An investigation of changes in NMDA-receptor evoked monoamine efflux following administration of antidepressant drugs: A microdialysis study *in vivo*

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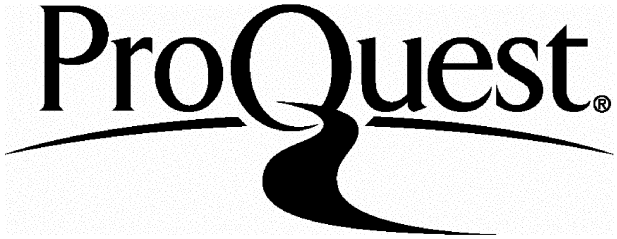

Abstract

It is widely accepted that the symptoms of depression are due, in part, to abnormal monoaminergic tone in the brain, primarily serotonin, noradrenaline and to a lesser extent dopamine. This constitutes the monoamine theory of depression. Antidepressants (ADs) work by increasing the extracellular concentration of monoamines at the synapse. Though, their mechanism is not fully understood, it has been suggested that chronic AD treatments can affect NMDA receptor function in the brain.

Using *in vivo* microdialysis in freely moving rats, the effects of acute, 7-day subchronic and chronic doses of the ADs paroxetine and clomipramine treatment on the NMDA-evoked efflux of extracellular DA, 5-HT and their metabolites, DOPAC and 5-HIAA respectively in the frontal cortex were investigated. The duration of these effects after 48 hours and 14 days of drug cessation, and the effect of the co-administration of NMDA antagonists with paroxetine on monoamine levels and their metabolites was also investigated.

Acute injection of paroxetine (10 and 20 mg/kg i.p.) did not affect dialysate DA or 5-HT content in the frontal cortex. Clomipramine at 10 and 20 mg/kg caused a decrease in extracellular DA without exerting any influence on dialysate 5-HT levels. Local infusion of 100μM NMDA into the frontal cortex decreased both extracellular DA and 5-HT levels in this region. 21 day treatment of rats with paroxetine and clomipramine increased 5-HT levels to 150% and 147% above basal levels respectively. The same treatment increased DA levels to 200% and 186% above basal levels. When NMDA infusion was preceded by a single injection of paroxetine/clomipramine no marked differences between NMDA and NMDA+paroxetine/clomipramine treated groups were observed. Subchronic (7-days) and chronic (21-days) treatment with paroxetine/clomipramine were able to abolish the NMDA-evoked decrease in dialysate DA and 5-HT levels. This effect lasted for a period of 48 hours but was abolished following a 14-day 'drug holiday'. This suggests that adaptive functional changes occur in NMDA receptor function during treatment with AD drugs. These results suggest that the NMDA receptor is subject to adaptive changes following chronic AD treatment. Interestingly, the co-administration of acute paroxetine with NMDA antagonists (amantadine, budipine, CGP 40116 and ifenprodil) causes an increase in extracellular 5-HT which may prove to have clinical implications.

For my family

Abla, Munir, Massa and Hazim

Pity the poor patient with a disease whose doctor rejects the whole notion of disease.... Psychodynamic theories are based on stories not facts... nevertheless every person is a story. For the patient, this is the most crucial fact of all.

Donald Goodwin
Professor of Psychiatry,

University of Kansas

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Publications

Pallotta M, Segieth J, Sadideen F, Whitton PS (2001). Repeated but not acute clomipramine decreases the effect of *N*-methyl-D-aspartate receptor activation on serotonergic transmission between the raphe nuclei and frontal cortex. *Neuropharmacology.* **41**, 294-300

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Abbreviations

AADC	**Aromatic L-amino acid decarboxylase**
ACPC	**1-aminocyclopropanecarboxylic acid**
ACSF	**Artificial cerebrospinal fluid**
AMPA	**α-amino-3-hydroxy-5-methyl-iosoxazole-4-proprionate**
Aman	**Amantadine**
ANOVA	**Analysis of variance**
AP5	**2-amino-5-phosphopentanoic acid**
BDNF	**Brain-derived neurotrophic factor**
BBB	**Blood-brain barrier**
cAMP	**Cyclic adenosine monophosphate**
CBT	**Cognitive behaviour therapy**
CGP40116	**(R)-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid**
CIM	**Clomipramine**
CMS	**Chronic mild stress**
CNS	**Central nervous system**
COMT	**Catechol-O-methyltransferase**
CREB	**Cyclic adenosine 3',5' monophosphate response element binding protein**
CRH	**Corticotrophin releasing hormone**
CSF	**Cerebrospinal fluid**
DA	**Dopamine**
5, 7 DCKA	**{^{3}H}-5, 7 dichlorkynurenic acid**
DOPAC	**3, 5-dihydroxyphenylacetic acid**
ECT	**Electroconvulsive therapy**
FC	**Frontal cortex**
GABA	**γ-aminobutyric acid**
GLU	**Glutamate**
GLY	**Glycine**
GP	**General practitioner**
HAM-D	**Hamilton depression rating scale**
5-HIAA	**5-hydroxyindoleacetic acid**
HPA	**Hypothalamic-pituitary-adrenocortical axis**

HPLC	**High performance liquid chromotography**
HPLC-ED	**HPLC with electrochemical detection**
5-HT	**5-hydroxytryptamine**
5-HTP	**5-hydroxytryptophan**
I.D.	**Inner diameter**
i.p.	**Intraperitoneal**
i.v.	**Intravenous**
KA	**Kainate**
KD	**Kilo dalton**
LC	**Locus coeruleus**
L-DOPA	**L-dihydroxyphenylalanine**
LTP	**Long term potentiation**
MAOI	**Monoamine oxidase inhibitor**
MHPG	**3-methoxy-4-hydroxy-phenylglycol**
MK801	**Dizocilpine**
mRNA	**Messenger ribonucleic acid**
NA	**Noradrenaline**
Nacc	**Nucleus accumbens**
NMDA	**N-methyl-D-aspartate**
NRI	**Noradrenaline reuptake inhibitor**
OB	**Olfactory bulbectomized**
O.D.	**Outer diameter**
8-OH DPAT	**8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide**
Parox	**Paroxetine**
PCP	**Phencyclidine**
PDE	**Phosphodiesterase**
PKA	**Protein Kinase A**
PKC	**Protein Kinase C**
RIMA	**Reversible inhibitors of monoamine oxidase type A**
RN	**Raphe nuclei**
s.c.	**Subcutaneous**
S.E.M	**Standard error of the mean**
SNRI	**Selective noradrenaline reuptake inhibitor**
ST	**Striatum**

SSRI	**Selective serotonin reuptake inhibitor**
$T_{1/2}$	**Half-life**
T_{max}	**Maximum rate of absorbance**
TCA	**Tricyclic antidepressant**
VTA	**Ventral tegmental area**
WAY 100635	**N-{2-{4-(2mehoxyphenly)-1-piperazineyl}ethyl}-N-(2-pyridil)cyclohaxanecarboxamide trihydrochloride**

Chapter 1
General Introduction

1.0 Introduction

1.1 Depression

Depression is a potentially life-threatening disorder that affects between 1-12% of the Western male and 3-25% of the Western female population (Angst, 1998). It may occur at any age from early childhood to late life. The impact of this major psychiatric disorder has been severely underestimated by traditional approaches that do not value disability. If disability rather than death is used as a measure of socio-economic burden, then major depression is the fourth highest most disabling ailment and has been predicted to rank second only to heart disease by 2020 (Skolnick, 1999). The annual direct and indirect costs of depression are estimated to be in excess of £3 billion in the United Kingdom and $43 billion in the United States. These figures include treatment, loss of earnings and productivity (Henry and Rivas, 1997).

Major depression is defined as a chronic state (>2 weeks) of a patient suffering from at least one core symptom and at least four secondary symptoms. The core symptoms are: (i) lack of motivation and loss of interest in daily activities and (ii) the inability to experience pleasure (anhedonia). The secondary symptoms are: (i) loss of appetite; (ii) insomnia (increased amount and decreased latency of rapid eye movement (REM) sleep, as determined by EEG measurement); (iii) motor retardation or agitation; (iv) feelings of worthlessness or guilt; (v) continuous fatigue; (vi) learning difficulties and (vii) suicidal thoughts [Diagnostic and Statistical Manual, 4th edition (DSM IV), American Psychiatric Association, 1994].

There are two main types of depression: unipolar and bipolar. Unipolar depression is more common than bipolar depression and more often related to adverse circumstances. This type of depression is more common later in life and is often associated with anxiety and aggression. In bipolar depression, mood and behaviour oscillate between depression and mania. In the manic state, an individual portrays excessive exuberance, enthusiasm and self-confidence. There is strong evidence for a hereditary link in the condition. This type of depression develops earlier in life and may have features in common with schizophrenia.

1.2 Epidemiology

The World Health Organization (WHO) estimates that 5-10 % of the population (500 000 000 people) worldwide on any one day may be depressed. Creed (1993), reported that over 80 million working days per year are lost in the UK through depression. This is 30 times the number lost due to industrial disputes. As stated above, the total costs of depression on the UK economy is £3 billion and this is 1 % of the gross national product (Taylor, 2000).

Epidemiological studies in the UK initiated by Shepherd in the 1960s identified substantial psychiatric morbidity in general care/primary care populations. The annual prevalence rate is 5-10% of the population or 30% of those seen in primary care. Lifetime prevalence of these disorders are estimated to vary between 20 and 50% of the population.

1.2.1 Social epidemiology of depression.

Social (occupational) class, domestic position, sex and genetic predisposition are factors that have been thought to contribute toward the manifestation of depression (Bebbington, 1998).

There are conflicting reports on the direct link between social (occupational) class and depression (Bebbington et al 1981 and Kessler et al, 1994). Generally, it is agreed that the frequency of depression is greater in the less privileged. This is generally thought to be a consequence of the lack of advantages they face in the everyday world.

One of the most obvious causes in the prevalence of depression is directly related to changes in domestic situations. These situations could be marital or relationship break up, bereavement of a loved one or being made unemployed (see Bruce, 2002). These domestic changes are indeed stress-related events that trigger up to 50% of all depression. Early life stress has been thought to play a role in increasing vulnerability to depression in later life stages (Marano, 1999).

Several studies have concluded that females are more likely to suffer depression than males (Bebbington, 1998). This finding may be due to the fact that women are more

likely to report depressive symptoms (Sher et al, 2001). Men suffer a suicide rate 2-3 higher than women, a figure which may be artificially high due to the inability of males to report their depressive condition (see Taylor, 2000).

There is a hereditary component for both unipolar and bipolar depression. The siblings of bipolar depressives show morbid risks of bipolar depression of 21% and of unipolar illness of 0.5%. Similarly, the prevalence of bipolar or unipolar depression in siblings of unipolar depressives is equal to 0.5% and 12.6% respectively (Leboyer et al, 1998).

1.3 Causes of depression.

Depression is a difficult illness to define precisely as it is difficult to differentiate between unpleasant feelings which are a normal consequence of stressful events and the state of abnormal functioning which could be classified as a depressive illness.

Depression has psychological, environmental and biological roots. Different drugs such as β- blockers, steroids, the contraceptive pill, opiates and L-DOPA have been shown to cause depression (Nutt et al, 1997). Depression has also been linked to the neuroendocrine system and chronic stress (Checkley, 1996) as well as the tryptophan hydoxylase gene (Bellivier et al 1998), changes in the biochemical activity of discrete brain regions (Figure 1.1) (Drevets et al, 1997), and there is a direct link between stress and the activation of the immune system and depression (see Leonard, 2000).

The areas of the brain related to mood and emotion contain a high density of monoaminergic neurones. According to the monoamine theory of depression (see section 1.7), which has formed the cornerstone of research into the illness since the 1960s, there is a deficit in transmission of noradrenaline, serotonin (Baldessarini, 1975) and to a lesser extent, dopamine (Willner, 1983).

1.4 Clinical diagnosis of depression

Although depression is common in general practice and primary care, very few people present clear-cut symptoms of depression. Many GPs feel there is a nervous component to many physical disorders. After looking at recent life events, physical illness and family history, there are diagnostic criteria which are a major indication for depressive

disorders. The current diagnostic criteria for this are stipulated in the Diagnostic and Statistical Manual, 4th edition (DSM IV). Five or more of the following symptoms must have been present nearly everyday during a 2 week period for a diagnosis of depression may be made (Jackson et al, 1997).

1. Depressed mood for most of the day
2. Markedly diminished interest in or pleasure from normal activities.
3. Significant weight change (either loss or gain)
4. Insomnia or hypersomnia
5. Psychomotor agitation or retardation
6. Fatigue or loss of energy
7. Feelings of worthlessness or excessive guilt
8. Reduced ability to concentrate
9. Recurrent thoughts of death or suicide
10. Decreased eye contact, tearfulness, decreased libido and reduced self confidence.

(Refer to table 1.1).

Table 1.1 Different types of depression: Severity (Data criteria for depressive disorders in DSM IV).

(Adapted from Freeman et al, 1997).

Severity	Diagnosis	Characteristics	Treatment
Mild	Adjustment disorder	Mild, depressive symptoms for ≥ 2 weeks, following a stressful event.	Cognitive behaviour therapy Counselling, Antidepressant drugs
	Dysthymic disorder	Depression lasting ≥ 2 years. Person feels constantly negative.	
Moderate	Major depression	Overwhelming depressed mood with significant sleep and appetite disturbances, including weight loss.	Cognitive behaviour therapy ECT Antidepressant drugs
Severe	Major depression with melancholia	Total loss of interest and pleasure. Psychomotor retardation.	Cognitive behaviour therapy ECT Antidepressant drugs

1.5 Treatment of depression

After the clinician has diagnosed a patient to be suffering from depression, a treatment plan is drawn up. Treatment of depression varies among individuals and depression is not solely treated by antidepressant drugs. There are other psychological treatments used to combat depressive symptoms (discussed later).

In depression, there are 3 phases of treatment:

- Acute- This treatment will resolve symptoms.
- Continuation- This treatment will ensure the maintenance of the response.
- Prophylaxis- This treatment will prevent relapse.

1.5.1 The pharmacological treatment of depression

There are several classes of drugs that act as antidepressants. Recently, a new class of non-tricyclic antidepressants (TCAs) (see section 1.5.2.2 for TCAs)-like drugs have come into clinical use, which are as effective as TCAs but have fewer side effects. These have been termed 'atypical ADs'. Their therapeutic actions are usually explained by the mechanism by which they enhance monoamine function in the brain. These treatments, their classification and mechanism of actions are summarised in table 1.2 below.

	Class	
1957-70	**TCAs** **MAOIs**	**Tricyclic antidepressants** **Non-selective monoamine oxidase inhibitors**
1980-90	**SSRIs**	**Selective Serotonin reuptake inhibitors**
1990-2000	**RIMAs** **SNRIs** **SNARIs**	**Reversible inhibitors of monoamine oxidase** **Serotonin and noradrenaline reuptake inhibitors** **Selective Noradrenaline reuptake inhibitors**

Table 1.2 The historical development of antidepressants (as categorised by Leonard and Healy, 2000).

In addition to these classes in the table above, there are a number of other 'atypical'

antidepressants which can be used in the treatment of depression (e.g. trazadone and mianserin).

However, the 3 main classes of antidepressants used clinically are:

1. TCAs e.g. clomipramine and imipramine
2. SSRIs e.g. paroxetine and fluoxetine
3. SNRIs e.g. Venlafaxine

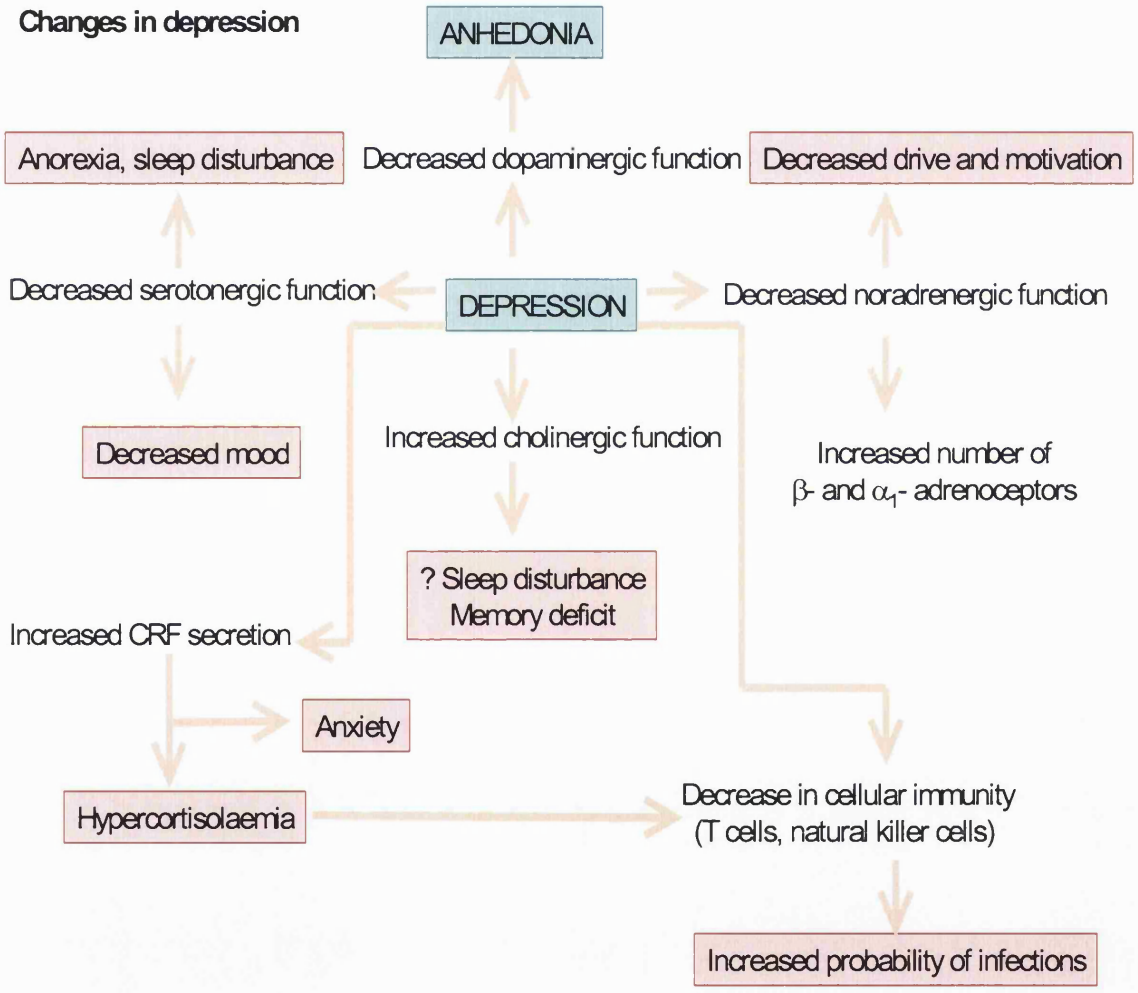


Figure 1.1 Relationship between the biochemical changes in depression and the mode of action of antidepressants (Adapted from Leonard and Healy, 2000).

1.5.2 Clinically used antidepressants:

1.5.2.1 Monoamine Oxidase Inhibitors (MAOIs)

In the 1950s it was found that isoniazid and its isopropyl derivative iproniazid, which were used as antimycobacterials, induced euphoria (regarded as an adverse reaction) in tubercular patients. It was subsequently found that these agents inhibit MAO, the main enzyme that metabolises monoamines (Crane, 1956). These observations in large part contributed to the foundation for the monoamine hypothesis of depression. The antidepressant action of MAO-inhibitors (MAOI) results from the increase in synaptic concentrations of amines due to increased monoamine release resulting from decreased enzymatic breakdown. There are two generations of MAOIs, subdivided on the basis of their binding characteristics to MAO. Most of the older clinically available MAOIs are unable to differentiate between the A or B isoform of MAO, which explains some of the side effects seen with this class of antidepressant. The first-generation MAOIs (e.g phenelzine, isocarboxazid and tranylcypromine) are irreversible inhibitors of MAO, and are associated with a high toxicity profile. Their toxicity arises from their interaction with primary amines, such as tyramine, contained in the diet (e.g. mature cheese, marmite and pickled herrings). This 'cheese reaction' can lead to a sudden increase in cardiac output and hypertension, may cause a cardiovascular crisis and even a stroke. In contrast, the second-generation MAOIs (e.g. moclobemide) bind reversibly to MAO_A, and have an improved toxicity profile compared to the former agents. Although the first developed irreversible, non-selective MAOIs are no longer the 1st choice in the clinic because of their numerous side effects, several newly developed, clinically cleaner, MAOIs remain valuable clinical tools today (for review see Thase et al, 1995).

1.5.2.2 Tricyclic antidepressants (TCAs)

Shortly after the discovery of the antidepressant action of MAOIs a new class of antidepressants was discovered; the TCA. TCAs are closely related and were initially developed as potential neuroleptics. Imipramine, the first TCA, was found to have no neuroleptic properties, but it proved effective in relieving the symptoms of depression. Subsequently, minor structural modifications resulted in drugs such as clomipramine, amitriptyline and desipramine. Contrary to the action of MAOIs, TCAs increase synaptic concentrations of monoamines not by inhibiting metabolism of the monoamine

but rather by blocking the reuptake carriers which clear the neurotransmitters from the synaptic cleft. The principal sites of action are the noradrenergic and the serotonergic uptake carriers. Due to their superior safety, the TCAs have largely supplanted the clinical use of MAOIs. Most side effects of both classes of antidepressants can be attributed to the non-specific interactions with peripheral and central cholinergic, adrenergic, dopaminergic, histaminergic and serotonergic receptors (Gareri et al., 2000) (Refer to (Fig 1.3) TCAs have been shown to be as effective as more novel antidepressants and may be more effective in the treatment of severe depression (Stahl, 1999). Clomipramine, as shown in Fig 1.3, displays highest potency at the 5-HT and NA reuptake site. It is also effective in the treatment of obsessive compulsive disorder. Unfortunately, high doses can lead to increased risk of seizures as with all TCAs.

1.5.2.3 Selective Serotonin reuptake inhibitors (SSRIs)

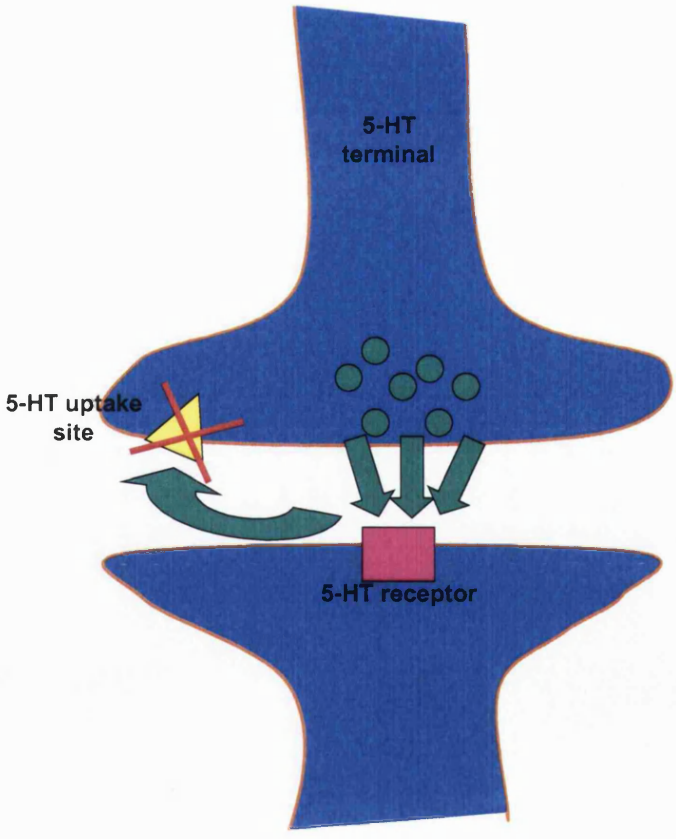


Figure 1.2 Action of SSRIs on serotonin reuptake. SSRIs have no direct action on 5HT-receptors or on other monoamine transmitters.

Since the development of the TCAs, research has focussed on developing agents that share the reuptake properties of the TCAs but lack the tricyclic structure, which is believed to be a major determinant of TCA related non-specific receptor binding characteristics. Among these are the selective reuptake inhibitors of serotonin (SSRI). SSRIs have revolutionised the treatment of depression. In comparison to TCAs and MAOIs, SSRIs have increased safety and tolerability with fewer side effects (Stahl, 1999). SSRIs act by selectively blocking the re-uptake of 5-HT into the presynaptic terminal (Figure 1.2 on previous page; Figure 1.3 below). Their side effects are well tolerated and most are resolved in approximately 3 weeks, except sexual dysfunction which may continue (Nutt et al, 1997). However, not all the effects of the SSRIs on sexual function are negative. Low acute doses of the shorter acting SSRIs can be used to treat premature ejaculation, which is accepted as a very common problem, not only in those patients with depression or anxiety.

The use of SSRIs have also been common in other known disorders which have a depressive symptom and 5-HT component such as anxiety, obsessive compulsive disorder (OCD) and panic syndrome. The effectiveness of SSRIs in severe depression is controversial (Hirschfeld and Schatzberg, 1994).

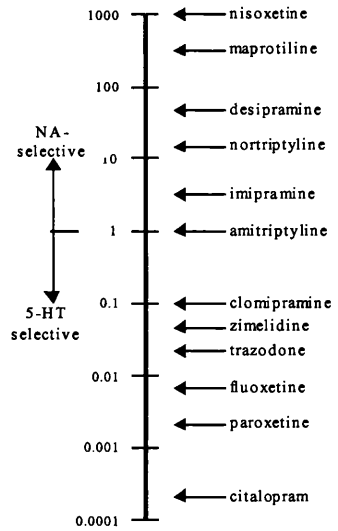


Figure 1.3 Selectivity of various antidepressants for NA and 5-HT uptake carriers *in vitro* (from Rang and Dale, 1997)

1.5.2.4 Selective serotonin and noradrenaline reuptake inhibitors (SNRIs)

SNRIs differ structurally from the TCAs and generally have no direct action on the muscarinic, histaminic or adrenergic receptors. Venlafaxine and milnacipran are the only SNRIs currently available. SNRIs block the uptake of both 5-HT and less so NA and possibly DA (Bolden-Watson and Richelson, 1993). The specificity of the reuptake block and the degrees of side effects of SNRIs are largely dose dependent. Low doses block serotonergic function while medium doses block both serotonergic and noradrenergic function and the highest doses block the reuptake of all 3 monoamines (see Roseboom and Kalin, 2000).

