding that fluoxetine increases NA efflux in the frontal cortex at low concentration by a mechanism not explained by inhibition 5-HT or NA uptake is striking. The nature of this mechanism is unknown. Future experiments could determine if any of fluoxetine's non-specific effects mediate this property. Thus, if co-administration of citalopram with a 5-HT_{2C} receptor antagonist (*e.g.* SB242,084) or a $GABA_A$ receptor antagonist (*e.g.* bicuculline), resulted in a 5-HT-dependent elevation of NA efflux in the frontal cortex, this would confirm that fluoxetine has effects on NA transmission in addition its established effect as an uptake inhibitor.

The possibility of down-regulation of α_2-adrenoceptors in NK1 receptor knockout mice could be tested by comparing the responses to ligands at this receptor in these mice. α_2-Adrenoceptor agonists (*e.g.* clonidine) would be preferable to antagonists, since their effects do not require there to be tonic activation of the receptor. Some simple behavioural models have been developed in mice such (*e.g.* locomotor sedation, Heal *et al*, 1989) that could be used to overcome the potential floor and ceiling effects that were problematic in experiments using α_2-adrenoceptor antagonists in Chapter 7.

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