1.5.2.5 Selective noradrenaline reuptake inhibitors (SNARIs)

Research has led to the development of SNARIs such as reboxetine (Dostert et al, 1997) which increases extracellular NA concentrations in the brain. SNARIs do not have the typical side effects associated with TCAs as SNRIs lack affinity for 5-HT and DA reuptake sites. SNARIs have low affinity for adrenergic, muscarinic and histaminergic receptors (Burrows et al, 1998; Wong et al, 2000).

Although the development of these newer agents has improved the physicians' choice of treatment and decreased treatment related side effects, there are still some key issues that have not been resolved; therapeutic lag persists, and up to a third of all patients remain unresponsive to treatment.

Table 1.3 Side effects of antidepressants

Antidepressant Class	Side Effect
TCAs	• Consequence of blockade of muscarinic receptors (atropine-like effect): dry mouth, blurred vision, raised intraoccular pressure, urinary retention, constipation, tachycardia, confusion. • Consequence of blockade of α_1 adrenoceptors: orthostatic hypotension, dizziness. • Consequence of blockade of H_1 receptors: sedation, weight gain • Reduced sexual dysfunction. • Cardiotoxicity, particularly in elderly patients and if taken in overdose, arises from cardiac conduction block (quinidine-like effect).
SSRIs	• Neurological side effects: agitation, akathisia, anxiety, insomnia, sexual dysfunction • Vascular side effects: Headache, migraine-like attacks • Gastrointestinal side-effects: nausea, vomiting, diarrhoea.
SNRIs	• Low dose: same as SSRI • Intermediate to high doses mediated by NA and DA as well as 5-HT. • Hypertension • Severe insomnia • Severe agitation • Severe nausea • Headache

1.6 Beyond pharmacological treatment

There are a number of alternative treatments that maybe used instead, or in combination with, antidepressant drug therapy such as Electroconvulsive Shock therapy (ECS) counselling and social intervention; cognitive behaviour therapy (CBT) and Psychotherapy. In addition to the above treatments there are several complementary and alternative therapies in the treatment of depression, such as exercise, acupuncture, and relaxation therapy. These therapies are beyond the scope of this thesis.

1.6.1 Electroconvulsive shock therapy

Electroconvulsive therapy (ECT) has been practiced for over 60 years and is still used today to successfully treat severe depression. ECT in man involves stimulation of the brain or parts of it by means of electrodes placed on the head, with the patient slightly anaesthetised and paralysed with neuromuscular blocking drugs so as to avoid physical injury. ECT is occasionally used in hospitalised patients who do not respond to conventional antidepressant therapy and/or have a high risk of committing suicide (Gareri. et al, 2000). Despite being very effective, it has several major disadvantages. These include the levels of distress experienced by the patient undergoing this therapy and chronic episodes of amnesia and cognitive abnormalities in addition to ECT being substantially more expensive than pharmacotherapy.

Electroconvulsive therapy (ECT) still remains the treatment that is considered to be the most potent in refractory depressive disorders. This treatment is usually restricted to patients hospitalised for severe depression. An advantage of this treatment is that it may be used in the elderly as it maybe safer than antidepressant drugs. Rates of responsiveness of up to 90% can be expected in delusional types of depression. There is therefore the temptation to resort to ECT in any non-responsive condition. Generally, ECT is a safe, effective treatment but there are risks of memory impairment in individuals with personality-based types of depression

1.7 The monoamine theory of depression

Because of the high prevalence of depression the pharmaceutical industry have placed great emphasis on unravelling its pathogenesis. This has resulted in several hypotheses of depression pathophysiology. The initial hypothesis proposed by Schildkraut (1965) suggested that depression was linked to a deficiency of the noradrenergic neurotransmitter system in the central nervous system (CNS). This theory contributed to what became known as the monoamine theory of depression. This hypothesis originated from the observation that agents that deplete monoamines cause depressive symptoms. This was first observed when a high proportion of patients taking the rauwolfia alkaloid 'reserpine', for the treatment of hypertension, developed symptoms of depression (Schildkraut, 1965). With ongoing research, it became evident that this theory had to be revised to include a deficiency in central serotonergic (Praag and Korf, 1971) and to a lesser extent, dopaminergic systems (Willner, 1983).

The monoamine hypothesis is supported by the fact that all clinically useful antidepressant therapeutic agents act by increasing synaptic levels of monoamines e.g. noradrenaline (NA), 5-hydroxytryptamine (5-HT) and dopamine (DA). However, this theory does not explain the discrepancies between the rapid effects on monoamine metabolism (i.e. release and reuptake caused by antidepressants) and the delayed onset of clinical action, which usually takes up to several weeks to develop. An additional drawback of this theory is that approximately 30% of patients do not respond to current antidepressant therapies (Skolnick, 1999). These findings suggest that additional factors contribute to the therapeutic mechanisms underlying antidepressant action.

1.8 Monoamine theory: Revised

The discrepancy between the onset of neurochemical actions and the onset of clinical action has led to the hypothesis that one or more adaptive changes must precede a clinical antidepressant response (Vetulani, 1991; Duman et al, 1997). This hypothesis was initially substantiated by the observation that activity of central β-adrenoreceptors in the cortex is decreased following chronic antidepressant treatment (Vetulani and Sulser, 1975; Stanford et al, 1983). Subsequently, it has been shown that chronic antidepressant treatment causes adaptive changes in the efficacy of the monoaminergic systems, including a decreased density of neurotransmitter uptake sites (Raisman et al.,

1980) and downregulation of receptors (e.g. β_1, 5-HT_2, D_1) (Papp et al, 1994). These observations have resulted in the monoamine hypothesis being revised to include the adaptive changes caused by chronic antidepressant treatment. Currently, the generally accepted mechanism of antidepressant action is that as a result of persistent elevated synaptic monoamine levels, with concomitant receptor activation, there is an adaptive decrease in receptor density or function. These adaptive changes are thought to underlie the clinical action of antidepressant drugs. However it is still poorly understood how these adaptive changes are linked to the clinical improvement.

Although there is general acceptance of the revised monoamine hypothesis, there are several shortcomings. Firstly, there is not one general pattern of neurological adaptive change in any of the monoaminergic systems that is shared by all antidepressant treatments. This is illustrated by the fact that β-adrenoreceptor downregulation is seen after treatment with tricyclic antidepressants (TCA's) but not after treatment with all the so-called selective serotonin reuptake inhibitors (SSRI's) (Vetulani, 1991). Secondly, there is a discrepancy between the time course of β-adrenoreceptor and 5-HT downregulation and the clinical onset of antidepressant action (Duman et al, 1997). Thirdly, attenuation of adrenergic functioning by β-adrenoreceptor antagonists fails to elicit any antidepressant affects and can aggravate them (see Hirschfeld, 2000).

Based on these findings, it may be hypothesised that the clinical efficacy of antidepressant drugs which alter monoaminergic transmission may act via a secondary common signal transduction mechanism, which may be evoked by an adaptive change in the monoaminergic neurotransmission system. Alternatively, the clinical efficacy of antidepressant treatment may be mediated by an adaptive change in an, as yet, uncharacterised neurotransmitter system.

1.9 Role of 5-HT in depression

5-HT has been implicated in the aetiology of depression. 5-HT is an indoleamine that was first identified as a vasoconstrictor (Rapport, 1949), and was then discovered in the CNS by Amin et al. (1954). The brain contains only 1-2% of the body's 5-HT. As 5-HT does not cross the blood brain barrier (BBB), it is synthesised in the brain from the essential amino acid L-tryptophan (Figure 1.4). The rate limiting step, tryptophan

hydroxylase, is responsible for converting L-tryptophan to 5-hydroxtryptophan (5-HTP). 5-HTP is then rapidly decarboxylated to 5-HT by aromatic L-amino decarboxylase (AADC) and then transported into storage granules within the serotonergic neurones.

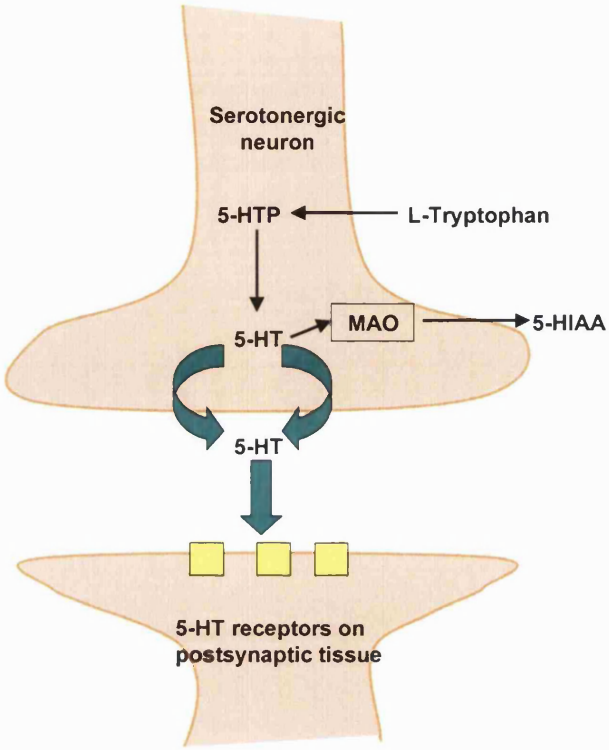


Figure 1.4 Biochemical events at serotonergic synapses. (5-HTP: 5-hydroxytryptophan; 5-HT: 5-hydroxytryptamine; 5-HIAA: 5-hydroxindoleacetic acid).

The cell bodies of serotonin neurones are known to be restricted to an area lying in or near the midline or raphe regions of the pons and the upper brain stem. Nine 5-HT nuclei (B_1-B_9) have been described by Dahlsrom and Fuxe and recently immunocytochemical localisation of 5-HT has also detected reactive cells in the area postrema and in the caudal locus coeruleus as well as in and around the interpeduncular nucleus. The more caudal groups project largely to the medulla and spinal cord. The more rostral (intermediate) groups (raphe dorsalis, raphe medianus and centralis superior), also called B_7-B_9, provides the extensive 5-HT innervation of the telencephalon and diencephalon.

The main serotonergic pathways originate from the raphe nuclei and have projections to the prefrontal cortex, the hippocampus and the striatum (Figure 1.5). The firing rate of

these neurones is controlled by somatodendritic autoreceptors in the raphe nuclei Romero and Artigas, 1997; Gobert and Millan, 1999). Additionally, presynaptic auto- and heteroreceptors regulate the 5-HT release from nerve terminals (Fink et al., 1996), Gobert and Millan, 1999). The dorsal raphe distributes 5-HT terminals to areas innervated by dopamine (e.g. the amygdala, basal ganglia and cortical areas), while the median raphe innervates the hippocampus and cortex in a similar but more limited distribution to NA (Azmitia and Segal, 1978).

DR ----------Amygdala, Basal Ganglia and Cortex

MRN----------Hippocampus and Cortex

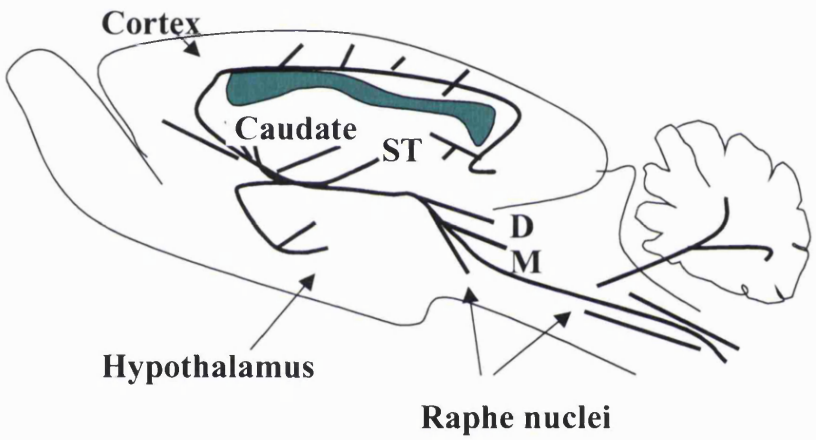


Figure 1.5 Serotonergic pathway in rat brain. ST: stria terminalis; D and M: dorsal and medial superior raphe nuclei. (Adapted from Ganong, 1999)

Multiple receptors for serotonin in the CNS have been suggested by physiological and molecular studies. In the last decade, a vast amount of new information has become available concerning the various 5-HT receptor subtypes and their functional and structural characteristics. Pre-synaptically, 5-HT receptors have been divided into 5-HT_{1A} and 5-$HT_{1B/D}$ subtypes. Post-synaptically, several receptors have been identified (5-$HT_{1A, 1B/D, 2A, 2C, 3, 4, 6, 7}$) (for review, see Barnes and Sharp, 1999). The presynaptic 5-HT_{1A} receptors are termed as somatodendritic autoreceptors. These receptors appear to possess a negative feedback mechanism that partially contributes to presynaptic 5-HT release. Presynaptic 5-$HT_{1B/D}$ receptors are called terminal autoreceptors and regulate 5-HT release in a similar manner to the 5-HT_{1A} receptors, though they only inhibit terminal release without having an effect on cell firing (for review see Adell et al, 2002).

During the last decade, preclinical and clinical evidence has accumulated indicating the involvement of the 5-HT system in the therapeutic action of antidepressant drugs (AD) (Figure 1.6). Impairment of 5-HT synthesis leads to a transient reappearance of depressive symptoms in patients in remission obtained with various SSRIs (Delgado et al., 1990). On the other hand, tryptophan and lithium, which both increase 5-HT function (Sharp et al., 1991), can potentiate the therapeutic effect of ADs (de Montigny et al., 1983). Thus, there seems to be a clear association between the AD response and enhanced 5-HT neurotransmission.

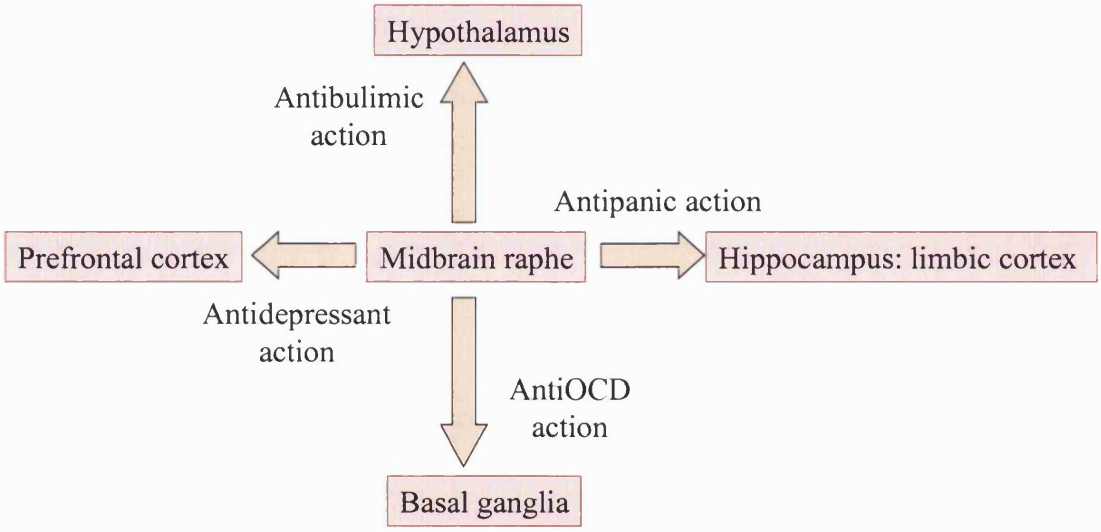


Figure 1.6 Schematic representation of the main serotonergic pathways involved in the main therapeutic actions of the SSRIs (Adapted from Leonard and Healy, 2000)

1.10 Role of dopamine in depression

Dopamine (DA) synthesis, similar to all catecholamines in the CNS, originates from the amino acid precursor tyrosine, which must be transported across the blood-brain barrier into the DA neuron. The rate-limiting step in dopamine synthesis is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase. DOPA is subsequently converted to dopamine by aromatic L-amino decarboxylase (AADC). Since tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of DA, this enzyme sets the pace for the formation of DA and is particularly susceptible to physiological regulation and pharmacological manipulation. Released DA is converted to dihydroxyphenylacetic acid (DOPAC) by intraneuronal MAO after reuptake by the nerve terminal. Released DA is also converted to

homovanillic acid (HVA), at an extraneuronal site through catechol-O-methyltransferase (COMT).

The main dopaminergic pathways originate from cell bodies situated in the substantia nigra (SN) and innervate the striatum, prefrontal cortex and the nucleus accumbens. The A9 group of the SN mainly innervates the basal ganglia while the A10 group of the ventral tegmental (VTA) mainly projects to the mesolimbic terminals (Figure 1.7).

A9 (SN)----------Basal Ganglia
A10 (VTA)----------Mesolimbic regions

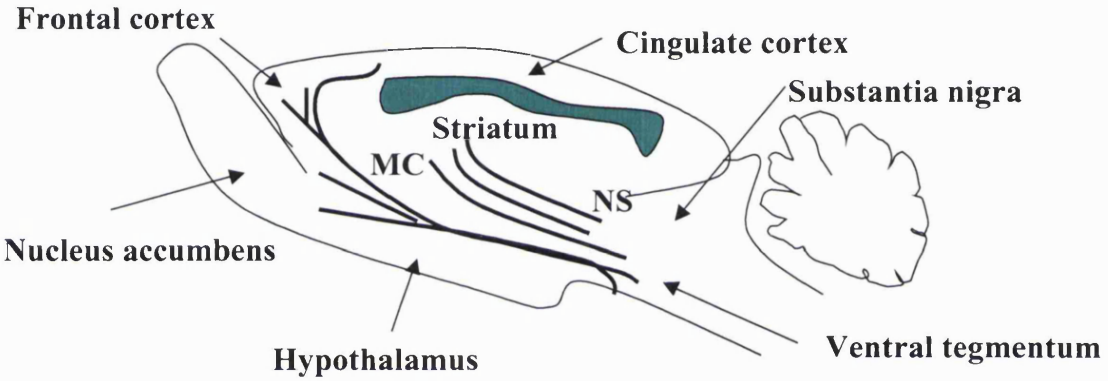


Figure 1.7 Dopaminergic pathway in rat brain. MC: mesocortical system; NS: nigrostriatal system (Adapted from Ganong, 1999)

DA receptors mediate dopaminergic transmission within the CNS. DA synthesis and release is influenced by neuronal dopaminergic activity. Postsynaptic dopamine receptors include D_1 and D_2 subtypes and are present in the projection areas of the midbrain DA neurons. They regulate the activity of neuronal feedback pathways e.g. postsynaptic receptors in the striatum regulate communication pathways between striatal neurones and DA cell bodies in the SN (see Starr, 1995; Tzscentke, 2001). D_2 autoreceptors exist on most portions of DA cells and this determines their effects. Activation of somatodendritic autoreceptors reduces the rate of neuronal firing whilst terminal autoreceptors inhibit DA synthesis and release (see Starr, 1995; Tzscentke, 2001).

Following chronic treatment with the TCA imipramine, it has been shown that there is a selective downregulation of dopamine D_1 receptors in the mesolimbic system of rats (Serra et al, 1990). However, this observation is not seen in AD-treated suicide postmortems (Bowden et al, 1997). In general, it would appear that dopaminergic transmission is increased by chronic antidepressant treatment, particularly in the mesolimbic area (Vetulani, 1991). However, it remains unclear whether changes in DA function are primary or secondary in depression.

1.11 Role of noradrenaline in depression

Noradrenaline (NA) synthesis is similar to that of DA. The final hydroxylation to convert DA to NA is catalysed by the non-specific enzyme DA-β-hydroxylase. This enzyme is restricted to NA producing cells. Similar to DA catabolism, MAO and COMT are the 2 principal enzymes involved in NA breakdown.

Two major clusterings of noradrenergic cell bodies have been described within the brain. The first is the locus coeruleus (LC), a compact cell group (A6) within the caudal pontine gray. The LC pathways terminate in the thalamus, cortex, amygdala, hippocampus and hypothalamus (Moore and Bloom, 1979) and are extremely important physiologically in the regulation of learning, memory, sleep, adaptation, arousal and stress (Leonard, 1997). The second group of cells (A1, A2, A3) lie outside the LC and consist of mainly descending fibres within the mesencephalon and spinal cord, although the more anterior tegmental levels innervate the forebrain and diencephalon (for review see Mongeau et al, 1997).

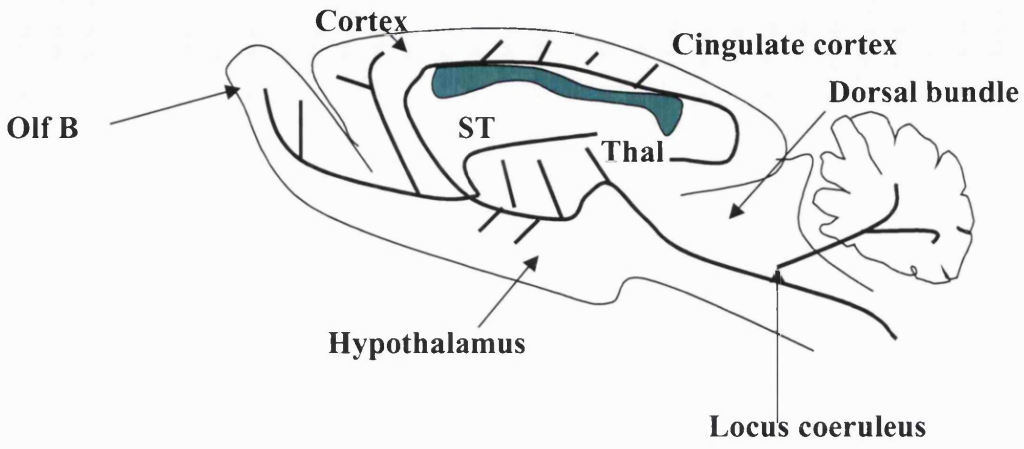


Figure 1.8 Noradrenergic pathway in rat brain (locus coeruleus system). Olf B: olfactory bulb; ST: stria terminalis; Thal: thalamus (Adapted from Ganong, 1999)

Noradrenergic transmission is regulated by a number of pre-and post-synaptic receptors. These receptors are adrenergic and termed α_1, α_2 , β_1 and β_2 . When activated by NA, these receptors trigger a molecular cascade in the postsynaptic neuron. The presynaptic α_2 receptor, acts as an autoreceptor and when activated by NA, reduces release of NA. This occurs physiologically to prevent excess NA acting on its receptors.

The role of NA in affective disorders is therefore regarded as highly significant, and it is known that as well as its involvement in depression, there are abnormalities in the central and peripheral noradrenergic systems in patients suffering from anxiety, panic disorders and post-traumatic stress (Leonard, 1997). Early AD drugs acted on noradrenergic systems whereas more recently the SSRIs target 5-HT reuptake without affecting noradrenergic pathways *in vitro*. There is considerable evidence to suggest that both drug types affect NA and 5-HT pathways in the CNS, and the two transmitter systems are inextricably linked to one another. For example, local (Hughes and Stanford, 1996) and chronic administration of an SSRI such as fluoxetine, selectively blocking the reuptake of 5-HT, will increase extracellular NA (Potter, 1996). Following these findings, extensive investigations into the ligand binding properties of α- and β-adrenoceptors in depressed patients and also measurements of the main NA metabolite 3-methoxy-4-hydroxyphenyl-glycol (MHPG) in plasma, urine and CSF were carried out but proved largely inconclusive (reviewed by Redmond and Leonard, 1997). Attention then turned toward tyrosine hydroxylase (TH), the rate limiting enzyme in NA

synthesis, and it was found that in depressed patients and animal models of depression, AD drug treatment caused downregulation of TH mRNA expression. However, like the metabolic studies, the findings were inconclusive of the involvement of TH in depression (Ordway et al., 1994).

1.12 Monoaminergic neurotransmitter interactions

As no one transmitter-system can fully explain the pathophysiology of depression, it must be noted that interactions between transmitter systems must occur in the CNS of depressed patients.

1.12.1 DA-5-HT interactions

Dopaminergic cells of the VTA project to limbic structures such as the nucleus accumbes, amygdala and lateral septum and receive convergent afferents from several parts of the brain, including the mesencephalic dorsal raphe nucleus (Steinbusch, 1981). Endogenous 5-HT, as well as synapses immunoreactive for 5-HT can be found in the VTA (Hervē et al, 1987). These 5-HT fibres contact dendrites of cells that may or may not contain TH, the DA (and NA) synthetic enzyme (Hervē et al, 1987). *In Vitro* electrophysiological studies indicate that 5-HT, via the stimulation of 5-HT_2 receptors, increases the firing of a large proportion of DA cells located in the VTA (Pessia et al, 1994). The overall effects, however are more complex, since indirect changes in DA cell-firing can result from 5-HT exciting or inhibiting local GABA-containing interneurones (Pessia et al, 1994). For example, Ugedo et al (1989) have found that the systemic administration of ritanserin, a 5-HT_2 antagonist, increases the firing rate of DA neurons. Thus, the discrepancy between the effects of 5-HT_2 agonists and antagonists, which both increase DA cell-firing emphasises that complex neuronal loops interact when compounds are injected systemically.

Ferre and Artigas (1993) report that stimulating D_2 receptors increases the local concentration of 5-HT. This leads to the activation of somatodendritic raphe nuclei (RN) 5-HT_{1A} autoreceptors thus reducing 5-HT release in the striatum. Several 5-HT receptor subtypes (e.g. 5-$HT_{1B/1D}$, 5-HT_2 , 5-HT_3) are found in high densities in DA-containing structures (Hoyer, 1990). Several authors (Benloucif & Galloway, 1991;

Chen et al, 1991) have reported 5-HT mediated regulation of mesencephalic dopaminergic systems at somatodendritic and terminal regions.

The above findings suggest that 5-HT neurons exert both direct and indirect, excitatory and inhibitory, controls on DA neurons. It is possible that 5-HT_2 receptors regulate, through indirect neuronal loops, the activity of DA cells controlling DA release. There is some indication that NA neurons may contribute to the link between the 5-HT and DA circuits (Figure 1.9; see Tassin et al, 2000).

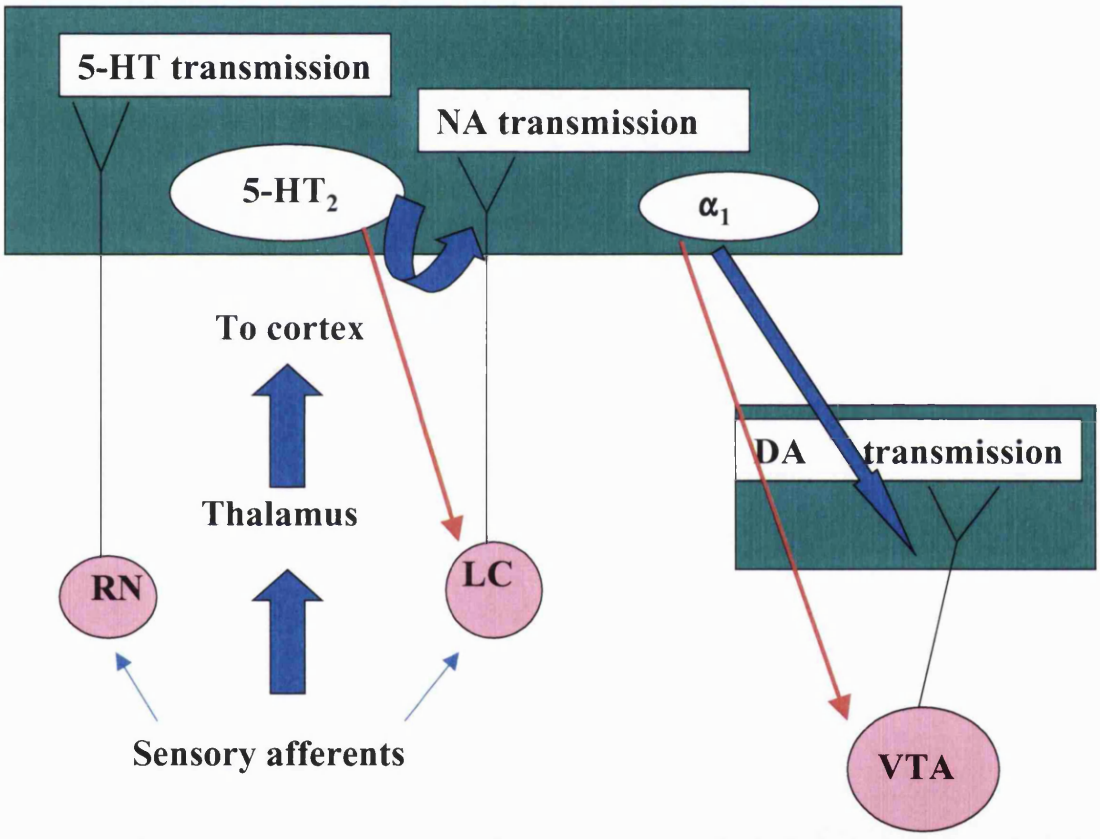


Figure 1.9 Schematic diagram of the monoaminergic cascade induced by ADs (Adapted from Tassin et al, 2000).

The reactivation of the 5-HT transmission by ADs facilitates the stimulation of 5-HT_2 receptors and restores the sensitivity of NA neurons to sensory stimuli. The NA neurons, via the stimulation of cortical α_1 receptors, may then enable a functional DA subcortical transmission. RN, raphe nuclei; LC, locus coeruleus; VTA, ventral tegemental area.

1.13 Depression and the glutamatergic system

1.13.1 The glutamatergic system

Glutamate is a non-essential amino acid and is the major excitatory neurotransmitter in the mammalian CNS. It is synthesised either de novo from glucose via the Krebs cycle, or from glutamine which is taken up into the nerve terminal after synthesis in glia from previously released glutamate. Its occurrence in the CNS is ubiquitous and at high levels (Fonnum, 1984).

A high affinity Na^+-coupled glutamate transporter found on the presynaptic nerve terminal and on glial cells is responsible for terminating the action of synaptic glutamate. The glutamate that is transported into the glial cells is converted back to glutamine by glutamine synthetase and is then transported into the nerve terminal to be converted back to glutamate.

Glutamate mediates its actions via activation of two major classes of glutamate receptors termed ionotropic and metabotropic receptors which are named on the basis of extensive studies using selective agonists (Cotman et al., 1989). The ionotropic receptors are further subdivided into N-methyl-D-aspartic acid (NMDA), amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and kainate receptors on account of the affinity of the ligands that were used initially to characterise them (Refer to table 1.6*)*.

Since it has not been possible to evince any monoamine related adaptive change that is uniform across all antidepressant therapies, recent research has focussed on adaptive changes beyond the monoaminergic systems. There is now an emerging body of evidence implicating a pivotal role for the glutamatergic system in the therapeutic action of antidepressant drugs and, by implication, in the pathogenesis of depression (reviewed by Skolnick et al, 1996).

1.13.2 NMDA receptors

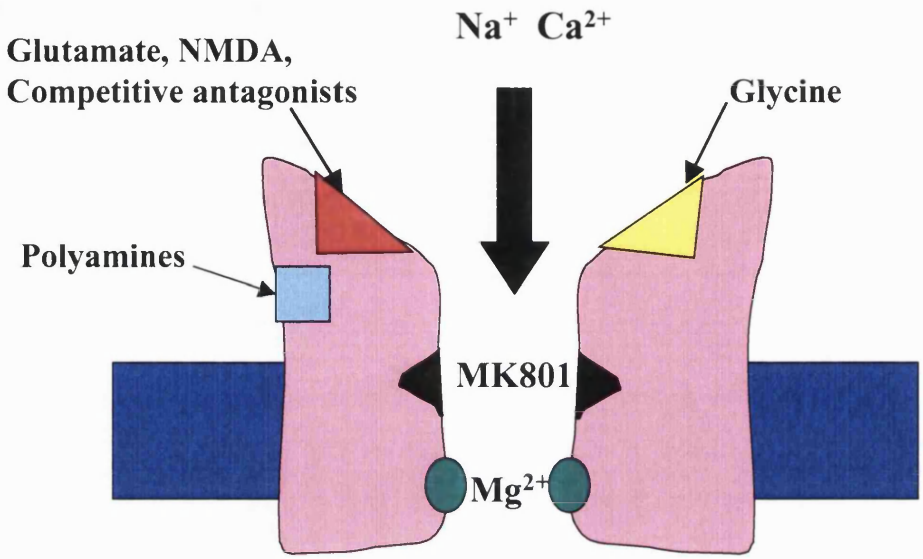


Figure 1.10 Diagram of NMDA receptor in neuronal membrane, shown with binding sites.

1.13.2.1 Pharmacology

As mentioned above, glutamate mediates its actions via activation of two major classes of glutamate receptors, ionotropic and metabotropic receptors. The ionotropic class have been subdivided into NMDA, AMPA and Kainate (Refer to table 1.4).

The NMDA receptor has multiple sites whereby ligands can bind to their active site and modulate the receptors ion channel properties (Figure 1.10 above). Activation of the receptor occurs when endogenous glutamate binds to its site in addition to strychnine-insensitive glycine acting as a co-agonist binding to its allosteric domain. Both these sites must be occupied before receptor activation can occur (Kleckner and Dingledine, 1988). If both criteria are fulfilled, in addition to the membrane potential being sufficiently depolarised from its resting state of ~-70 mV, the voltage-operated ion channel blockade governed by magnesium ions is removed allowing the free passage of both calcium and sodium cations into the cell (Foster and Wong, 1987). Over-stimulation of glutamate receptors has been implicated in neurodegeneration with levels of intracellular calcium being raised to such a level that cell death ultimately occurs. Blockade of the glutamate recognition site can be achieved following the synthesis of

ligands such as CGP40116 (Sills et al, 1991), AP5 (Watkins et al, 1990), AP7 (Watkins and Olverman, 1987) and CPP as can the allosteric site by action of drugs such as (+)-HA 966.

In addition, the NMDA receptor can be modulated by a polyamine regulatory site (Williams et al, 1991) with agonists such as spermidine and antagonists such as ifenprodil and eliprodil. The polyamine site has generated much interest in recent years after it was shown that this class of drug aids in preventing the widespread excitotoxicity that follows an insult in certain neurodegenerative conditions such as cerebral ischaemia (Scott et al, 1993).

Within the ion channel itself there are sites where drugs can act to mimic the magnesium blockade that exists under conditions when the receptor is physiologically quiescent. Drugs which act within the ion channel include MK801, PCP, ketamine, dextromethorphan, budipine, memantine and amantadine (Starr, 1995). Drugs which act by blocking the ion channel are termed 'use-dependent' blockers because they can only act once the receptors are stimulated and the endogenous magnesium block is removed. MK801 and PCP are experimental agents, while drugs such as amantadine and budipine are now employed clinically.

1.13.3 Molecular biology

The NMDA receptor has been characterised electrophysiologically in Xenopus oocytes and it is now apparent that multiple receptor subtypes exist with unique biochemical and pharmacological properties (Foster and Fagg, 1987). Each of these subtypes is hetero-oligomeric in structure and cloning studies have demonstrated the existence of at least one of eight NMDAR1 subunits (NMDAR1A-H) (Moriyoshi et al., 1991; Yamazaki et al., 1992) and at least one of four NMDAR2 subunits (NMDAR2A-D) (Kutsuwada et al., 1992) in each receptor-ion channel complex. NMDAR1 is required for receptor activation and unlike the NMDAR2 subunits, has the capability to form functional homomeric receptors in expression systems (Yamazaki et al., 1992), albeit resulting in lower agonist-induced current flow when compared to the native heteromeric form.

Several potential configurations for the arrangement of subunits have been suggested, but for optimum channel activation the binding of two glutamate molecules and two

glycine molecules is required (Clements and Westbrook, 1991). This is consistent with a tetrameric configuration of two NR1 and two NR2 subunits. Thus for a functional ion channel to be formed, there must be a pairing of NR1 and NR2 subunits. Function is characterised by a high Ca^{2+} conductance (Boyer et al, 1998). Thus, overactivation of the channel results in a sustained rise in the cytosolic Ca^{2+} concentration: this triggers neurotoxicity. Ideally, the prevention of elevated Ca^{2+} levels following NMDA receptor activation may therefore limit the neuropathological toxicity.

NMDA receptor subunits are differentially expressed throughout the CNS (Monyer et al, 1994). The mRNA for the NR1 and NR2A subunits are ubiqitously expressed throughout the adult brain. The NR2 subunit mRNA expression has more restricted, region-specific distribution (Boyer et al, 1998). NR2B subunit expression is restricted to forebrain regions such as the cortex, hippocampus and the striatum while the NR2C subunit is expressed in the cerebellum. Finally, the NR2D subunit is restricted to the midbrain regions.

Receptor Type	Distribution/Function
Ionotropic **NMDA**	• Widely distributed in the CNS-enriched in hippocampus and cerebral cortex. • Usually recognised as a slow component in repetitive activity generated primarily by non-NMDA receptors. • Important in synaptic plasticity
AMPA	• Widespread in the CNS. Parallel distribution to NMDA receptors. • Involved in the generation of fast component of EPSPs in many excitatory pathways.
Kainate	• Concentrated in a few specific areas of CNS, complementary to NMDA/AMPA distribution (eg hippocampus). • Difficult to distinguish from AMPA receptors pharmacologically due to nonspecificity of kainate in electrophysiological experiments. • Present specifically (in absence of AMPA receptors) on dorsal root C fibres and dorsal root ganglion.
Metabotropic **ACPD**	• Linked to IP_3 formation. • Activated by glutamate, quisqualate, ibotenate and ACPD but not by NMDA, AMPA or kainate. • Not antagonized by NMDA or non-NMDA antagonists but sensitive to pertussis toxin. • Possibly involved in developmental plasticity.

Table 1.4 Distribution and function of excitatory amino acid receptors in the mammalian CNS.

1.13.4 NMDA receptor antagonists

Antagonists that act on the NMDA receptor can either be classified as:

- *Competitive.* These antagonists act on the glutamate recognition site. Examples are CGP 40116, 2-amino-5-phoshopentanoic acid (D-AP5) and CPP.
- *Non Competitive.* These antagonists can either be *channel blockers* such as MK-801 (potent channel blocker) and amantadine and memantine (weak channel blocker) or *glycine site antagonists* such as HA-966 and D-cycloserine.

Excessive release of glutamate leads to the over-activation of the NMDA receptor and neurotoxicity (McCulloch, 1992). Thus, NMDA antagonists could reduce or prevent this neurotoxicity. Early competitive antagonists were highly polar and were poorly absorbed across the BBB when given peripherally. The channel blockers e.g. dizocilpine (MK801) and PCP penetrate easily into the CNS but are not free from undesirable side effects such as psychotomimetic effects (Tricklebank et al, 1989) and cardiovascular effects (Lewis et al, 1989). NMDA antagonists which act on excitatory amino acid receptors, e.g. NMDA receptors, have few peripheral side effects as their receptors are primarily located in the CNS, unlike other transmitter systems e.g. serotonergic and noradrenergic neurons. However, there is some evidence for the presence of excitatory amino acid receptors in the PNS (Bertrand et al, 1992).

Because of the NMDA antagonists having therapeutic potential but having a major disadvantage of their side effects, they have only successfully been used as neuroprotectants in animal models of neurodegeneration.

1.14 NMDA and depression

During the past decade several important studies have implicated NMDA receptors in the aetiology of depression. This was initially supported by findings that functional antagonists of the NMDA receptor mimic the effects of clinically effective antidepressant drugs in a preclinical animal model predictive of antidepressant activity (Trullas and Skolnick, 1990). Subsequently it was shown that chronic treatment with NMDA antagonists induces antidepressant activity in putative animal models of depression (Papp and Moryl, 1994). Additional evidence supporting a role for NMDA receptors in the mechanism of antidepressant action follows from the observation that

there is an important interaction between the glutamatergic and the monoaminergic systems in the brain (Pallotta et al., 1998;1999). The therapeutic relevance of this interaction is underscored by the finding that chronic treatment with NMDA antagonists produces a down-regulation of cortical β-adrenoreceptors comparable to that produced by chronic treatment with the TCA imipramine (Paul et al., 1992). The role of NMDA receptors in the aetiology of depression is also supported by post-mortem data, showing a glutamatergic dysfunction in the frontal cortex of untreated depressed suicide victims (Nowak et al, 1995). Finally, Nowak et al., (1993) have shown that chronic treatment with imipramine alters ligand binding to the NMDA receptor. The altered ligand binding would be expected to dampen NMDA functioning and is manifest as; (i) a reduced potency of glycine to inhibit [^{3}H] 5,7-dichlorokynurenic acid (DCKA) to strychnine-insensitive glycine receptors; (ii) a diminution in the number of glycine displaceable [^{3}H]CGP 39653 binding sites and (iii) a reduction in basal [^{3}H]MK-801 binding that was reversed by the addition of positive modulators such as glycine and glutamate.

The former observations support the role of NMDA receptors in the mechanism of action of antidepressants. These studies have led to the hypothesis that conventional antidepressant drug therapies aimed at altering the efficacy of monoaminergic systems ultimately exert their therapeutic effect by altering the efficacy of the glutamatergic system. Further evidence in support of this hypothesis came when Paul and co-workers (1994) showed that chronic (14 days) but not acute (1 day) treatment with 17 out of 18 clinically active antidepressants were able to reduce DCKA binding. It is still difficult to determine whether these effects form the basis of antidepressant action or are merely a secondary side effect of antidepressant drugs. However, it is clear that these neurochemical effects have a superior indicative value of antidepressant activity compared to β-adrenoreceptor down-regulation.

1.14.1 Molecular evidence for NMDAR involvement in depression

Based on recombinant DNA studies it has become clear that subunit composition plays a crucial role in determining the pharmacological and physiological properties of ligand gated ion channels, such as the NMDA receptor (Monaghan et al, 1989). Recently, *in situ* hybridization studies have revealed that chronic treatment with the antidepressants imipramine and citalopram produces region-specific and treatment-specific changes in

mRNA levels encoding NMDA receptor protein subunits (Boyer et al., 1998). These observations form the basis for alternative hypothesis for mechanisms underlying the adaptive changes in NMDA receptor functioning.

Although it is clear that chronic antidepressant treatment with compounds from each major class of antidepressants causes an adaptation of NMDA functioning, it is still unclear what the functional consequences of these adaptive changes are and how these changes are connected to the monoaminergic systems and depression.

1.14.2 NMDA, monoamines and intracellular second messengers

The exact mechanisms by which antidepressants cause an adaptive change in the functioning of the NMDA receptor are as yet unclear. However, since conventional antidepressants modulate monoaminergic transmission, it has been hypothesised that the adaptations in NMDA functioning are secondary to antidepressant actions at monoaminergic terminals (Skolnick, 1999). Direct evidence for this theory comes from experiments showing that lesions in the noradrenergic system block the adaptive changes caused by chronic desipramine treatment (Harkin et al., 2000). Additionally, there is considerable evidence suggesting that the monoaminergic and the glutamatergic systems are strongly synaptically linked to one another (Pallotta et al, 1999).

Most currently used antidepressants, when administered chronically, increase synaptic levels of monoamines with concomitant stimulation of adenylyl cyclase via G-protein coupled receptors (e.g. β-adrenergic, 5-$HT_{4,6,7}$) (for review see Duman et al, 1997). The resulting increased formation of cAMP leads to activation of cAMP dependent protein kinase (PKA), with subsequent phosphorylation of substrate proteins. A possible substrate for PKA is the cAMP response element binding protein (CREB), which is capable of regulating gene expression (Duman et al, 1997). Phosphorylation of CREB dramatically increases its ability to regulate gene expression. CREB is capable of up-regulating the expression of specific genes containing the cAMP response element, one of which is the brain-derived neurotrophic factor (BDNF) (Nibuya et al., 1995). It is demonstrated that the expression of BDNF is increased after chronic but not acute treatment with antidepressants from several different classes (Nibuya et al., 1995, 1996). This effect is believed to be mediated by CREB, since there is also an elevation of CREB mRNA and protein after chronic treatment with antidepressants (Nibuya et al,

1995). Further evidence that the cAMP system mediates the induction of BDNF is provided by the observation that inhibitors of phosphodiesterase (PDE) IV, a key enzyme responsible for the breakdown of cAMP, increase the expression of both CREB and BDNF in rat hippocampus (Fujimaki et al., 2000).

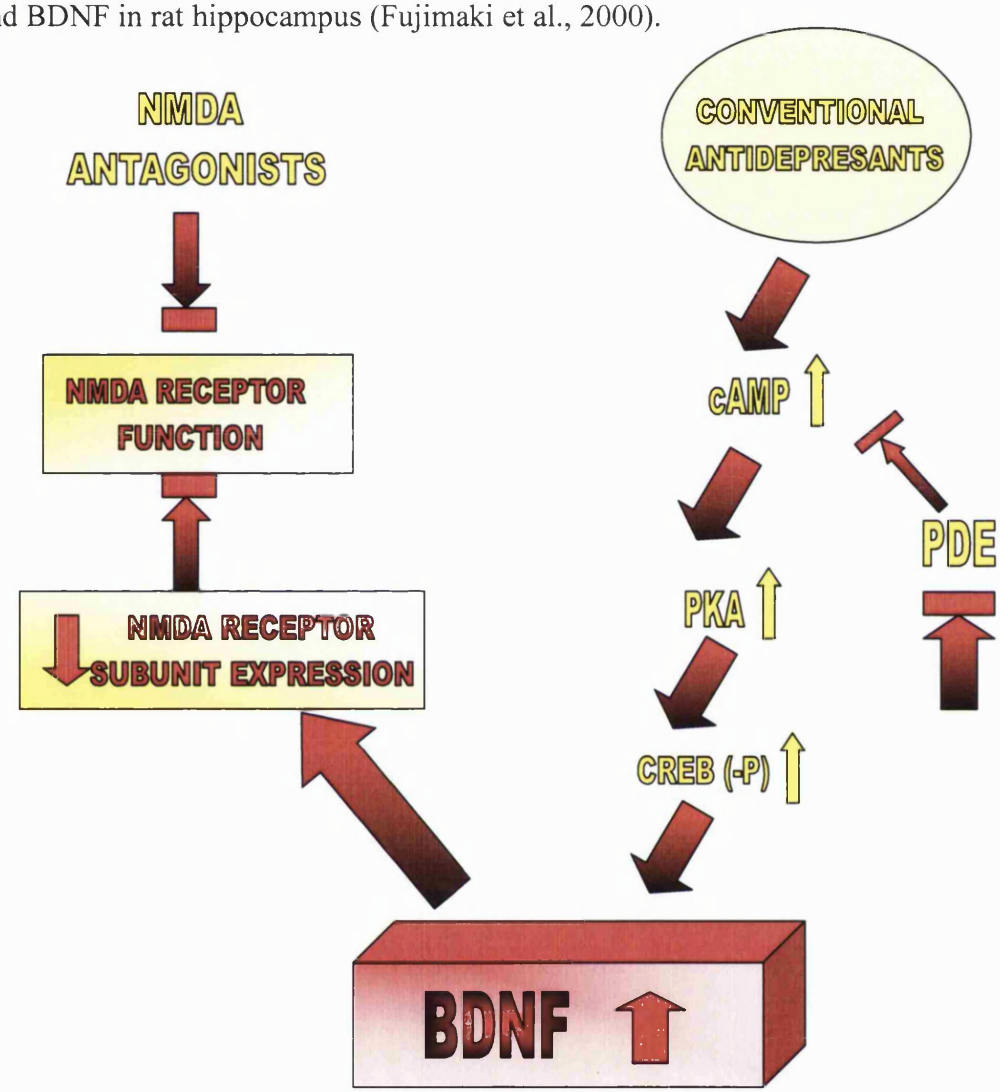


Figure 1.11 (Adapted from Skolnick, 1999)

The relationship between increased BDNF levels and alterations in NMDA functioning has been explored by a series of pivotal experiments performed by Brandoli et al (1998). They have shown that chronic, but not acute, infusion of BDNF into the rat brain reduces the expression of NMDA receptor protein, to an extent comparable to that seen after chronic imipramine treatment (Brandoli et al, 1998). Thus, in addition to its neurotrophic effect, BDNF is able to dampen NMDA receptor mediated transmission. This is confirmed by the observation that chronic BDNF attenuates the intracellular Ca^{2+} response to NMDA treatment (Brandoli et al., 1998) an effect that is readily demonstrated by treatment with an NMDA antagonist.

The aforementioned observations support the hypothesis that the alterations in NMDA functioning, which occur after chronic antidepressant treatment, are elicited by the primary rise in synaptic concentrations of monoamines. In addition they form a plausible explanation for the molecular mechanisms underlying this adaptation. Altogether, it is evident that future research will provide tools to develop new treatment strategies aimed at intervention beyond the monoaminergic systems, thereby reducing side effects and therapeutic lag as well as increasing the efficacy of treatment.

1.15 Principle drugs used in this study: Mechanisms of action

1.15.1 Paroxetine

Paroxetine (commercially known as Seroxat®, Paxil®), a phenyl-piperidine derivative (Figure 1.12), belongs to the AD class of SSRIs and it has been successfully used in the treatment of depression for over a decade. Paroxetine proves to be an effective treatment for anxiety disorders [eg. panic disorder, obsessive compulsive disorder (OCD), etc.]. However this property is not unique for paroxetine but is a general feature of SSRIs. Paroxetine is a more potent inhibitor of 5-HT reuptake *in vitro* than most SSRIs, having a K_i of 1.1, 350 and 2000 nM for the serotonin, noradrenaline and dopamine transporters, respectively (Thomas et al., 1987). In contrast to the TCAs, paroxetine displays low affinity for monoamine and histamine H_1 receptors (Thomas et al, 1987; Gunasekara et al, 1998). However, the relative affinity for the cholinergic muscarinic receptor is greater than other SSRIs. The relative low affinity of paroxetine for neurotransmitter receptors is believed to account for the superior side-effect profile compared to TCAs and MAOIs.

Numerous clinical trials have compared paroxetine with other antidepressants from different classes (reviewed by Gunasekara et al, 1998, Fujishiro et al, 2002). Paroxetine was found to be superior to fluoxetine on measures of agitation and psychic anxiety after only one week of treatment (Chouinard et al, 1999). The antidepressant potency of paroxetine is equivalent to that of TCAs and other SSRIs (Gunasekara et al, 1998; Feighner et al , 1993). The most common adverse effects occurring during treatment are nausea, headache, somnolence and anticholinergic effects such as dry mouth and constipation. However, most of the adverse effects associated with paroxetine tend to be mild and transient and it is generally well tolerated by elderly patients (Gunasekara et

al, 1998). Having a long half-life of approximately 24 hours in human, with no active metabolites, paroxetine is well suited for a once daily treatment regime of 40 mg/day.

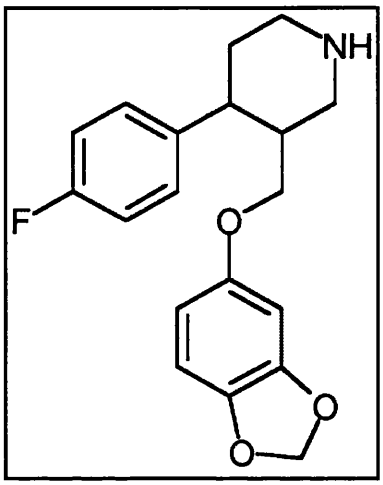


Figure 1.12 Chemical structure of paroxetine

1.15.2 Clomipramine

Clomipramine (Figure 1.13) is a tricyclic antidepressant that inhibits NA and 5-HT re-uptake and causes β, α_2 and 5-HT_2 receptor downregulation. After oral administration, it is absorbed in 2-3 hours, undergoes hepatic metabolisation to demethylclomipramine (Fujita et al, 1991), which is pharmacologically active with a higher affinity for the NA transporter than the parent drug (Benfield et al, 1980). Its plasma half life is about 20-50 hours and it is excreted in the urine and faeces. The most important side effects are postural hypotension, anticholinergic effects, weight gain, sedation and cardiotoxic effects (Gareri et al, 2000).

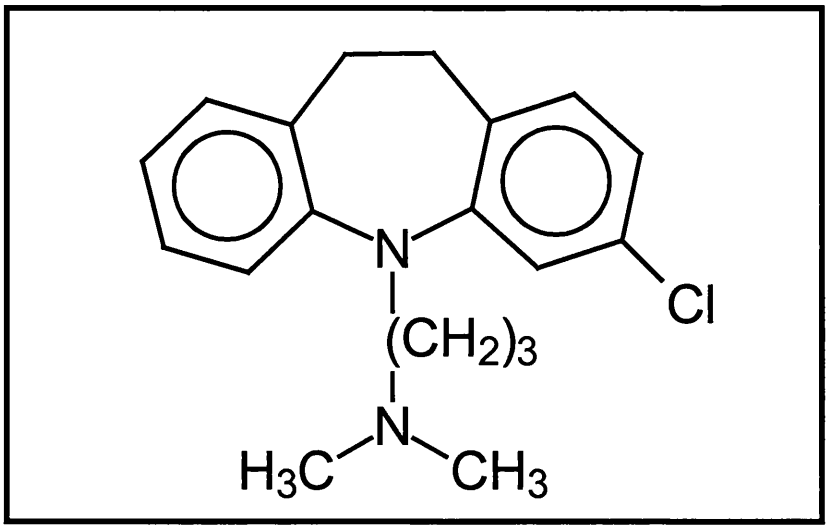


Figure 1.13 Chemical structure of clomipramine

1.16 Aims of this study

The psychopathology and treatment of depression is outlined in the introduction above along with evidence supporting a crucial role for the NMDA receptor in this disorder. Whatever AD is used to treat clinical depression, there is in general a 3-week delay or 'therapeutic lag' before a clear therapeutic effect is obtained.

Information on the effects of chronic ADs on NMDA receptors stems from the work of Skolnick and Colleagues (1994) using radioligand binding assays. This group observed that chronic, but not acute treatment with a range of antidepressants including SSRIs decreases the binding affinity of NMDA receptors (Paul et al, 1994). The purpose of this present study was to investigate the effects of acute, subchronic (7 days) and chronic (21 days) paroxetine or clomipramine treatment on NMDA-evoked DA and 5-HT efflux in the frontal cortex and their respective metabolites, DOPAC and 5-HIAA using *in vivo* microdialysis. This was prompted by the work of Paul and colleagues (1994) who report that AD-induced properties of the NMDA receptor complex require 7 to 14 days of treatment and persist for 5 to 10 days after the cessation of treatment. The effects on AD cessation on NMDA-evoked monoamine changes were also investigated.

As it has been suggested that chronic AD treatments can affect NMDA receptor function in the brain, this study was aimed to answer the following questions:

- Do ADs of different classes e.g. SSRI and TCA, have similar effects on extracellular DA/5-HT efflux in the frontal cortex when given acutely, subchronically and chronically? (Chapter 3 and 4)
- What are the effects of acute, subchronic (7-days) and chronic (21-days) treatment on local NMDA-evoked monoamine efflux in the frontal cortex? (Chapter 3 and 4)
- Are these changes reversed following AD cessation? (Chapter 3 and 4)
- What are the effects of polypharmacy studies involving the administration of NMDA antagonists as adjuncts to paroxetine? (Chapter 5)

Chapter 2

Materials and Methods

2.0 Materials and Methods

2.1 Determining levels/concentrations of brain extracellular fluids: In Vivo methods

Over the past 50 years, there have been many methods developed to monitor monoamine release and uptake. The earliest techniques relied upon measurement of tissue monoamine content, monoamine synthesis or measurement of monoamine metabolites to indicate the underlying changes in neurotransmission. However, these techniques lacked accuracy as 'actual' extracellular monoamine concentrations could only be inferred and not measured directly. Subsequent techniques developed to study brain biochemistry in intact (normal) and neuropathological states, permit the direct measurement of the concentration of extracellular monoamines. The main aim of the criteria used to develop these techniques are as follows:

- Reliable measurement of neurotransmitter and metabolite levels in specific brain regions with sensitivity and selectivity.
- Non-invasive: Normal tissue physical structure and biochemical states (metabolism) remain unchanged during and following application of the technique.
- Determine concentration versus time profiles within individual animals.

2.2 *In Vivo* sampling techniques

One of the first established *in vivo* sampling techniques used was the cortical cup (Macintosh and Oborin, 1953). This technique involved drilling a hole in the skull, exposing the brain surface and then placing a cylinder, or 'cup' containing artificial cerebrospinal fluid (aCSF) on the surface of the cortex. Monoamines and other compounds of interest pass from the surface of the cortex into the aCSF in the cup which can then be sampled and analysed (e.g. Milby et al, 1981). The advantages of this technique are that it can be used in awake rats and does not involve tissue penetration. However, the main drawback is that sampling is only possible from the surface of the cortex, i.e. other internal brain structures are inaccessible. The use of

the push-pull cannula, originally designed by Gaddum (1961), involved the implantation of two concentric tubes in or near the brain region of interest. Sampling is accompanied by perfusing perfusion fluid at a high flow rate of 10-30 μL/min via a tube into the tissue and withdrawing samples from the extracellular fluid via another tube (Gaddum, 1961; Reisine et al, 1984). This method has fallen out of favour, except for neuropeptide monitoring, because of the tissue damage caused by the perfusion of relatively large volumes directly into the tissue. However, there have been technical improvements in the design of the push-pull system such as miniaturization of the microbore cannuale which have minimised associated tissue damage (for review, see Myers et al, 1997).

The problems associated with the push-pull technique, such as perfusion with a high flow rate and anatomical problems associated with the use of large push-pull needles placed in deep structures of the brain, lead to significant improvements and the development of the microdialysis technique. Bito et al (1966) were the first to implant 'dialysis sacs' containing dextran into the brain of dogs and, 10 weeks later, analysed their amino acid content. In 1972, Delgado and colleagues constructed a cannula in which its tip was covered with a porous semipermeable membrane. This dialysis bag or 'dialytrode' was later replaced by a hollow fiber of dialysis membrane (Delgado et al, 1984). Thus, the microdialysis probe was applied to experiments identical in purpose to those utilising a push-pull cannula device. Microdialysis involves the insertion of a microdialysis probe (Figure 2.4) into a specific area of the brain. Substances around the semipermeable part of the probe will diffuse into or out of the perfusate (i.e. aCSF), down their concentration gradient. Subsequently the dialysate is collected for analysis.

Microdialysis has become a widely used method for the analysis of the extracellular fluid composition in various organs (Benveniste & Huttemeier, 1990). It has found numerous applications monitoring the release of neurotransmitters in the brain of anaesthetised or freely moving animals. Microdialysis is especially suited for monitoring monoamines, DA, NA and 5-HT, which can be separated and quantified using highly sensitive assay procedures such as HPLC with electrochemical detection (HPLC-ECD) (Martin-Fardon et al, 1997). Microdialysis enables the

modification of the extracellular environment by drugs that can enter the CNS, either by infusion through the microdialysis probe or administered systemically. Not only is microdialysis used to measure neurotransmitter efflux but it can also be used as a drug delivery system and as a pharmacokinetic tool.

The microdialysis technique has a number of advantages over previous experimental procedures: (for review, see de Lange et al, 1997) (1) distinct brain regions can be examined; (2) there is only modest tissue trauma; (3) concentration versus time profiles can be obtained from (freely moving) individual animals; (4) a wide range of substances can be analysed. It also has some associated disadvantages. For instance, (1) it requires sensitive analytical methods to detect small concentrations; (2) and requires *in vivo* recovery of the drug to calculate true concentrations in the extracellular fluid of the surrounding tissue (Refer Section 2.7). The advantages and disadvantages of microdialysis compared to other *in vivo* techniques can be summarised in table 2.1.

Another *in vivo* method for measuring the concentration of central monoamines which is a more recent and entirely different approach to estimate the concentrations of electroactive compounds in the extracellular space is *in vivo* voltammetry (for review, see Stamford, 1985). Here, a three electrode system (consisting of a carbon working, reference and auxillary electrode) is implanted in a discrete brain region. By applying a potential difference, between the working (carbon) and reference (Ag/AgCl) electrode, oxidation of the electroactive species occurs. This results in a concentration dependent flow of electrons which can be detected with the auxillary (usually platinum, Pt) electrode. The advantage of voltammetry is the high time resolution (ms) and this method is a promising tool for the study of continuous changes in transmitter release and metabolism. However, the disadvantage is that detection is performed *in situ*, without prior separation of solutes, so it can be difficult to identify the compound peaks as different compounds can oxidise at similar potentials [e.g. 5-HT is oxidised at the same potential as its metabolite, 5-HIAA (Cespuglio et al, 1981)].

2.3 Microdialysis: What is being measured?

Microdialysis is especially adapted for measuring changes in the extracellular concentration of particular substances within the brain such as monoamines. Thus microdialysis does not measure the concentration of monoamine in the synapse, but rather the amount of monoamine which overflows into the ECF. Monoamines in the ECF diffuse down their concentration gradient through the dialysis membrane into the probe. Thus, the concentrations of monoamines measured reflect the combined effect of synthesis, release, uptake and metabolism of monoamines. Thus the term monoamine 'release' has never been correct and has subsequently been modified to reflect these simultaneous events and is now termed monoamine 'efflux'.

Table 2.1 The advantages and disadvantages of microdialysis in comparison to other *in vivo* techniques

Technique	Advantages	Disadvantages
***In Vivo* Voltammetry**	• Examination of all brain regions • Time resolution 1 ms-sec	• Only detects oxidisable compounds • Selectivity poor without prior HPLC analysis
Microdialysis	• Regional specificity allowing distinct brain regions to be examined. • Blood brain barrier (BBB) remains intact following implantation of the microdialysis probe. • Modest tissue trauma • Wide range of substances can be analysed • Freely-moving animals used	• Highly sensitive, specific assay required. • Anatomical resolution limited by the external diameter of the membrane (250-350μm) • Transient changes missed as sampling times extended.
Push-Pull Cannula	• Examination of all brain tissues • Freely-moving animals used • Blood brain barrier (BBB) remains intact following implantation	• Enzymatic degradation of collected compounds • Tissue trauma following implantation due to size of cannula (O.D. >1mm) and • Turbulance caused by high flow rates used
Cortical Cup	• No tissue penetration • Used in conscious animals	• Time resolution > 10 min • Only cortex can be analysed.

2.4 Animals used for dialysis experiments

Male albino Wistar rats (Bantin & Kingman Ltd., Hull, UK) weighing 220-250 g were used in all the experiments. The animals were initially housed in groups of six at 22°C under fluorescent lighting from 07:00-17:00 h and allowed free access to food and water.

Once the animals had undergone stereotaxic surgery, they were individually housed in clear Perspex cages wrapped in paper towel to prevent post-operative hypothermia and they were observed until full recovery from anaesthesia was achieved. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act, 1986.

2.5 Stereotaxic surgery and probe implantation

In the dialysis experiments, the probes were unilaterally implanted in the rat frontal cortex. The microdialysis probes (see section 2.1.4) were implanted under isoflurane anaesthesia (4% v/v in O_2 for induction and 2.5% v/v in O_2 for maintenance) using a stereotaxic frame (David Kopf, USA). Once the rat was secured in the stereotaxic frame, a medial incision was made in the scalp of the rat. The skin was retracted using crocodile clips to expose the skull surface. The bregma was marked as a reference point to determine the location of the frontal cortex using the stereotaxic atlas of the rat brain (Paxinos and Watson, 1986). The co-ordinates for the frontal cortex were in mm: A +3.2 mm from bregma; LM ±3.0 mm from the midline and -5.5 mm ventrally. A dental drill was used to drill burr holes at the appropriate stereotaxic co-ordinates exposing the dura, which was scraped off with the tip of a sterile hypodermic needle before probe implantation. An additional burr hole was drilled in the skull several mm anterior to lambda to insert a screw which was used to anchor the dental acrylic (Duralay) in which the probe was sealed. Additional acrylic was applied to the shaft of the probe to aid durability.

The microdialysis probes were perfused with deionised water at a rate of 0.8 μL/min using a microinfusion pump (Harvard Apparatus, syringe infusion pump 22) and a

500 μL gas-tight syringe. This was carried out in order to limit compression of the dialysis membrane by the surrounding tissue and to prevent membrane collapse once the probe was in place. The perfusion of deionised water during implantation was to prevent overnight blockage of the probe that can occur with aCSF, due to the formation of salt crystals. It is not believed that any excess oedema will result from this process due to the small volumes involved as well as the recovery time allowed before the initiation of dialysis. The probes were slowly lowered vertically into the frontal cortex and secured using dental acrylic. Once secure, the inlet and outlet of the probe were sealed using dental acrylic to prevent the formation of air blocks within the probe.

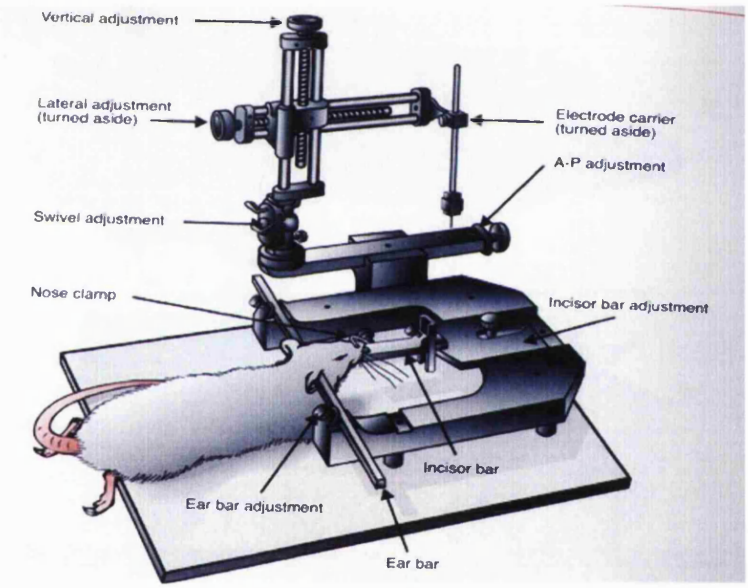


Figure 2.1 The stereotaxic frame [Replicated from The Laboratory Rat by Krinke (2000) with kind permission from *Academic Press*]

After completion of surgery, the animals were given 1mL/kg saline (0.5M) subcutaneously (s/c) to compensate for fluid losses during the operation period and placed individually in Perspex cages and allowed to recover. The rats were wrapped in paper towels to limit post-surgical hypothermia.

Animals were allowed an overnight postoperative recovery period of 18-24 hours prior to the start of the microdialysis experiments. This time period has proved to be essential as several studies have raised concerns about the time between probe

implantation and the start of experimental sampling. For instance, it is thought that acutely implanted

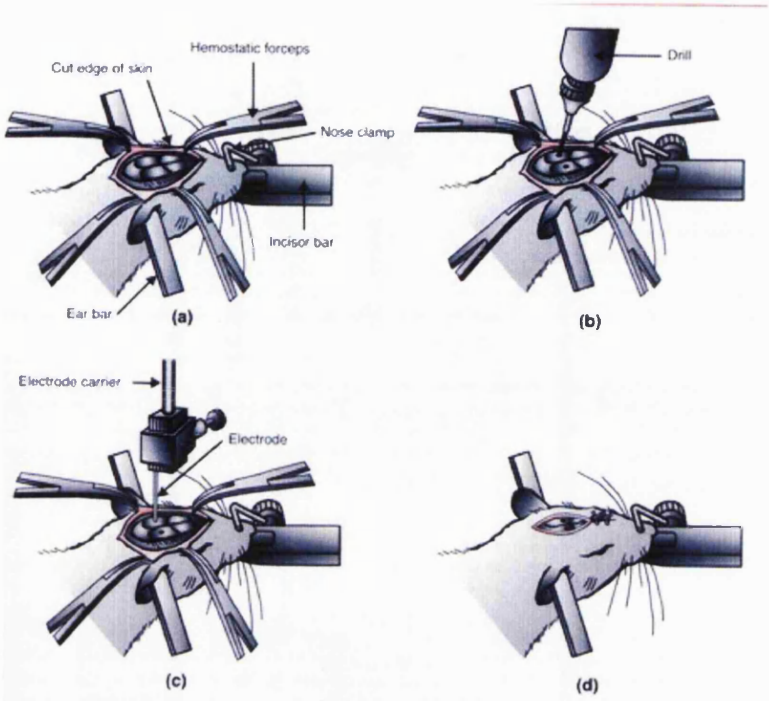


Figure 2.2 Stereotaxic surgery [Replicated from The Laboratory Rat by Krinke (2000) with kind permission from *Academic Press*]

microdialysis probes can destabilise the cerebral tissue surrounding the probe due to changes in cerebral blood flow and metabolism (Benveniste et al, 1987) and transient disruption of the BBB (Morgan et al, 1996). Thus, acutely implanted probes often recover excessive quantities of anylates shortly after implantation which do not reflect true extracellular basal levels of monoamines. Westerink and de Vries (1988) have shown that following acute probe implantation, the dialysate neurotransmitters recovered following acute probe implantation are largely derived from damaged nerve terminals as opposed to impulse-evoked release from neuronal stores. A study in this laboratory by Whitton et al (1994a) reports that after 18 hours of implantation, the monoamine levels return towards normal with DA and 5-HT efflux totally abolished by the Na^+ channel blocker tetrodotoxin (TTX).

After a period of 18-24 hours, the microdialysis probes were infused with artificial cerebrospinal fluid (aCSF) composed of (mM): NaCl 125; KCl 2.5; $CaCl_2$ 1.26 and $MgCl_2$ 1.18 (pH 7.0) through 30-50cm of pp10 polyethylene tubing. The aCSF was infused initially at 2.0 μL/min before and shortly after connection to the probe in order to remove any trapped air bubbles. FEP tubing (Carnegie Medicin, Sweden: 1.2 μL/100mm), which has a low internal volume was fitted to the probe output and the system was flushed with aCSF. This outlet tubing transported the dialysate to the collection vial. Subsequent perfusion throughout the experiment was carried out at 0.8 μL/min (this rate has been used previously in the lab with similar dialysis probes) for 1 hour before dialysates were collected to reach a stable baseline. Further dialysate samples were collected at 30 minute intervals for 2 hours to establish extracellular basal levels of monoamines. At this time point, drug(s) were infused through the probe or injected (i.p.) and further samples were taken every 30 minutes for 4 hours. Methodological differences between separate studies are described in detail in the appropriate results chapters.

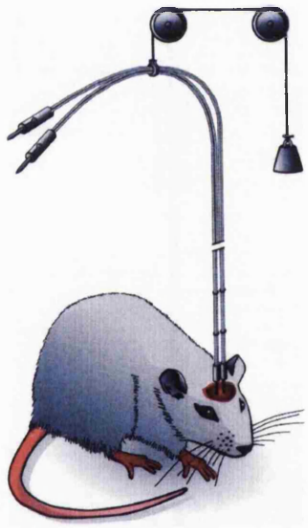


Figure 2.3 Microdialysis: The technique [Replicated from The Laboratory Rat by Krinke (2000) with kind permission from *Academic Press*]

2.6 Microdialysis probe construction

A binocular microscope was used as a visual aid. (For diagram of probe, refer to Figure 2.4). Two lengths of fused silica tubing (25mm) were inserted in a steel cannula (24 guage), which acted as the body of the probe. One length was fed through the cannula with several mm emerging from the opposite end, while the second piece of silica was inserted approximately half way through the cannula. Araldite adhesive (Ciba-Geigy) was used to attach the silica to the cannulae tip. After the araldite had fully dried (minimum 1 hour) lengths of polythene tubing (pp10 tubing, OD 0.68mm; Portex Ltd, UK) were pushed along the lengths of smaller cannulae which then were carefully pushed along the length of both portions of silica attached to the cannula. Again, a drop of araldite was used to secure the polythene tubing to the body of the probe. This results in a Y-shaped probe. After leaving the araldite to dry, the length of the silica which emerged from the opposite end of the cannula was trimmed to 4mm. A Cuprophan hollow fibre dialysis membrane, type f18, with a 0.2 mm O.D. and a 10kD MW cut-off point (Gambro, Hechingen, Germany) was placed over the trimmed silica and carefully inserted into the cannula (ensuring the membrane doesn't bend) until resistance was felt. The dialysis membrane was trimmed so that 1mm protruded from the end of the silica and was secured to the cannula with a minimal amount of araldite. To complete the construction of the probe, the open end of the dialysis membrane was sealed with araldite. The probe was then left overnight before it was used for implanting to ensure all the araldite had fully dried. Probes which failed to allow free passage of fluid throughout the entire length of the probe were discarded. Successful probes were retained in a moist atmosphere within a sealed container to eliminate dust and to prevent the membrane from drying out before implantation.

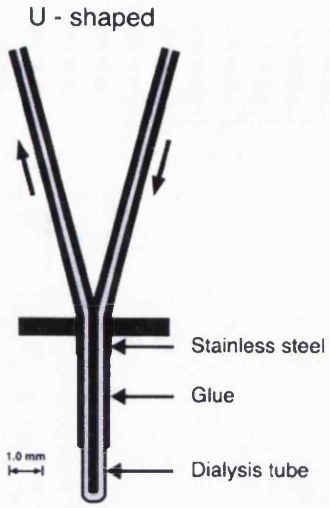


Figure 2.4 Microdialysis probe [Replicated from The Laboratory Rat by Krinke (2000) with kind permission from *Academic Press*]

2.7 The *in vitro* recovery of perfusate from constructed microdialysis probes

One of the most important questions in microdialysis is how to relate the dialysate concentrations to the true concentrations outside the probe i.e. to what extent is the compound of interest (monoamines in this thesis) recovered by the dialysate. Because of the constant flow of dialysate through the probe, the monoamine concentration in the probe will not be in equilibrium with the extracellular concentration. Thus *in vivo* and *in vitro* recovery rates differ. To overcome this problem, it is possible to estimate accurately the extracellular concentration of monoamines with the no-net-flux method by Lonnroth et al (1987). The principal of this method consists of perfusing increasing concentrations of the analysed substances through the probe. At lower concentrations in the perfusate than in the extracellular space, the diffusion gradient leads to an enrichment in the dialysate relative to the perfusate. Conversely, at higher concentrations in the perfusate, the diffusion gradient is inverted, and the amount of monoamine in the dialysate is diminished relative to the perfusate. By plotting the difference between the 'in' and 'out' concentrations against the 'in' concentrations, the exact extracellular concentration can be estimated at the no-net-flux point (where the line crosses the X-axis at the value where the 'in' concentration equals the 'extracellular' concentration. This technique has been mainly used to determine the concentration of DA and its

metabolite, DOPAC (Justice, 1993) but can be equally applied to other released compounds.

Ungerstedt (1991) suggested that there are several factors that can affect the recovery of perfusate.

Flow rate: The flow rate of the perfusion medium affects the recovery and the resolution of the measurements over time. Recovery varies inversely with flow rate. The inner volume of the inlet and outlet tubes creates a dead volume in the system that must be calculated in order to change the dialysate fractions at the right time point. It must be noted that when using a low perfusion flow rate, it may take a few minutes from the time the perfusate reaches the probe in the tissue and even longer until it reaches the collection vial. On the other hand, although a high flow rate will yield a greater perfusate volume to analyse using HPLC, it may also affect the pressure inside the probe. If this pressure is higher than in the surrounding ECF, net fluid transport over the membrane will occur. Thus a low enough flow rate that yields sufficient dialysate for analysis should be used.

Length and material of dialysis probe: An increase in the length of the dialysis membrane leads to an increase in the surface area of the dialysing surface, thus recovery increases linearly with length and surface area of the dialysis membrane. Tao and Hjorth (1992) clearly demonstrated the importance of the properties of the dialysis membrane. The dialysis membrane should be as inert as possible in order not to interfere with the passage of molecules.

Composition of the perfusion solution: Microdialysis perfusion medium is contiguous with the ECF and may therefore influence local ECF composition. The exact composition of the ECF in the brain is unknown and hence a number of approximations of perfusion medium compositions have been used in microdialysis experiments (Benveniste and Huttemeier, 1990).

There are 2 types of recovery:

- **Relative Recovery**: The solute concentration in the perfusate when it leaves the probe expressed relative to its concentration in the surrounding medium. Relative recovery *in vivo* remains constant as long as the perfusion conditions remain constant.
- **Absolute Recovery**: The total amount of a substance recovered during a set time period expressed in moles/L. Absolute recovery *in vivo* will vary depending on the production or release of the substance of interest.

To determine the 'real/absolute' concentrations of substances from the dialysis probe perfusate, the microdialysis probe must be calibrated before its use *in vivo.*

Before the start of the microdialysis experiments, *in vitro* recovery experiments were carried out in order to obtain an estimate of the extracellular concentrations of monoamines and their metabolites. This was done by connecting the probe to a syringe using a length of pp10 tubing and continuously perfusing the probe at a constant flow rate of 0.8μL/min. The perfusion fluid (aCSF) was identical to the bathing medium (aCSF) with the exception that the bathing medium contained the monoamine of interest. For the monoamines, a freshly prepared 1μM solution of DA or 5-HT was made up in the aCSF. To limit oxidation, L-cysteine (20mg/100ml) was added (Chai and Meltzer, 1992). The probes were inserted into the bathing medium and perfused for a minimum of 60 minutes before samples were collected at 30 minute intervals. Monoamine levels in the outflow were immediately determined using HPLC.

The relative recoveries for the monoamines and their metabolites were calculated using the relationship devised by Benveniste, (1989) (Refer to table 2.2).

Monoamine/Metabolite	***Mean In Vitro* recovery (%) n=5**
DA	**38 ± 4.9**
DOPAC	**30 ± 5.3**
5-HT	**27 ± 6.2**
5-HIAA	**31 ± 3.9**

Table 2.2 *In Vitro* recovery of monoamines and their metabolites from standard solutions.

$$\textit{In Vitro}\ \text{recovery} = C_{out}/C_{in}$$

C_{out} = substance concentration in the outflow

C_{in} = substance concentration in the bathing medium

The same procedure is then performed *in vivo* and the extracellular concentration of the substance can be calculated as:

$$C_I = C_{outflow}/\textit{In Vitro}\ \text{recovery}$$

C_I= true extracellular concentration

$C_{outflow}$=concentration of the substance in the *in vivo* outflow solution.

These relationships are based on the assumption that the conditions *in vitro* and *in vivo* are equivalent. Without doubt, this is not the case. Because of the constant flow of dialysate through the probe, the concentration of monoamine in the probe will not equilibrate with the extracellular concentration. We still assume the conditions to be approximately equal to allow us to make estimates of actual brain extracellular levels from microdialysis experiments.

2.8 HPLC-ElectroChemical Detection (ECD) parameters and hardware

A Spark-Holland refrigerated autosampler was connected to an Antech Intro electrochemical detector using a Vt03 flow cell with V_{cell} +625 mV, filtered to 5abu with the range set on 1nA/volt for full scale deflection. A C_{18} ODS 3 μm reverse phase column heated to 40°C was connected to a Bischoff compact HPLC pump (model no. 2250) with the flow rate set to 650 μL/min. Data capture was achieved and analysed by means of a computer equipped with ChromPerfect software. Mobile phase was not recirculated and was composed of the following (mM): sodium acetate 90, citric acid anhydrous 35, octane sulphonic acid 0.06 and EDTA 0.34 (all of high analytical grade (Fluka, Germany) with the addition of methanol (5.5% v/v) at pH 4.2.

2.9 Chromatograph calibration

Monoamine standards and their metabolites (DA, DOPAC, 5-HT and 5-HIAA) at a known concentration were used regularly to ensure consistent identification of the correct peaks following injection of dialysate samples.

Typical chromatograms showing the separation of the monoamines using HPLC-ECD are shown (see figure 2.6a/b). Typical calibration plots of the monoamines are shown in Figs 3a-3d. Thus, the DA, DOPAC, 5-HT and 5-HIAA content of dialysates were identified and quantified by comparing their respective elution times and peak heights with those obtained from the 'chromatograph calibration' reference standards. The detection limit for DA, DOPAC, 5-HT and 5-HIAA was approximately 1 fmol/10 μL sample. This sensitivity was approximately *19-fold* higher than the limit of detection for 5-HT to be measured without the addition of an uptake inhibitor in the perfusion medium.

2.10 Experimental drugs

The drugs and their suppliers that were used in this study are listed below:

Paroxetine hydrochloride (Glaxo-SmithKline, UK), CGP 40116 (Ciba-Geigy, UK), Ifenprodil tartrate (RBI, USA), NMDA, Amantadine hydrochloride, Clomipramine hydrochloride (Sigma, UK) and Budipine (Byk Gulden).

2.11 Experimental protocol

Chapter 3: On the day following probe implantation once spontaneous efflux was established, dialysate samples were collected every 30 minutes. Four consecutive basal samples (unless otherwise stated) were collected before administering test drugs. A further 8 post treatment samples (unless otherwise stated) were then collected.

In the first set of experiments, paroxetine (10 or 20 mg/kg) was dissolved in sterile water and systemically injected (i.p.) at 1 mL/kg of rat body weight. Control rats received an equivalent volume of vehicle (sterile water). Paroxetine, infused locally (90 minutes) into the frontal cortex, was dissolved in aCSF at a concentration of 100 μM. After 90 minutes infusion paroxetine was replaced with normal aCSF.

Clomipramine (10 or 20 mg/kg) was dissolved in 0.9% saline. Control rats received an equal volume of vehicle (saline). In experiments involving NMDA administration, drug was dissolved in aCSF at a concentration of 100 μM and infused into the frontal cortex.

In the second set of experiments, rats were treated acutely with paroxetine or clomipramine (10 mg/kg) i.p. or vehicle (0.9% saline) and after 30 minutes, rats were infused with NMDA (100 μM).

In the subchronic and chronic studies, rats were treated i.p. once daily with paroxetine or clomipramine (10 mg/kg) for a period of 7, 14 or 21 days. The final

dose of paroxetine or clomipramine was administered the day prior to dialysis. NMDA (100 μM) was then infused following collection of the 4th sample.

For the 'drug holiday' experiments, rats were treated once daily i.p. with paroxetine (10 mg/kg) for 21 days or clomipramine (10 mg/kg) for 14 days. Dialysis with NMDA (100 μM) after sample 4 was carried out 48 hours and 14 days respectively after the last drug injection.

Chapter 4: The same protocols as used in the experiments for chapter 3 were applied in the study of 5-HT and 5-HIAA efflux.

Chapter 5: On the day following probe implantation once spontaneous efflux was established, dialysate samples were collected every 30 minutes. Four consecutive basal samples (unless otherwise stated) were collected before administering test drugs. A further 8 post treatment samples (unless otherwise stated) were then collected.

Rats were treated i.p. with either amantadine (40 mg/kg), budipine (10 mg/kg), CGP 40116 (1 mg/kg) and ifenprodil (0.9 mg/kg) following sample 4. Following sample 5, rats were dosed (i.p.) with paroxetine (10 mg/kg) or vehicle (sterile water).

In the subchronic study, paroxetine (10 mg/kg) was administered i.p. for a period of 7 days prior to dialysis, with the final dose being administered the day prior to dialysis. Amantadine (40 mg/kg) or vehicle (sterile water) was administered following sample 5.

2.12 Data analysis and statistics

Data included in this study (not corrected for *in vitro* recovery) was expressed as percentage of basal analyte level (% basal; means ± S.E.M.) in acute studies. The average of the first four baseline samples was taken as 100%. However, in the subchronic and chronic studies, data was expressed as absolute values (fmol/10μL; means ± S.E.M.) using the calibration graphs (Figs 2.5a-2.5d).

All statistics were performed, using the program SPSS^{TM}, on raw data. Statistical significance was set at the 95% confidence level ($p<0.05$). The effects of drugs on experimental and control groups were compared using a two way ANOVA for repeated measures with treatment was a 'between subjects factor' and time as a 'within-subjects factor' i.e. as a repeated measure. A significant overall group, time or interaction was followed by the Newman-Keuls test. The effects of drugs at individual time points were compared with baseline values using one-way ANOVA with repeated measures followed by Dunnett's test, two tailed.

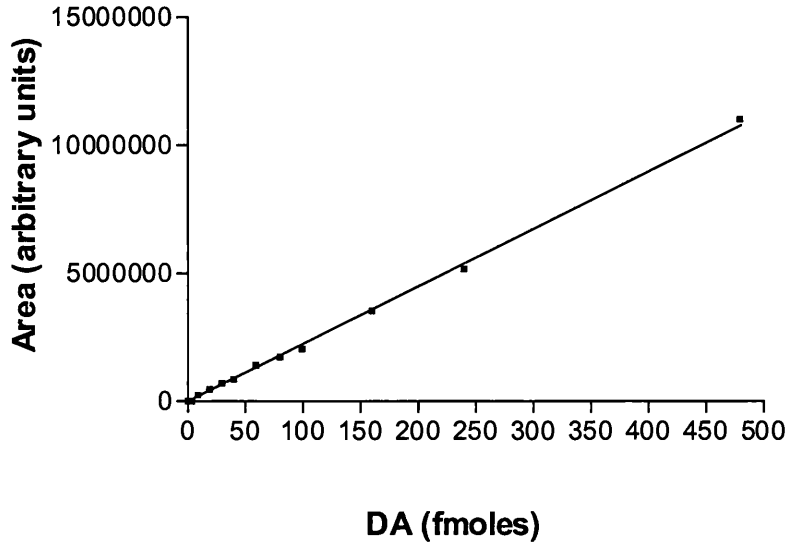


Figure 2.5a Calibration curve for DA

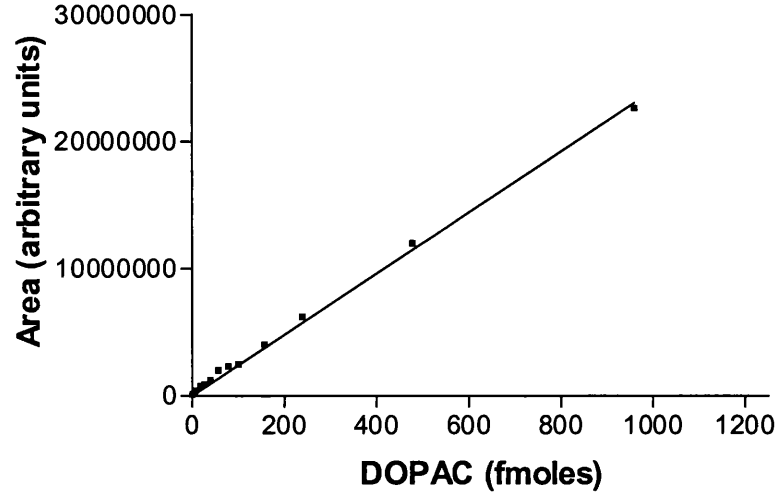


Figure 2.5b Calibration curve for DOPAC

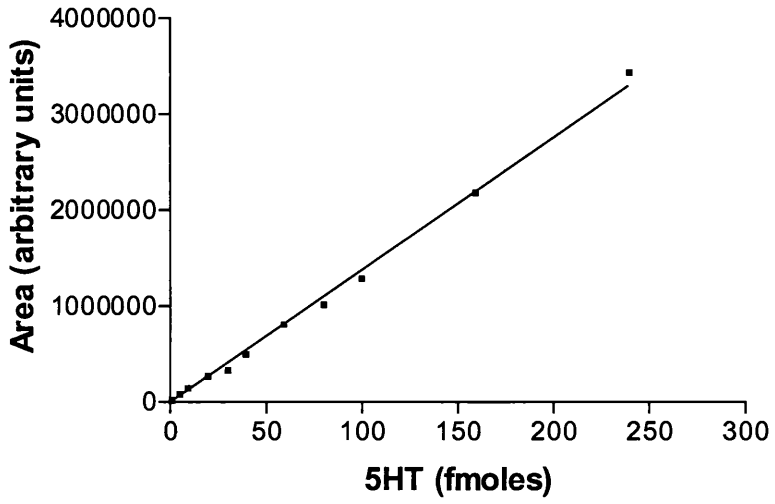


Figure 2.5c Calibration curve for 5-HT

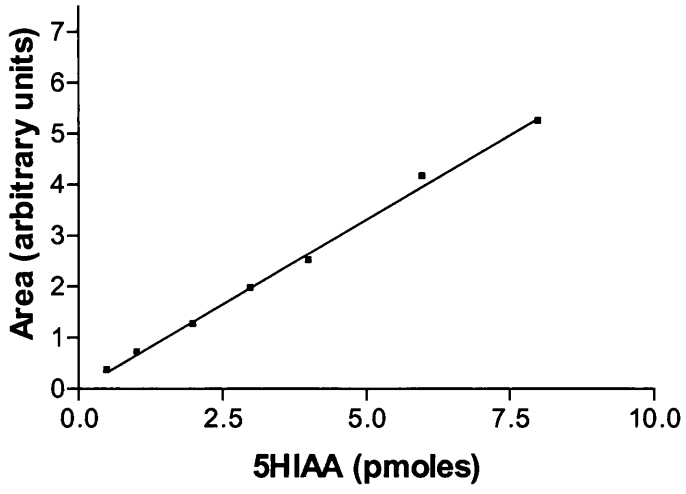


Figure 2.5d Calibration curve for 5HIAA

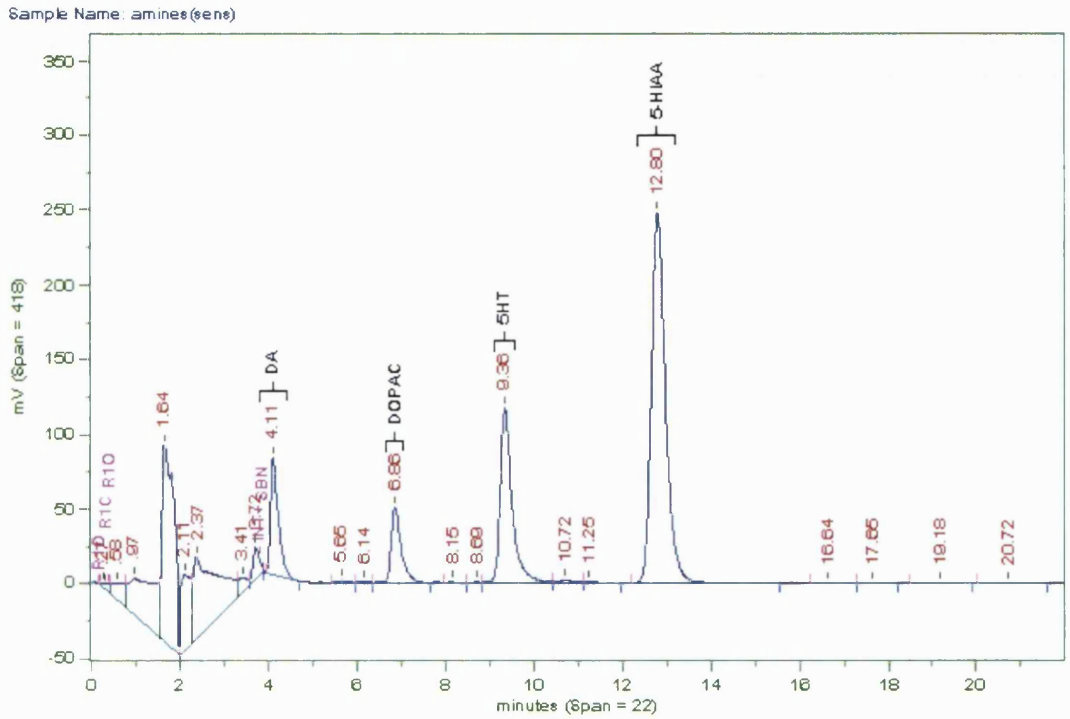


Figure 2.6a Typical chromatogram of a standard monoamine dialysate

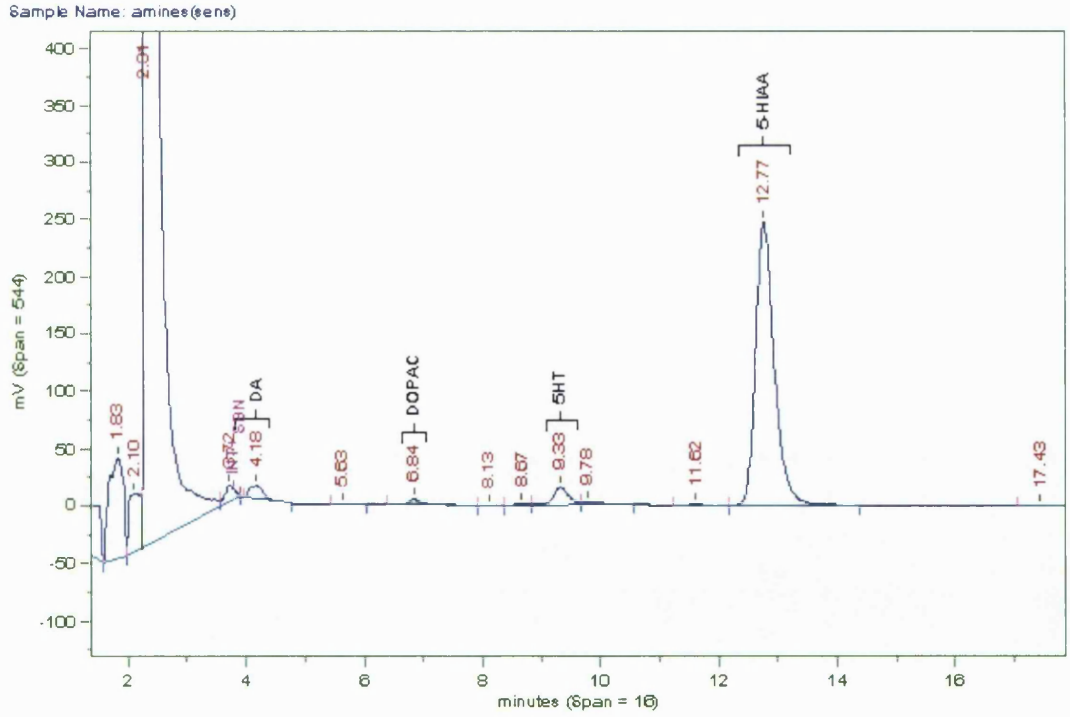


Figure 2.6b Typical chromatogram of a sample dialysate

Chapter 3

The effect of acute, subchronic and chronic paroxetine and clomipramine treatment on DA and DOPAC efflux in the frontal cortex.

3 The effect of acute, subchronic and chronic paroxetine and clomipramine treatment on NMDA- evoked extracellular DA and DOPAC changes in the frontal cortex

3.1 Introduction

The monoamine theory of depression has dominated research and treatment of depression for over 30 years. The neurotransmitters 5-HT and NA are believed to play a crucial role while that of DA has been less clear. Evidence exists to suggest DA plays some role in the pathogenesis of depression (Willner, 1983). Both direct acting DA agonists, [e.g. piribedil (Post, et al., 1978) and bromocriptine (Theohar et al, 1981)], and an indirect DA agonist, amphetamine (Silberman et al, 1981), which is reported to increase DA release in the nucleus accumbens (Arnold et al, 2000), have been reported to have antidepressant effects.

Three important lines of experimental evidence led to investigations on the possible role of DA both in the pathogenesis of depression and in the mechanism of action of antidepressant treatments. Firstly, drugs which increase DA levels, such as cocaine and amphetamine, cause euphoria and excitement (see Fleckenstein et al, 2000). In addition, drugs which either reduce DA levels, such as reserpine, or block DA receptors, such as neuroleptics, can either induce dysphoria or depressed mood (Jimerson, 1987). Secondly, several studies have shown that the DA metabolite, HVA, is reduced in the CSF of untreated depressed patients (Banki, 1977). Thirdly, it has been demonstrated by several studies that dopaminergic neurones originating in the VTA and projecting their nerve terminals into different telencephalic areas, (including the prefrontal cortex and the nucleus accumbens), are involved in the control of reward-related behaviour and incentive motivation (Fibiger and Phillips, 1987; Wise, 1989; Blackburn et al, 1992).

Dopamine is thought to play an important role in the associative functions of the prefrontal cortex (Goldman-Rakic, 1987). The frontal cortex is a brain structure implicated in depression (Petty et al, 1992; Sulivan et al, 1998) and evidence suggests that alterations in the normal functioning of the dopaminergic system may be significant in the aetiology of depression (Willner, 1983).

There is considerable electrophysiological, behavioural and neurochemical evidence that chronic administration of both the TCAs (eg clomipramine) and the SSRIs, (e.g. paroxetine) increase brain serotonergic activity (Willner, 1985, Heninger and Charney, 1987; Blier et al, 1987; Chaput et al, 1991; Beasley et al, 1992). It has also been reported that long-term treatment with TCAs increases brain dopaminergic activity. For example, chronic treatment with desipramine enhances the ability of amphetamine to increase extracellular DA levels in the nucleus accumbens (Brown et al, 1993). The properties of TCA treatment are attributed to the modulation of both noradrenergic and serotonergic function while the SSRIs are potent and selective inhibitors of the serotonin neuronal reuptake system and more recently are also reported to inhibit NA reuptake *in vivo* (Jordan et al, 1994; Hughes and Stanford, 1996).

As with TCAs, the SSRIs may affect other neurotransmitters indirectly as well. For example, the SSRI fluoxetine has been reported to acutely increase cortical extracellular DA levels without affecting extracellular DA levels in the nucleus accumbens (Jordan et al, 1994; Tanda et al, 1994; Clark et al, 1996). Similarly, the TCAs including clomipramine, imipramine, desipramine and amitriptyline, as well as atypical ADs, such as mianserin and maprotiline, have also been reported to acutely increase cortical extracellular DA levels without affecting extracellular DA levels in the nucleus accumbens (Gresch et al, 1995; Kihara and Ikeda, 1995; Tanda et al, 1994, 1996). Furthermore, striatal extracellular DA levels are reported to be increased by clomipramine and imipramine (Ichikawa and Meltzer, 1995) but decreased by SSRIs including paroxetine and sertraline (Meltzer et al, 1993), citalopram (Dewey et al, 1995), or unchanged by the TCA, amitriptyline (Meltzer et al, 1993). There are conflicting results with fluoxetine as Meltzer and colleagues (1993) report that fluoxetine decreases extracellular DA levels in the striatum while Maj and colleagues (1996) report that extracellular DA levels remain unchanged.

On a clinical level, in one of the first patients treated with fluoxetine, it caused extrapyramidal symptoms (EPS) (for example, akathisia and tremor) and decreased cerebrospinal fluid levels of HVA, suggesting this drug could decrease nigrostriatal dopaminergic activity in humans (Meltzer, 1979). Similar side-effects have been reported by others (Dave, 1994; Scheepers and Rogers, 1994) including after paroxetine treatment in man (see Caley and Weber, 1993). Although the relationship between SSRIs and EPS is not clear (see Caley and Weber, 1993), it appears to be initiated by

excessive stimulation of the 5-HT_2-receptors in the serotonergic pathway which project to the basal ganglia. It is postulated that the 5-HT_2-receptors inhibit the release of DA leading to these EPS (Lane, 1998; Scheepers and Rogers, 1994). These findings may support a role that both TCAs and SSRIs therapeutic effects or possibly side effects such as EPS may be due to an overall change in brain extracellular DA levels.

It has been found that NMDA receptor antagonists can give a positive result in a screen for antidepressants in a rodent behavioural model (Trullas and Skolnick, 1990). It has also been observed that chronic, but not acute treatment with a range of antidepressants including SSRIs decreases the sensitivity of NMDA receptors (Paul et al, 1994). This may suggest that a dysfunction in NMDA receptor-mediated transmission in the CNS of depressed patients may play a role in the pathophysiology of depression. This is supported by the finding that NMDA receptors are abnormal in the frontal cortex of depressed suicide victims (Nowak et al, 1995). As an important role exists for the NMDA receptor in the regulation of DA release in several brain regions (see Whitton, 1997), it may be likely that antidepressant related changes in NMDA receptors may alter NMDA-evoked changes in dopaminergic transmission.

In this part of the study, using *in vivo* microdialysis in freely moving rats, it was determined whether the clinically used SSRI, paroxetine, and TCA, clomipramine, alter NMDA receptor-mediated DA efflux following acute, subchronic (7-day) and chronic (21-day) treatment and how long these changes last after cessation of drug treatment ('drug holiday').

The following questions were addressed in this part of the study:

- What are the effects of paroxetine administered (systemically and locally) on extracellular DA efflux in the frontal cortex?
- What are the effects of NMDA on DA efflux in the frontal cortex?
- What are the effects of acute, subchronic and chronic treatment with paroxetine on NMDA-evoked changes on DA efflux?
- If any changes result from the above treatments, are these changes reversed following AD (paroxetine) cessation and what is the time scale?
- Are these changes related specifically to the SSRI, paroxetine, or can the findings be replicated in a different class of AD, in this case by using the TCA, clomipramine?

3.2 Results

3.2.1 Extracellular basal levels of DA and DOPAC measured in the frontal cortex

Basal dialysate DA and DOPAC values were 88 ± 4.2 and 535 ± 6.9 fmoles/10 μL (n=48). These values are similar to published results from our laboratory (Whitton et al, 1992a, b).

3.2.2 The effect of acute paroxetine on extracellular levels of DA and DOPAC in the frontal cortex

Acute administration of paroxetine i.p. (10, 20 mg/kg) had no effect on extracellular DA [$F(1,9)=4.41$, $p<0.08$] or DOPAC levels in the frontal cortex (Figure 3.1A/B).

Local infusion into the frontal cortex of 100 μM paroxetine caused a maximal increase in extracellular DA at 240 minutes to 179% of basal levels (Figure 3.2A). Two-way repeated measures ANOVA revealed significant effects over time [$F(11,66)=4.97$, $p<0.01$] and treatment [$F(1,6)=22.14$, $p<0.001$]. This returned to 128% basal level at the end of the experiment (360 minutes).

The effect of the local infusion of paroxetine (100 μM) on extracellular DOPAC levels was a profound transient increase. This increase in extracellular DOPAC was only observed after 90 minutes of the start of paroxetine infusion with a maximal increase of 300% basal at 240 minutes (Figure 3.2B). After this time point, extracellular DOPAC levels decreased rapidly with the levels returning to slightly higher than basal (150% basal).

3.2.3 The effect of acute clomipramine on extracellular levels of DA and DOPAC levels in the frontal cortex

Acute administration of clomipramine i.p. (10/20mg/kg). led to a dose-dependent decrease in extracellular DA [$F(2,9)=136.11$, $p<0.05$] and DOPAC levels in the frontal cortex which at the higher dose lasted for the duration of the experiment (Figure 3.3A/B).

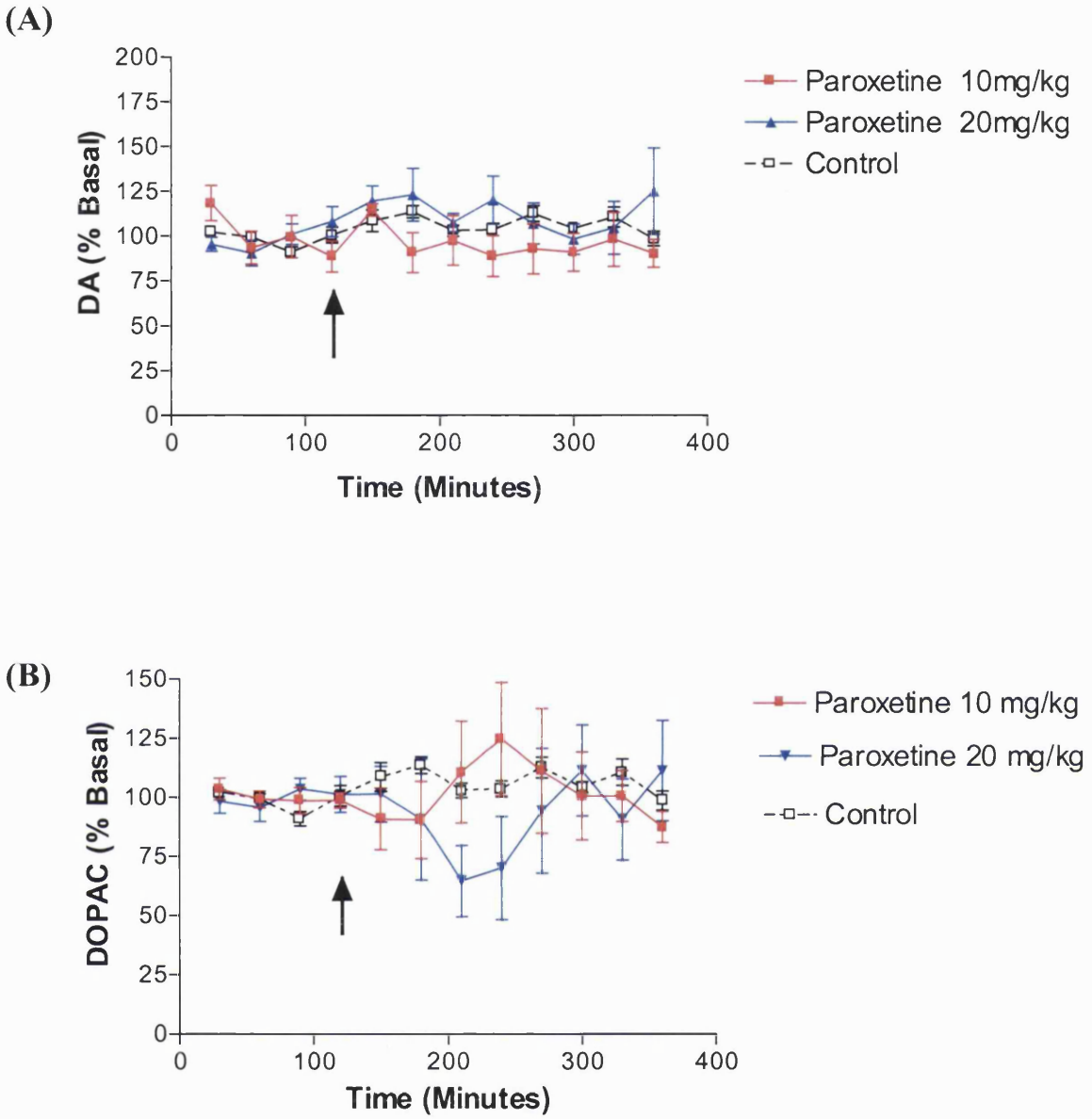
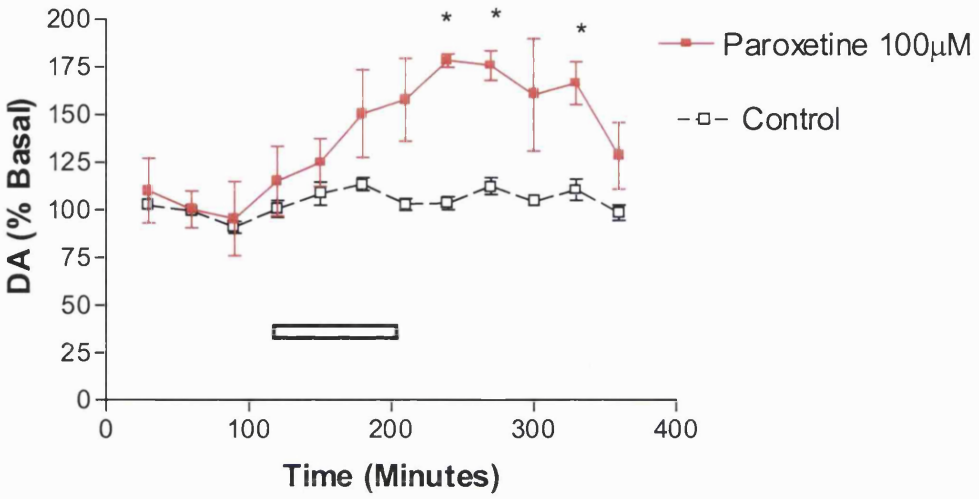


Figure 3.1. The effect of acutely administered paroxetine on extracellular levels of A) DA and B) DOPAC in the frontal cortex. The arrow indicates the time at which paroxetine (i.p) was administered. Data are the mean ± SEM of six-eight animals in each group.

(A)



(B)

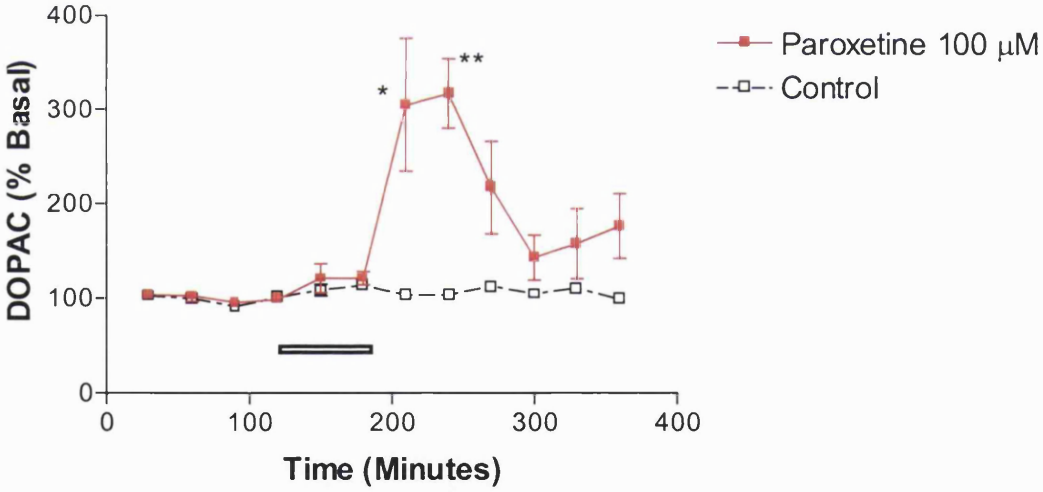
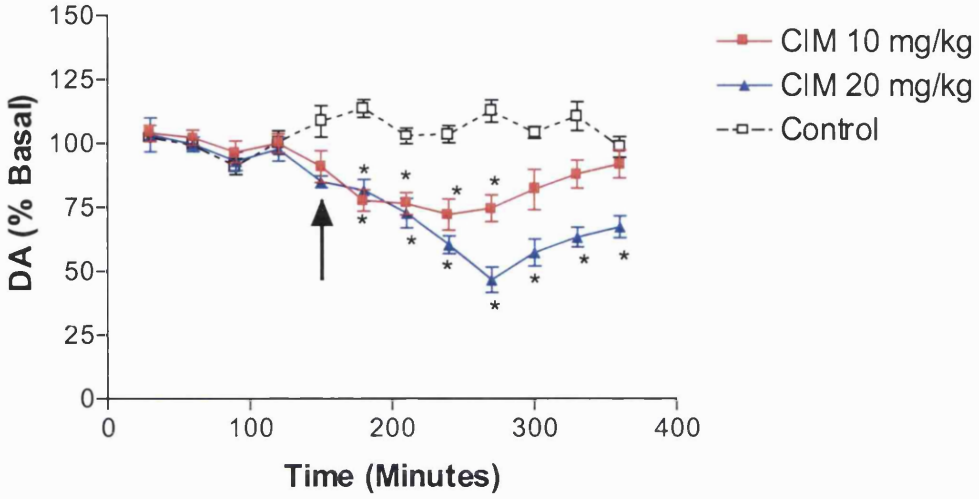


Figure 3.2. The effect of localised infusion of paroxetine on extracellular A) DA and B) DOPAC in the frontal cortex. Paroxetine was infused into the frontal cortex and extracellular DA measured. The solid box indicates the period of paroxetine infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$); **($p<0.01$) from control.

(A)



(B)

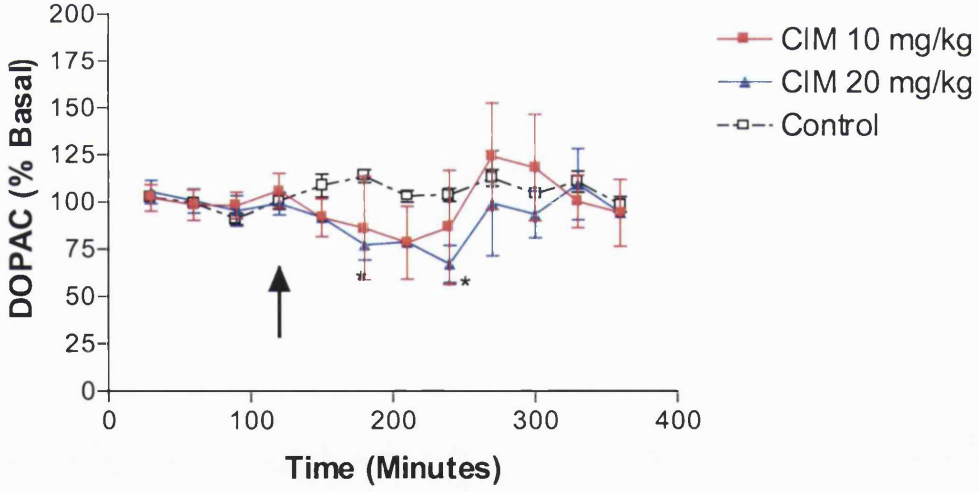


Figure 3.3 The effect of acutely administered clomipramine (CIM) on extracellular levels of A) DA and B) DOPAC in the frontal cortex. The arrow indicates the time at which clomipramine (i.p) was administered. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

3.2.4 The effect of NMDA infusion into the frontal cortex on extracellular DA and DOPAC

Direct, localised infusion of NMDA (100μM) for 30 minutes into the frontal cortex caused a statistically significant decrease of extracellular DA and DOPAC levels.

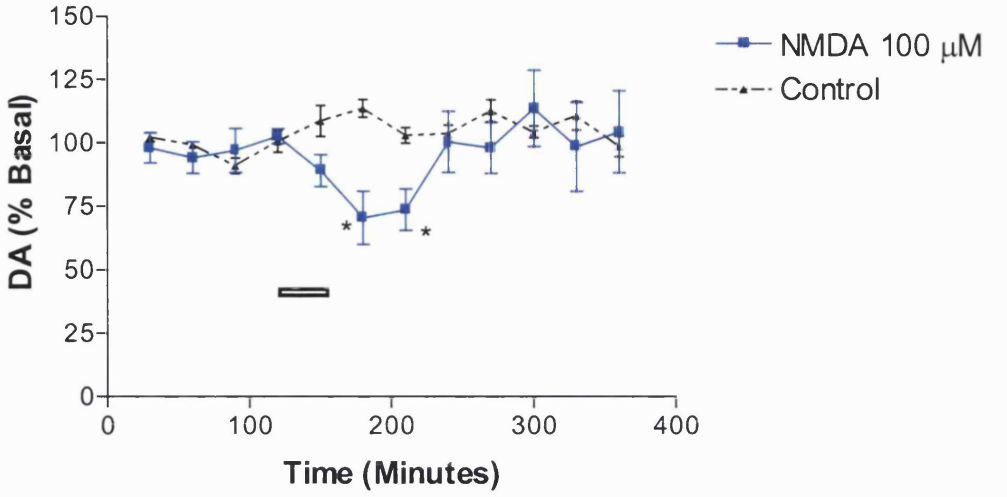
NMDA (100μM) administration caused an immediate significant [$F(1,6)=7.63$, $p<0.05$] decrease in extracellular DA to 70% of basal levels. This effect lasted for 60 minutes with levels returning to basal levels thereafter (Figure 3.4A). The infusion of NMDA (100μM) for 30 minutes also produced an immediate, significant [$F(1,6)=43.93$, $p<0.05$] decrease in extracellular DOPAC levels to 63% of basal levels which remained so for the duration of the experiment (Figure 3.4B).

3.2.5 Effect of acute paroxetine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC in the frontal cortex

When 100 μM NMDA was infused into the frontal cortex of rats treated acutely with paroxetine (10 mg/kg), extracellular DA in this region fell significantly below basal values (75 % of basal) [$F(2,9)=6.99$, $p<0.05$] and remained so for the duration of the experiment [$F(11,99)=2.74$, $p<0.01$] (Figure 3.5A).

Following NMDA infusion, animals acutely pre-treated with paroxetine (10 mg/kg) displayed an apparent increase in extracellular DOPAC levels which was not significant ($p>0.05$) (Figure 3.5B).

(A)



(B)

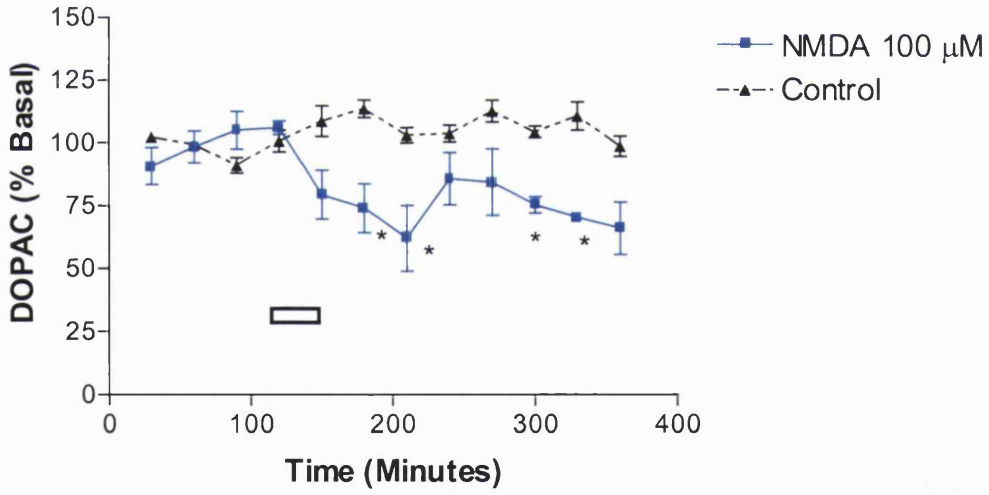
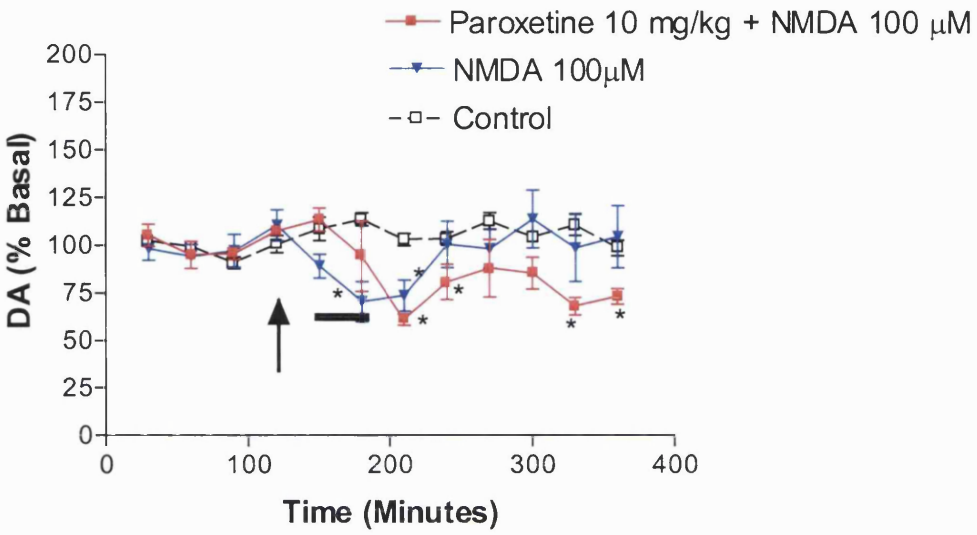


Figure 3.4. The effect of NMDA infusion in the frontal cortex on extracellular A) DA and B) DOPAC in the frontal cortex. The open bar indicates the time at which paroxetine (i.p) was administered. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

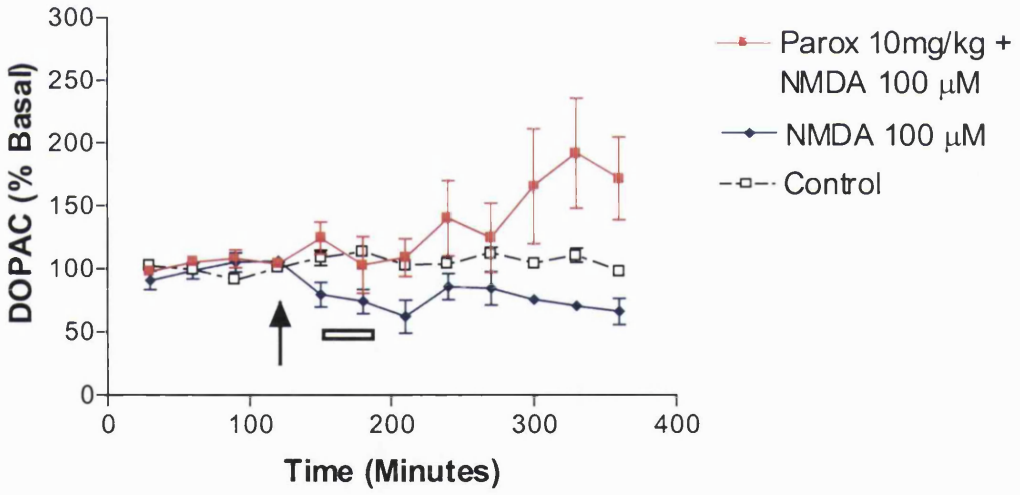


Figure 3.5. Effect of acute paroxetine (Parox) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. NMDA was infused into the cortex and extracellular DA and DOPAC measured. The arrow indicates the time at which paroxetine or vehicle was administered. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

3.2.6 Effect of acute clomipramine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC in the frontal cortex

When 100 μM NMDA was infused into the frontal cortex of rats treated acutely with clomipramine (10 mg/kg), extracellular DA in this region fell significantly below basal values (75 % of basal) [$F(2,9)=17.61$, $p<0.001$] and remained so for the duration of the experiment (Figure 3.6A) [$F(11,99)=2.50$, $p<0.01$]. The decrease in DA efflux was similar in magnitude to that seen after NMDA infusion alone.

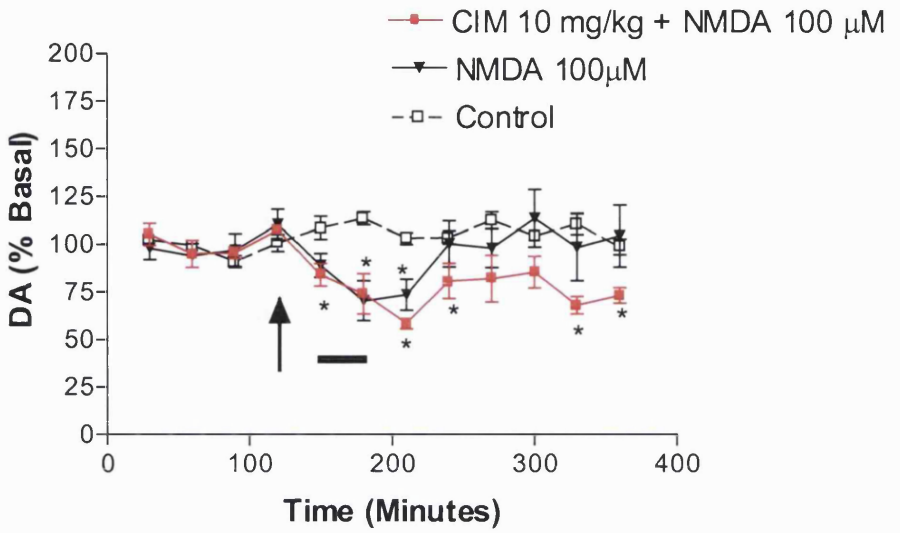
Following NMDA infusion, animals acutely pre-treated with clomipramine (10 mg/kg) displayed a similar significant [$F(2,9)=29.93$, $p<0.05$] decrease in extracellular DOPAC levels to that seen of NMDA alone which remained significantly [$F(11,99)=9.87$, $p<0.05$] so for the duration of the experiment (Figure 3.6B).

3.2.7 Effect of 7-day (sub-chronic) dosing of paroxetine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC levels in the frontal cortex

7-day dosing of paroxetine (10mg/kg) resulted in significant reductions in extracellular DA and DOPAC levels.

After a 7-day sub-chronic treatment with paroxetine, extracellular DA levels were reduced significantly to 67% of basal levels [$F(2,9)=23.54$, $p<0.001$]. Extracellular DA levels did not appear to decrease upon NMDA infusion, as was seen with saline-treated rats [$F(11,99)=0.802$, $p<0.638$]. In fact, no change was evident with NMDA infusion (Figure 3.7A). Extracellular DOPAC levels were observed to decrease significantly [$F(2,9)=18.04$, $p<0.01$] to 47% basal following 7-day paroxetine treatment. In these rats, infusion with NMDA was followed by a gradual increase in extracellular DOPAC levels which peaked at the end of the experiment near control levels (Figure 3.7B).

(A)



(B)

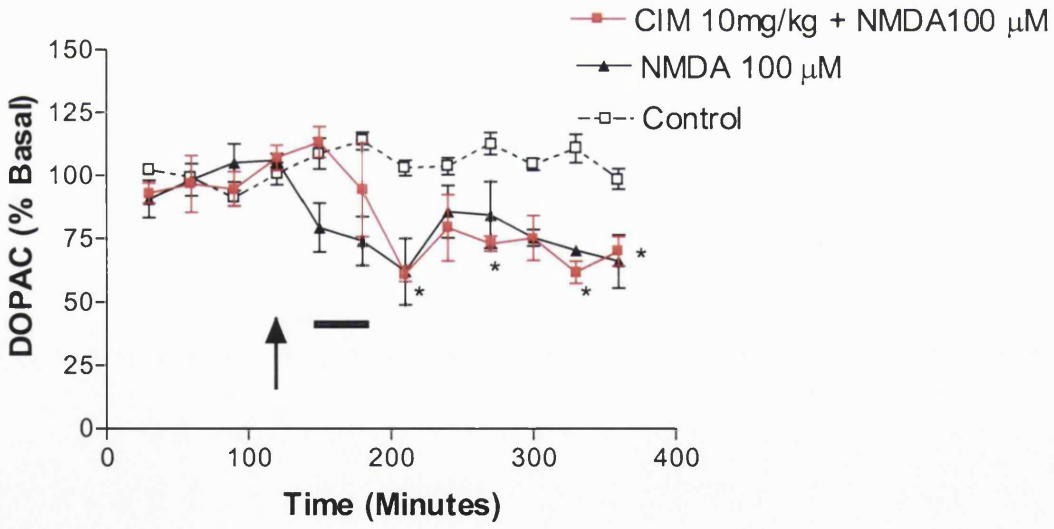
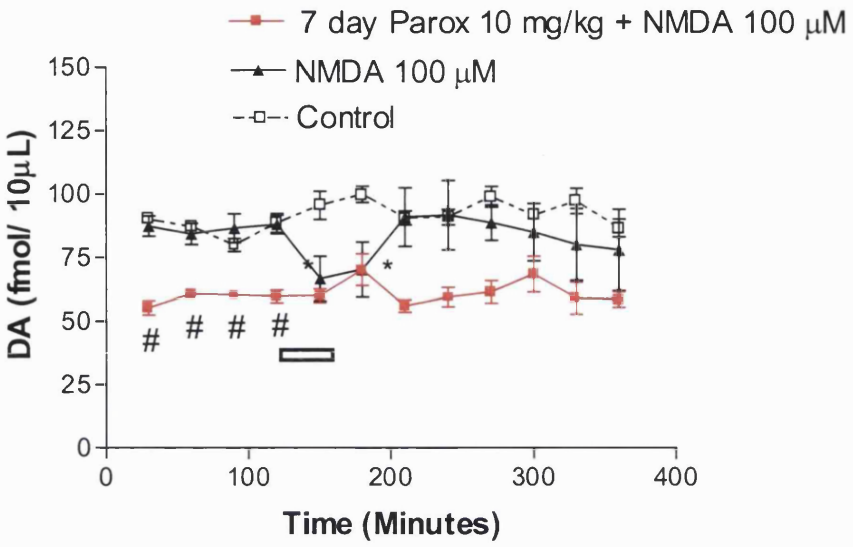


Figure 3.6. Effect of acute clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. NMDA was infused into the cortex and extracellular DA and DOPAC measured. The arrow indicates the time at which clomipramine or vehicle was administered. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

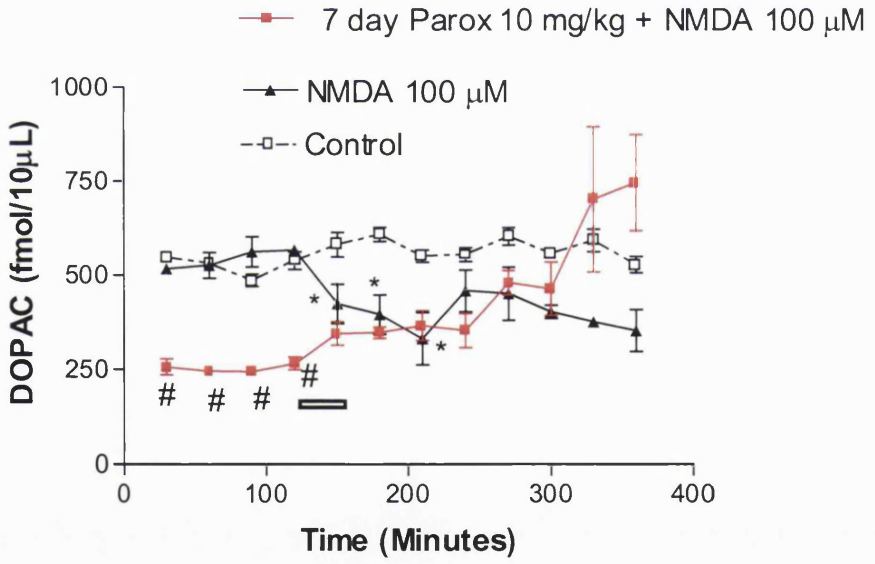


Figure 3.7. Effect of 7-day (sub-chronic) dosing of paroxetine (Parox) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

3.2.8 Effect of 21-day (chronic) dosing of paroxetine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC levels in the frontal cortex

After a 21 day chronic treatment with paroxetine (10 mg/kg), two marked changes were observed. Firstly, extracellular DA levels in this brain region were 175% (154 fmol/10μL) above basal values compared with vehicle treated controls. (Figure 3.8A). Secondly, infusion of 100 μM NMDA into the frontal cortex of chronically-treated rats was reversed causing a highly statistically significant increase in DA efflux which remained greater than control for the duration of the experiment. This increase was significant over main factor 'time' [$F(16,96)=173.99$, $p<0.001$] and between treatments [$F(2,6)=87.24$, $p<.001$]. The maximal increase in DA efflux was six-fold of basal values (Figure 3.8A).

When paroxetine was administered repeatedly for 21 days extracellular DOPAC levels decreased significantly ($p<0.05$) to 75% of basal levels. The effect of NMDA infusion, as with DA, was a significant increase in extracellular DOPAC levels that reached a peak after 150 minutes after infusion (Figure 3.8B). The maximal peak in extracellular DOPAC levels was 585fmols/10μL. Although this peak was significantly different from points prior to infusion, it was not significantly different from control levels.

3.2.9 Effect of 21-day (chronic) dosing of paroxetine with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular DA and DOPAC levels in the frontal cortex

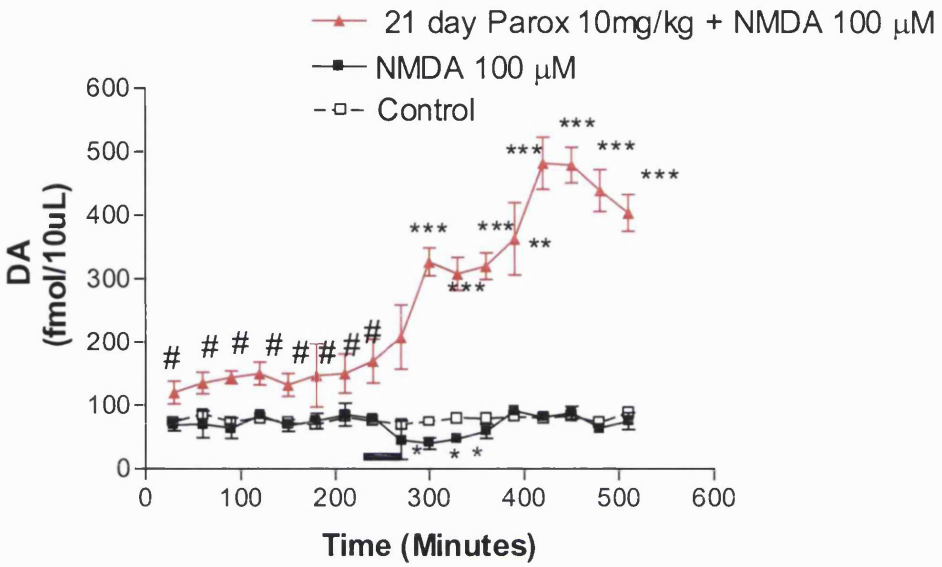
Chronic dosing for 21 days with paroxetine followed by 48 hours of 'drug holiday' caused an increase in extracellular DA while not affecting DOPAC levels.

After a 21 day chronic treatment with paroxetine (10 mg/kg) with 48 hours of 'drug holiday', two marked changes were observed. Firstly, extracellular DA levels in this region were 176% (140 fmol/10μL) above basal values compared with vehicle treated controls. (Figure 3.9A). Secondly, the effect of infusion of 100 μM NMDA into the frontal cortex of these rats was reversed compared to that of controls and an increase in dialysate DA was observed. This increase in extracellular DA efflux was significant between drug treatments [$F(2,9)=32$, $p<0.001$] but not on main factor 'time' [$F(11,99)=1.87$, $p=0.052$].The increase in extracellular DA was immediate and transient

lasting for 60 minutes after which extracellular DA levels returned to basal levels. The maximal increase in extracellular DA was 250fmol/10μL which occurred 60 minutes after the start of infusion.

The repeated treatment with paroxetine (10 mg/kg) with 48 hours of 'drug holiday' failed to cause any change in extracellular DOPAC levels. However, the NMDA infusion into the frontal cortex reversed the decrease in extracellular DOPAC levels seen in saline treated rats. When NMDA was infused into these chronically treated rats a significant [$F(2,9)=41.94$, $p<0.01$] increase in extracellular DOPAC levels was observed. These increases were gradual and the maximal increase (850fmol/10μL) was observed at 150 minutes after the start of NMDA infusion (Figure 3.9B). This increase lasted for the duration of the experiment with all samples up to 300 minutes being statistically significant [$F(11,99)=2.55$, $p<0.01$] while the extracellular DOPAC levels for last 60 minutes of experiment rested above those of control.

(A)



(B)

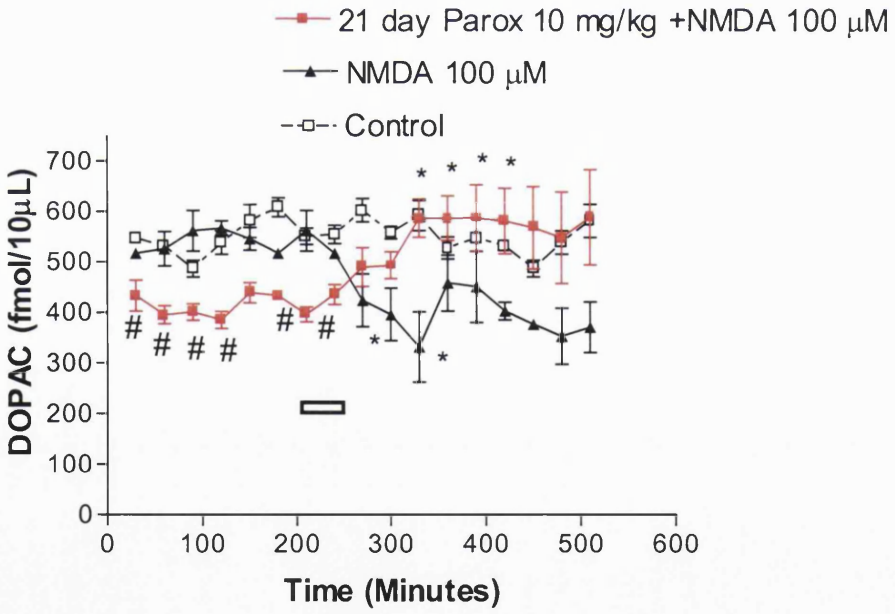
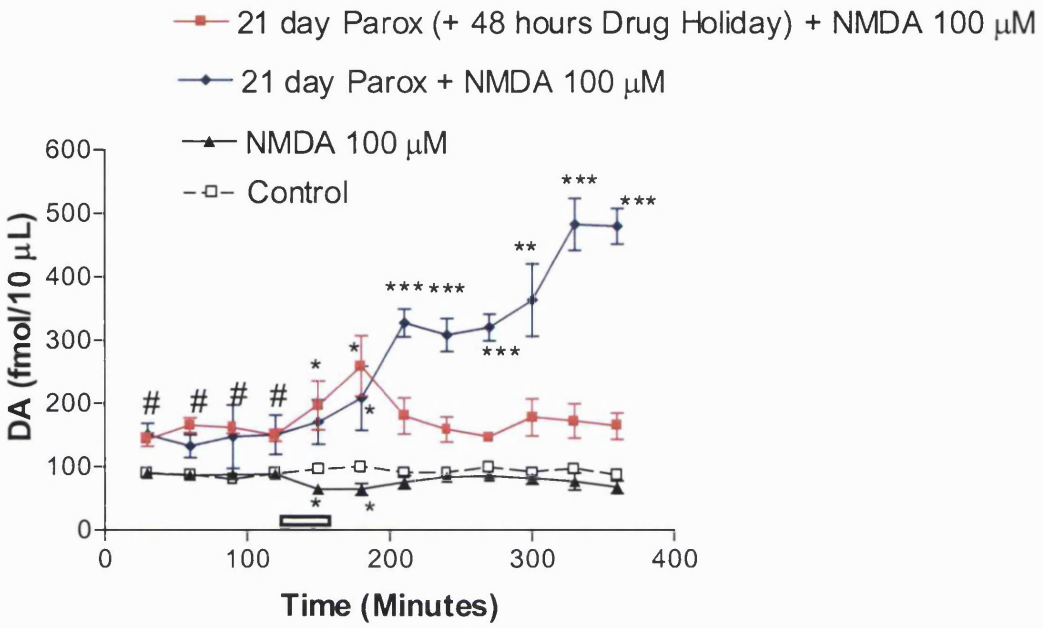


Figure 3.8. Effect of 21-day (chronic) dosing of paroxetine (Parox) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$) from control. # indicates significantly different from treatment basal ($p<0.05$).

(A)



(B)

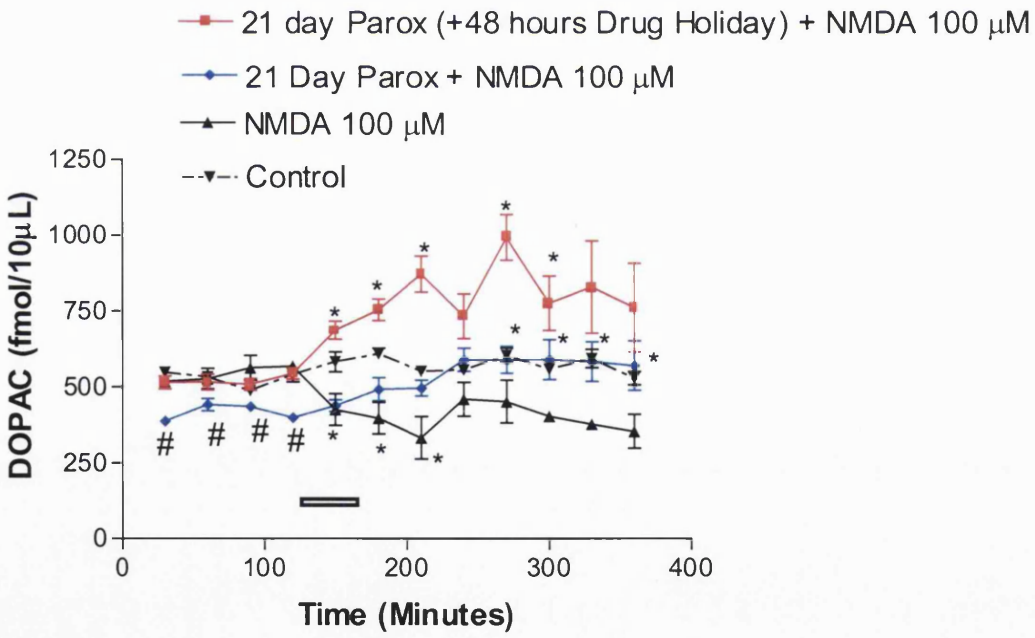


Figure 3.9. Effect of 21-day (chronic) dosing of paroxetine (Parox, 10 mg/kg) with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

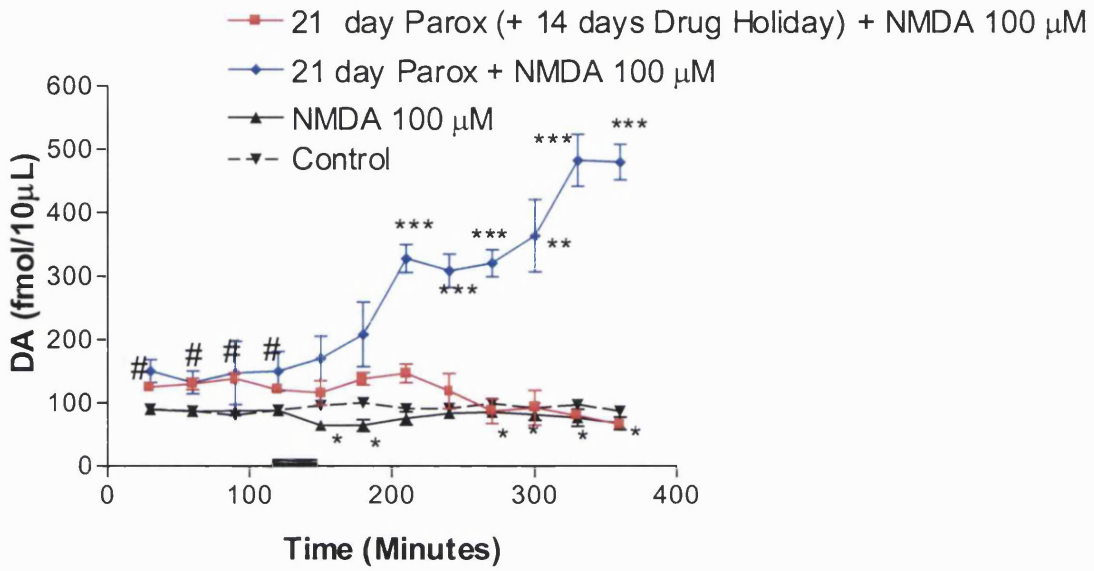
3.2.10 Effect of 21-day (chronic) dosing with paroxetine followed by 14 days 'drug holiday' on 100 μM NMDA-evoked changes in DA and DOPAC levels in the frontal cortex

Chronic dosing for 21 days with paroxetine with 14 days of 'drug holiday' caused an increase in extracellular DA and DOPAC levels.

After a 21 day chronic treatment with paroxetine (10 mg/kg) with 14 days of 'drug holiday', two marked changes were observed. Firstly, extracellular DA levels in this region were 147% (135 fmol/10μL) above basal values compared with vehicle treated controls. (Figure 3.10A). Secondly, the infusion of NMDA into the frontal cortex of these rats, produced a significant decrease in extracellular DA levels. Two-way ANOVA revealed significant differences over time [$F(11,99)=3.44$, $p<0.01$] and between treatments [$F(2,9)=14.04$, $p<0.01$]. Although this decrease was delayed, only occurring after 150 minutes after the start of NMDA infusion, it persisted until the end of the experiment with extracellular DA levels falling to 50% (75fmol/μL) after drug infusion.

The apparent increase in extracellular DOPAC levels to 125% basal levels following this drug treatment was not significant ($p=0.057$). However, when NMDA was infused into the frontal cortex there was a reversal of the decrease seen in saline-treated rats. There was a slightly delayed increase in extracellular DOPAC levels. This 28% increase in extracellular DOPAC was only observed after 150 minutes following NMDA infusion (Figure 3.10B).

(A)



(B)

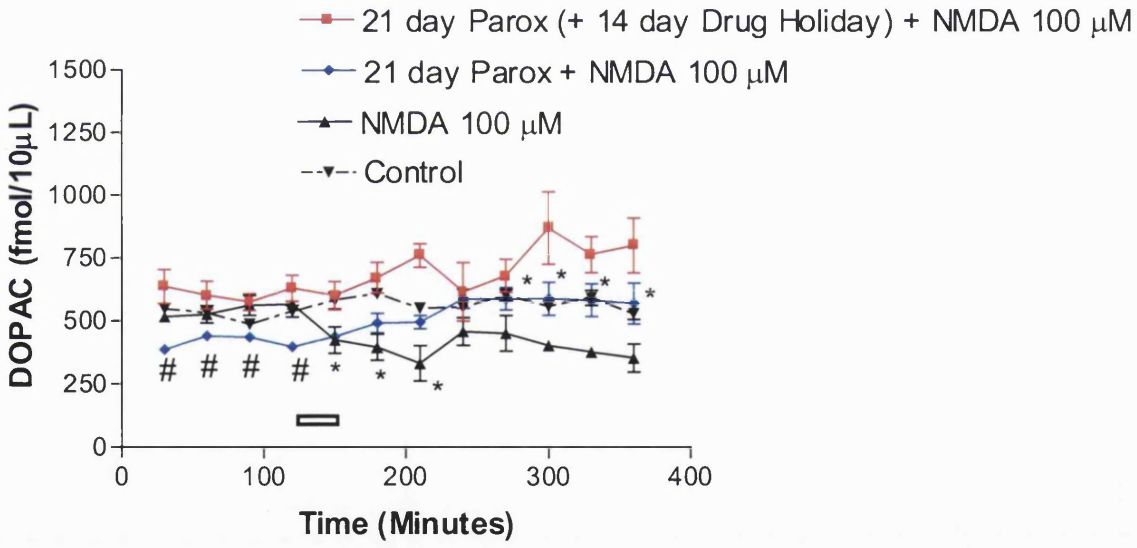


Figure 3.10. Effect of 21-day (chronic) dosing of paroxetine (Parox, 10 mg/kg) with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six-eight animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

3.2.11 Effect of 7-day (sub-chronic) dosing of clomipramine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC levels in the frontal cortex

7-day dosing of clomipramine (10mg/kg) resulted in significant reductions in extracellular DA and DOPAC levels.

After a 7-day sub-chronic treatment with clomipramine, extracellular DA levels were reduced significantly to 77% basal (79fmol/10μL) ($p<0.05$). Extracellular DA levels did not appear to decrease upon NMDA infusion compared with saline-treated rats ($F(11,99)=1.076$, $p<0.39$]. In fact, there was no apparent change with NMDA infusion alone (Figure 3.11A).

Extracellular DOPAC levels decreased drastically to 26% (150 fmol/10μL) basal [$F(2,9)=243.6$, $p<0.01$] following 7-day clomipramine treatment. In these rats, infusion with NMDA did not have any significant effect on extracellular DOPAC levels [$F(11,99)=2.42$, $p=0.07$] (Figure 27). This is in contrast when NMDA is infused in saline-treated rats in which a persistent decrease in extracellular DOPAC is observed (Figure 3.11B).

3.2.12 Effect of 21-day (chronic) dosing of clomipramine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC extracellular levels in the frontal cortex

After 21 days chronic treatment with clomipramine (10 mg/kg), two marked changes were observed. Firstly, extracellular DA levels in this region were 186% (170 fmol/10μL) above basal values compared with vehicle treated controls (Figure 3.12A). Secondly, the effect of infusion of 100 μM NMDA into the frontal cortex of chronically-treated rats was reversed: there was an increase in DA efflux which remained so for the duration of the experiment. This increase in extracellular DA efflux was significant between drug treatments [$F(2,6)=617$, $p<0.001$] and on main factor 'time' [$F(16,96)=617$, $p<.0001$]. The maximal increase in DA efflux was five-fold that of basal values (600fmol/μL) (Figure 3.12A).

When clomipramine was administered repeatedly for 21 days, extracellular DOPAC levels increased to 120% of basal levels. NMDA infusion did not cause any

change in extracellular DOPAC levels to these chronically treated rats (Figure 3.12B). NMDA infusion to the saline-treated rats caused a significant decrease in DOPAC efflux.

3.2.13 Effect of 14-day (chronic) dosing of clomipramine on 100 μM NMDA-evoked changes in extracellular DA and DOPAC in the frontal cortex

Chronic dosing for 14 days with clomipramine caused an increase in extracellular DA levels while not affecting extracellular DOPAC levels.

After a 14 day chronic treatment with clomipramine (10 mg/kg) extracellular DA levels in this region were significantly increased to 213% (187 fmol/10μL) above basal values compared with vehicle treated controls. [$F(2,9)=159.46$, $p<0.001$] (Figure 3.13A).

The repeated treatment with clomipramine (10 mg/kg) had no significant effect on extracellular DOPAC levels. However, the NMDA infusion into the frontal cortex of CIM-treated animals reversed the decrease in extracellular DOPAC levels seen in saline treated rats (Figure 3.13B). Thus when NMDA was infused into these chronically treated rats a significant [$F(2,9)=52.65$, p,0.05] increase in extracellular DOPAC levels was observed. These increases were gradual and the maximal increase (919 fmol/10μL) was observed at 90 minutes after the start of NMDA infusion and lasted for the duration of the experiment.

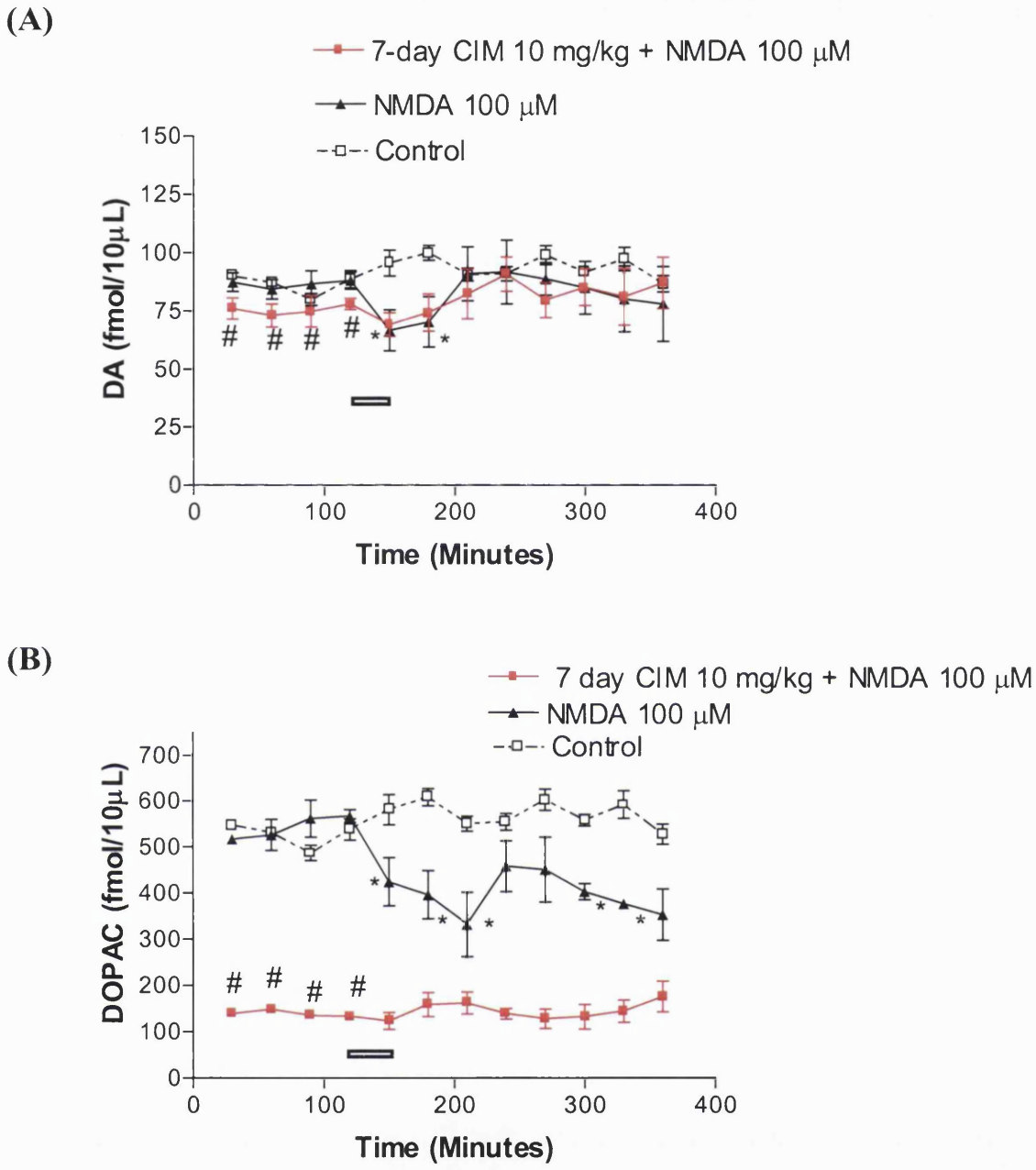
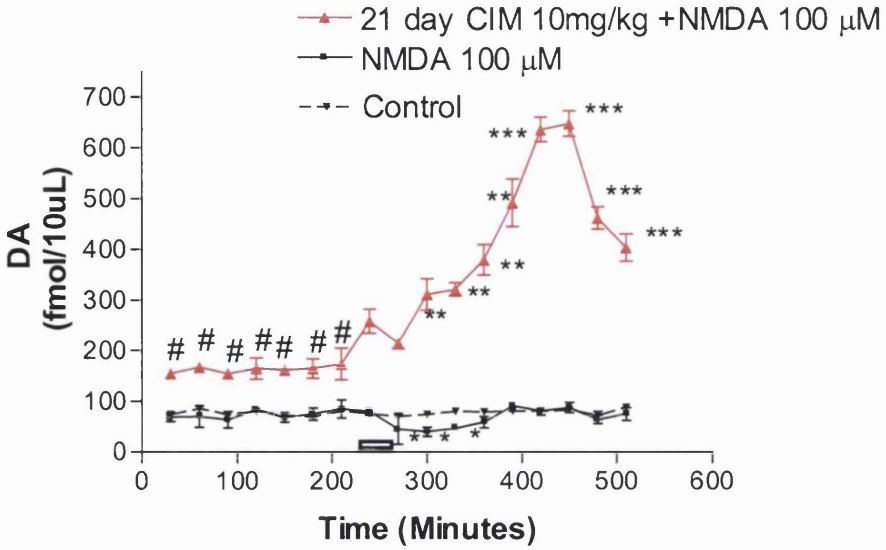


Figure 3.11. Effect of 7-day (sub-chronic) dosing of clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different from control ($p<0.05$). # indicates significantly different from treatment basal ($p<0.05$).

(A)



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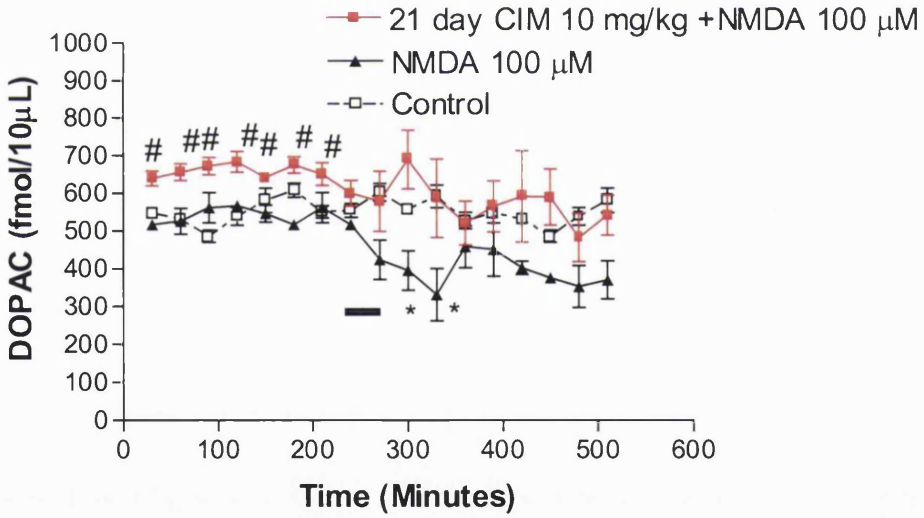
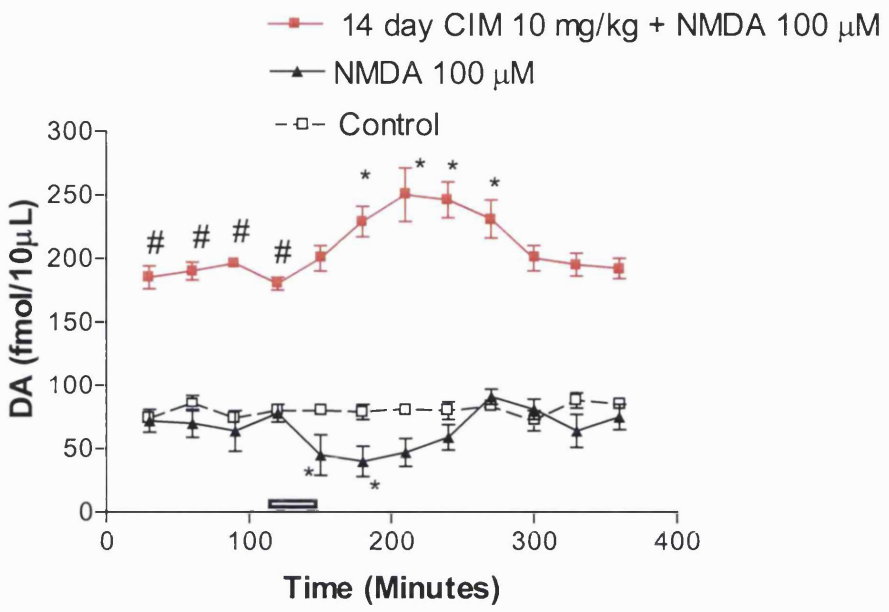


Figure 3.12. Effect of 21-day (chronic) dosing of clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$) from control. # indicates significantly different from treatment basal ($p<0.05$).

(A)



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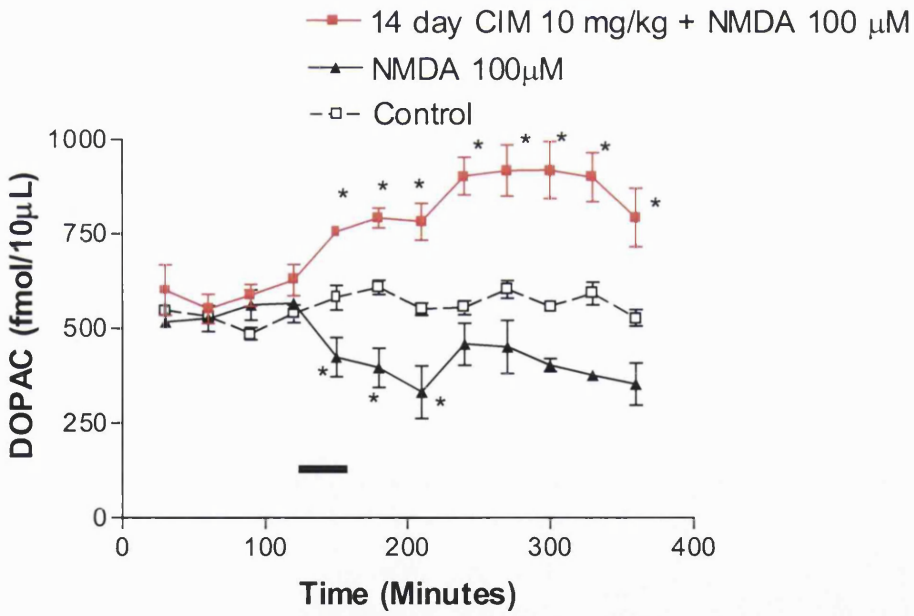


Figure 3.13. Effect of 14-day (chronic) dosing of clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$). # indicates significantly different from treatment basal ($p<0.05$).

3.2.14 Effect of 14-day (chronic) dosing of clomipramine with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular DA and DOPAC in the frontal cortex

With chronic dosing of 14 days with clomipramine with 48 hours of 'drug holiday' there was still an increase in extracellular DA while still having no effects on extracellular DOPAC levels.

After a 14 day chronic treatment with clomipramine (10 mg/kg) with 48 hours of 'drug holiday', extracellular DA levels in this region were significantly increased to 268% (236 fmol/10μL) above basal values compared with vehicle treated controls. [$F(2,9)=159.46$, $p<0.001$] (Figure 3.14A). The repeated treatment with clomipramine (10 mg/kg) with 48 hours of 'drug holiday' did not effect basal extracellular DOPAC levels. However, the NMDA infusion into the frontal cortex of CIM-treated animals reversed the decrease in extracellular DOPAC levels seen in saline treated rats (Figure 3.14B). Thus when NMDA was infused into these chronically treated rats a significant [$F(2,9)=52.65$, p,0.05] increase in extracellular DOPAC levels was observed. These increases were gradual and the maximal increase (790 fmol/10μL) was observed at 90 minutes after the start of NMDA infusion. This increase lasted for the duration of the experiment with all points beyond 210 minutes being statistically significant [$F(11,99)=2.02$, $p<0.05$].

3.2.15 Effect of 14-day (chronic) dosing of clomipramine with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in DA and DOPAC levels in the frontal cortex

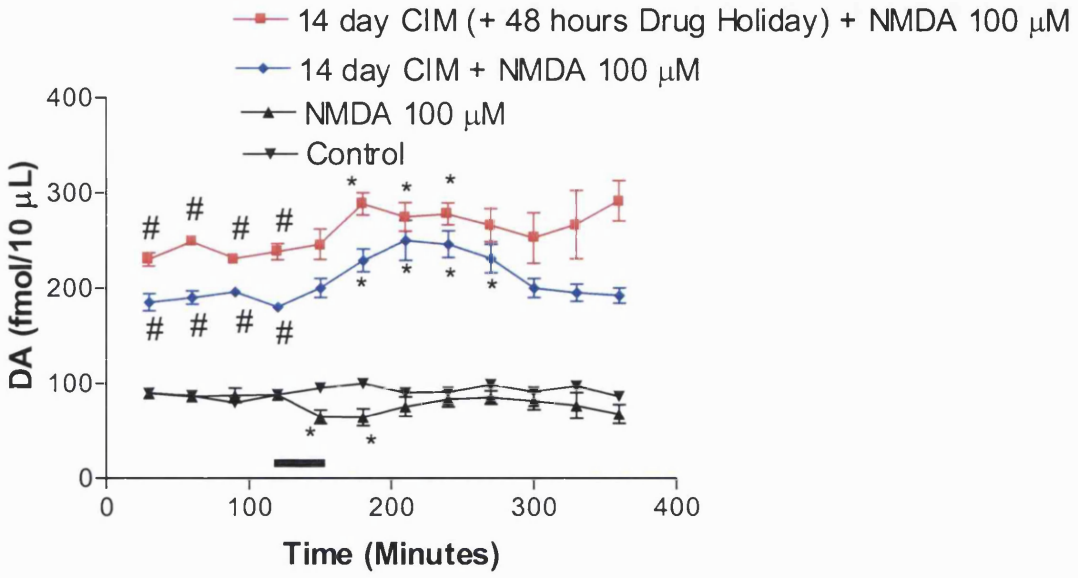
Chronic dosing of 14 days with clomipramine with 14 days of 'drug holiday' caused an increase in basal extracellular DA and DOPAC levels.

After a 14 day chronic treatment with clomipramine (10 mg/kg) with 14 days of 'drug holiday', two marked changes were observed. Firstly, basal extracellular DA levels in this region were 156% (150 fmol/10μL) above basal values compared with vehicle treated controls. (Figure 3.15B). Secondly, the infusion of NMDA into the frontal cortex of these rats, produced an immediate, persistent significant decrease in extracellular DA levels. Two-way ANOVA revealed significant differences between

treatments [F 2,9=14.55, $p<0.001$] and over time [F(11,99)=7.76, $p<0.001$]. This decrease persisted for the duration of the experiment with the maximal decrease at the last time point of 360 minutes. Extracellular DA levels here were 30% (55 fmol/10μL) of basal levels at the end of the experiment.

Basal extracellular DOPAC levels were observed to increase to 156% basal following this drug treatment. However, when NMDA was infused into the frontal cortex there was a reversal of the decrease seen in saline-treated rats. There was a delayed increase in extracellular DOPAC levels was observed 120 minutes after the start of NMDA infusion and persisted for the duration of the experiment (Figure 3.15B). The maximal increase in extracellular DOPAC levels was seen at the last time point (360 minutes). This significant [F(2,9)=70.03, $p<0.05$] increase was 150% basal (1700fmol/10μL).

(A)



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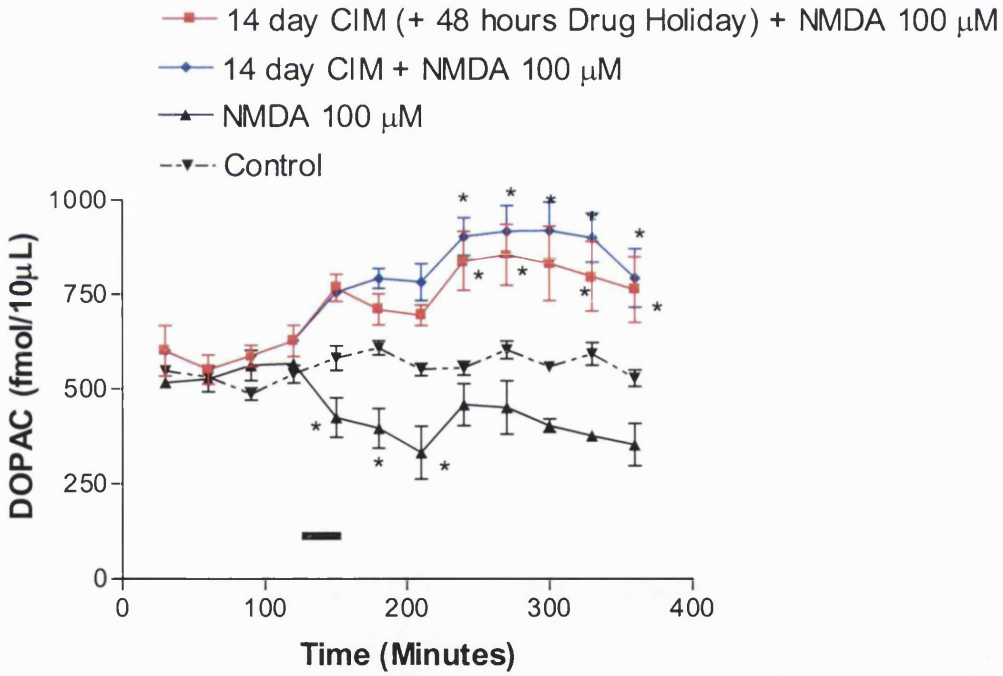
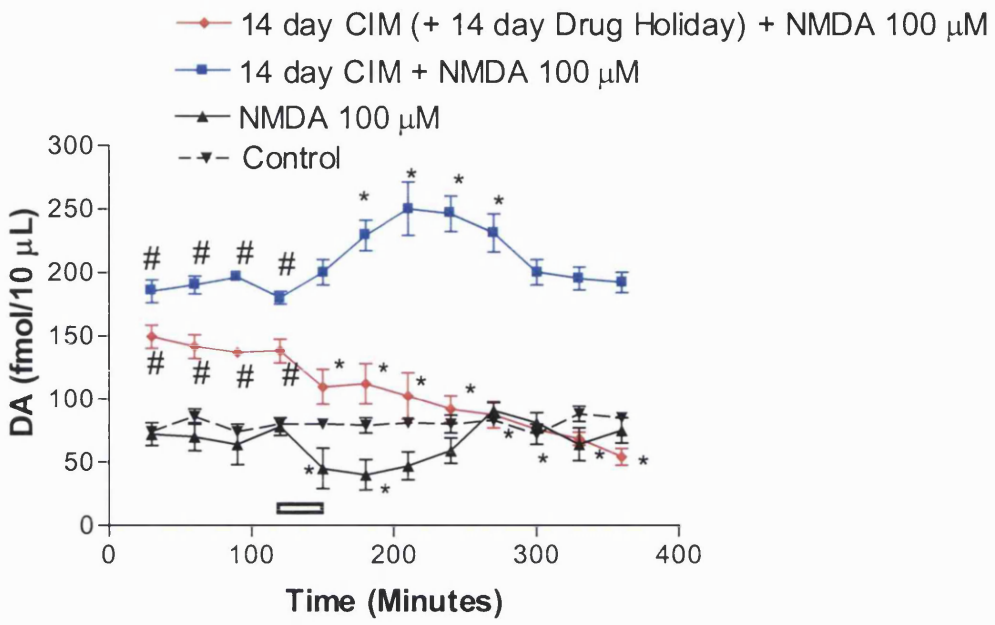


Figure 3.14. Effect of 14-day (chronic) dosing of clomipamine (CIM, 10 mg/kg) with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular DA and DOPAC in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six-eight animals in each group.* indicates significantly different ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$) from control. # indicates significantly different from treatment basal ($p<0.05$).

(A)



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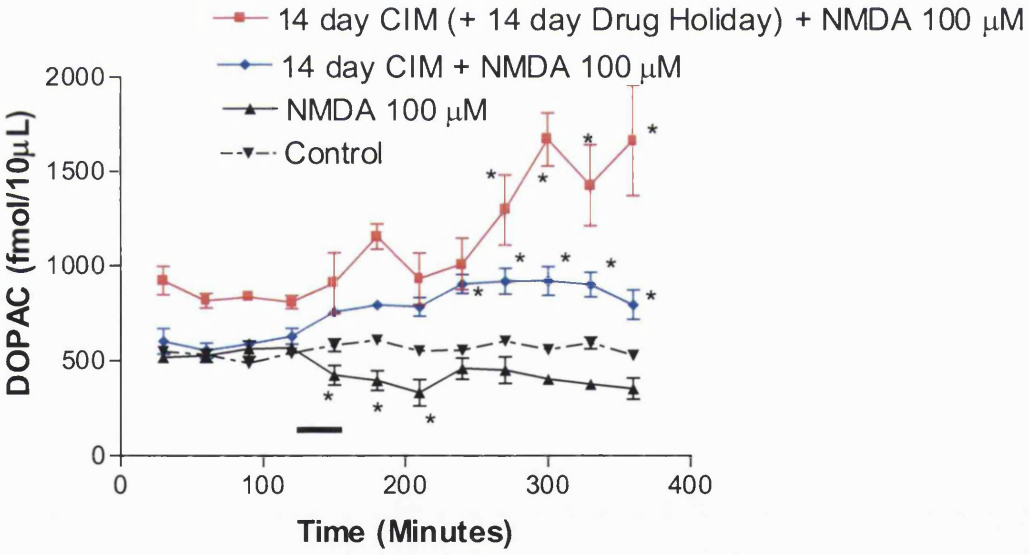


Figure 3.15. Effect of 14-day (chronic) dosing of clomipramine (CIM, 10 mg/kg) with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) DA and B) DOPAC in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

Drug Treatment	DA	DOPAC
Paroxetine 10 mg/kg	N/E	↑
Paroxetine 20 mg/kg	N/E	↓
Paroxetine 100 μM	↑	↑
NMDA 100 μM	↓	40% ↓
NMDA 25 μM *	20 % ↓	↓
Paroxetine 10 mg/kg + NMDA 100 μM	↓	↑
Paroxetine 20 mg/kg + NMDA 100 μM *	N/E	N/E
Clomipramine 10 mg/kg	↓	↓
Clomipramine 20 mg/kg	↓↓	↓
Clomipramine10 mg/kg + NMDA 100 μM	↓	↓
7 day Paroxetine 10 mg/kg + NMDA 100 μM	67% Basal Transient ↑	47% Basal Delayed ↑
21 day Paroxetine 10 mg/kg + NMDA 100 μM	200 % Basal ↑ 6 fold	75% Basal ↑
21 day Paroxetine 10 mg/kg + NMDA 100 μM. Drug Holiday of 48 hours	176% Basal ↑ 2 fold	97 % Basal Transient ↑
21 day Paroxetine 10 mg/kg + NMDA 100 μM. Drug Holiday of 14 days	147% Basal ↓	125 % Basal Delayed Minimal ↑
7 day Clomipramine 10 mg/kg + + NMDA 100 μM	77 % Basal N/E	26 % Basal N/E
14 day Clomipramine 10 mg/kg + NMDA 100 μM	186 % Basal ↑	120 % Basal ↑
21 day Clomipramine 10 mg/kg + NMDA 100 μM	186 % Basal ↑ 5 fold	120 % Basal N/E
14 day Clomipramine 10 mg/kg + NMDA 100 μM. Drug Holiday of 48 hours	268 % Basal N/E	115 % Basal ↑
14 day Clomipramine 10 mg/kg + NMDA 100 μM. Drug Holiday of 14 days	156 % Basal ↓	148 % Basal Delayed ↑

Table 3.1 Summary of chapter 3 results

↑ and ↓ Increase and decrease in efflux respectively.
N/E No effect in efflux.

* Data not shown

3.3 Discussion

3.3.1 The effect of acute, subchronic (7-day) and chronic (21-day) paroxetine/clomipramine treatment on extracellular DA and DOPAC changes in the frontal cortex

3.3.1.1 Acute paroxetine and clomipramine treatment

Acute systemically administered paroxetine had no effect on DA efflux in the frontal cortex in this study whereas acute administration of clomipramine led to a dose-dependent decrease in extracellular DA levels. During the final preparation of this manuscript, a similar microdialysis study by Bymaster and colleagues (2002) investigating the effect of paroxetine (3 mg/kg s.c) on DA efflux in the frontal cortex confirmed that paroxetine does not affect DA efflux in the frontal cortex. Although, this dose is lower than that used in the present study (10 mg/kg), the s.c. route, however, reduces hepatic metabolism of paroxetine therefore exposing a greater concentration of the drug to the brain. These authors report that at this dose plasma levels of paroxetine were 129 ng/mL and as many patients responding to paroxetine treatment for depression and OCD exhibit serum concentrations between 10-200 ng/mL (Eap et al, 1998; Owens et al, 2000), the dose (10 mg/kg) used in this present study was within the therapeutic range of paroxetine.

Similar microdialysis experiments have also used paroxetine 10 mg/kg (Ramaiya et al, 1997; Roberts et al, 1998) and this dose was used by others examining the effect of paroxetine on uptake transporter function (Piñeyro et al, 1994). However, Meltzer et al (1993) reported that paroxetine (10 mg/kg) produced a significant decrease in extracellular DA in the nucleus accumbens and striatum. In the light of these findings and those of this present study, it is evident that there is considerable regional diversity with regard to the effect of acute paroxetine on DA efflux.

The clomipramine data presented here supports previously published data from this laboratory (Pallotta et al, 1999). Clomipramine has previously been shown to alter DA efflux in the frontal cortex (Pallotta et al, 1999) and in other brain regions (Ichikawa and Meltzer, 1995) using similar doses to those used in the present study. Friedman and Cooper (1983) found a maximal clomipramine concentration of 1.1 μM in rat cortex

after the i.p. administration of 15 mg/kg clomipramine, while Adell and Artigas (1991) estimate after clomipramine (20 mg/kg i.p.) a maximal concentration of 1.5 μM can be assumed. Estimates from the dose used in this study (10 mg/kg) would give a maximal concentration of 0.7 μM in the brain. Ichikawa and Meltzer (1995) have observed that acute clomipramine did not alter extracellular levels in the nucleus accumbens but increased DA efflux in the striatum. In light of these findings and those of this current study, it is evident that, like paroxetine, there is also considerable regional diversity with regard to the effect of acute clomipramine on DA efflux.

Paroxetine and other SSRIs such as fluoxetine and sertraline have not been reported to have any significant affinity for DA receptors or DA uptake sites (Paroxetine IC_{50}=5100 nmol/L see Kent, 2000) as the affinity of paroxetine and other SSRIs for the dopamine transporter (DAT) is much lower than the 5-HT transporter (SERT) (Thomas et al, 1987). In contrast to paroxetine and other SSRIs, clomipramine has been shown to have a relatively high affinity for central D_2 receptors, in addition to an affinity for H_1 and α_1 adrenergic receptors. There are no reports to suggest the decrease in extracellular DA levels seen in these experiments when clomipramine was administered systemically is due to a direct effect of clomipramine on DA uptake sites (K_i = 1.8 μM) except for the relatively weak affinity of clomipramine for D_2 receptors (K_i = 190 nM) (Richelson and Nelson, 1984; Richelson and Pfenning, 1984).

Collectively, these data indicate that the effects of acutely administered clomipramine are unlikely to be mediated via an inhibitory interaction with the DA transporter but are more likely to be the result of secondary changes in the reuptake of other transmitters, presumably 5-HT, the uptake of which is more potently altered by clomipramine. Moreover, it is known that 5-HT decreases DA release in certain brain regions (Westfall and Tittermay, 1982).

The subsequent decrease seen in DA efflux may be due to the breakdown of clomipramine to its metabolite, desmethylclomipamine (Fujita et al, 1991), which has been shown to have a higher affinity for the NA transporter (Benfield et al, 1980) and which may decrease uptake by the noradrenaline transporter (NET). Pozzi et al, (1994) have provided evidence that extracellular concentrations of DA are regulated by noradrenergic neurones, via the noradrenergic uptake mechanism, in the frontal cortex of rats. Studies by Carboni et al (1990) and Izenwasser et al (1990) suggest that DA

uptake blockers have no effect on DA efflux in the frontal cortex as most of the DA is taken up by noradrenergic terminals. On the other hand, acute administration of other antidepressants such as fluoxetine, imipramine and desipramine have been reported to cause modest increases in extracellular DA in the frontal cortex upon acute i.p. treatment (Tanda et al, 1994, 1995 1996). From the above observations, it can be seen that, in general, the mechanism of action of ADs and possibly their therapeutic effect may involve the dopaminergic system.

Acute paroxetine did not appear to affect extracellular DOPAC efflux while acute clomipramine decreased extracellular DOPAC efflux. No change in extracellular DOPAC levels following paroxetine treatment would also be in agreement with microdialysis studies by Bymaster et al (2002) following acutely administered paroxetine and a wide range of SSRIs. In addition, Di Chiara and Imperato (1988) report that acutely administered imipramine had no effect on DA and DOPAC efflux in the nucleus accumbens. However, acute clomipramine administration caused a decrease in both DA and DOPAC efflux which is consistent with a study by Garrett and Soares-Da-Silva (1990) who report that DOPAC in the brain is derived from the deamination of DA by MAO_A, and so a decrease in DA efflux would be expected to be paralleled by a decrease in DOPAC levels.

There are conflicting reports on the value of DOPAC measurements to assess the degree of DA release, but several authors strongly suggest that the deamination of DA to form DOPAC occurs before DA is released from presynaptic terminals (Zetterström et al, 1988; Garrett and Soares-da-Silva, 1990; Soares-da-Silva and Garrett, 1990) and it was concluded that DOPAC efflux is more indicative of DA synthesis than of DA release. Therefore, a decrease in DOPAC efflux following clomipramine administration suggests a decrease in DA synthesis which is subsequently shown by a decrease in DA efflux. Interestingly, the rate of utilisation of DA in the frontal cortex is greater than in the mesolimbic and nigrostriatal DA terminal areas (Sharp et al, 1986; Soares-da-Silva and Garrett, 1990). This has also been proven by electrophysiological studies that have shown that the rate of firing of dopaminergic neurones in the frontal cortex is greater than in the mesolimbic and nigrostriatal pathways suggesting increased DA release (for review see Bannon and Roth, 1983). These differences in the metabolism of DA to DOPAC are largely derived from the rates of formation of DA and not differences in MAO activity.

3.3.1.2 Local infusion of paroxetine

The local infusion of 100 μM paroxetine directly into the frontal cortex produced a slight increase in both extracellular DA and DOPAC efflux whereas systemic paroxetine failed to increase extracellular DA efflux. The increase in DA efflux seen with infused paroxetine may seem unusual as systemic paroxetine did not affect DA efflux. However, Jordan and colleagues (1994) report similar increases in extracellular DA efflux to 600%, 360% and 200% above basal levels following fluoxetine, fluvoxamine and imipramine infusion, respectively, into the frontal cortex. The pronounced increase in extracellular DA levels achieved with fluoxetine compared to paroxetine used in the current study is most probably due to the high dose (1 mM) used by these authors. The paroxetine dose of 100 μM used in this study is similar to that used to infuse the ADs imipramine, desipramine and citalopram into the frontal cortex (Golembiowska and Zylewska, 1999).

A thorough literature review does not reveal paroxetine's receptor affinity (K_i) for the DA uptake transporter (DAT) in the frontal cortex, however, as mentioned previously, a review by Kent (2000) reveals paroxetine's potency (IC_{50}) for inhibiting DA uptake to be in the range of 5.1 μM. A calculation from the K_i values published by Hughes and Stanford, (1996) and a review (Stanford, 1996) generates a value for DA uptake affinity of paroxetine to be in the range of 0.9-8.4 μM in the striatum. These values may differ from those for the frontal cortex (see Stanford, 1996) but the dose of 100 μM paroxetine infused into the frontal cortex and surrounding the probe will be well within the range of the K_i for inhibition of DA uptake assuming a probe efficiency of at least 10%. It is thus quite difficult to make dose-effect comparisons between drugs applied locally and those applied systemically because of the variance in the exact concentration of drug in the extracellular environment surrounding the probe.

It would have been interesting to measure NA levels upon paroxetine infusion as an increase in NA efflux is the most likely explanation causing an increase in DA efflux here. This hypothesis is supported by a study of Owens and colleagues (1997) who report paroxetine, compared to other SSRIs, possesses moderate affinity for the rat noradrenaline transporter/uptake sites (NET) with a K_i of 59 nM. Infusion of the potent SSRIs, fluoxetine (10 μM) and citalopram (100 μM) into the frontal cortex causes an increase in both NA and DA efflux. A possible explanation for this could be the

interaction between noradrenergic and dopaminergic neurotransmission as it has been demonstrated that the blockade of the noradrenaline transporter (NAT) increases extracellular DA levels in the frontal cortex, providing evidence that NET is also involved in clearing DA in the frontal cortex of the rat (Carboni et al, 1990; Pozzi et al, 1994). Noradrenergic innervation is also denser than the dopaminergic innervation within the frontal cortex (Slopsema et al, 1982). Thus it may be that in this study infused paroxetine blocked the NAT resulting in an increase in DA efflux in the frontal cortex. Recently work by Mazei et al (2002) reported that NAT can clear extracellular DA, especially in regions that have much less DAT than NAT, such as in the prelimbic cortex.

Alternatively, the increase in DA efflux could be due to increases in 5-HT efflux which in turn affect extracellular NA and DA efflux (Figure 3.16). As an evident increase is seen in 5-HT efflux upon paroxetine infusion in Chapter 4 an explanation for the increase in DA efflux could be the facilitation of DA release by 5-HT in rat frontal cortex mediated by 5-HT_{1B} (Iyer and Bradberry, 1996) or 5-HT_3 receptors (Tanda et al, 1995). 5-HT may also elevate DA efflux via a non-exocytotic mechanism by penetrating into DA terminals through DA uptake sites and displacing DA from its vesicular pool (De Deurwaerdere et al, 1996).

3.3.1.3 Subchronic and chronic paroxetine/clomipramine treatment

Interestingly, there were different results after a 7-day subchronic and a 21-day chronic treatment with both paroxetine and clomipramine (10 mg/kg). Extracellular DA and DOPAC levels in the frontal cortex were decreased upon the 7-day subchronic treatment with paroxetine or clomipramine treatment. The 21-day chronic treatment with paroxetine or clomipramine increased extracellular DA levels whereas extracellular DOPAC levels were modestly decreased with paroxetine treatment and increased with the clomipramine treatment. Both effects on extracellular DA and DOPAC levels in the frontal cortex following subchronic and chronic treatments were similar in magnitude.

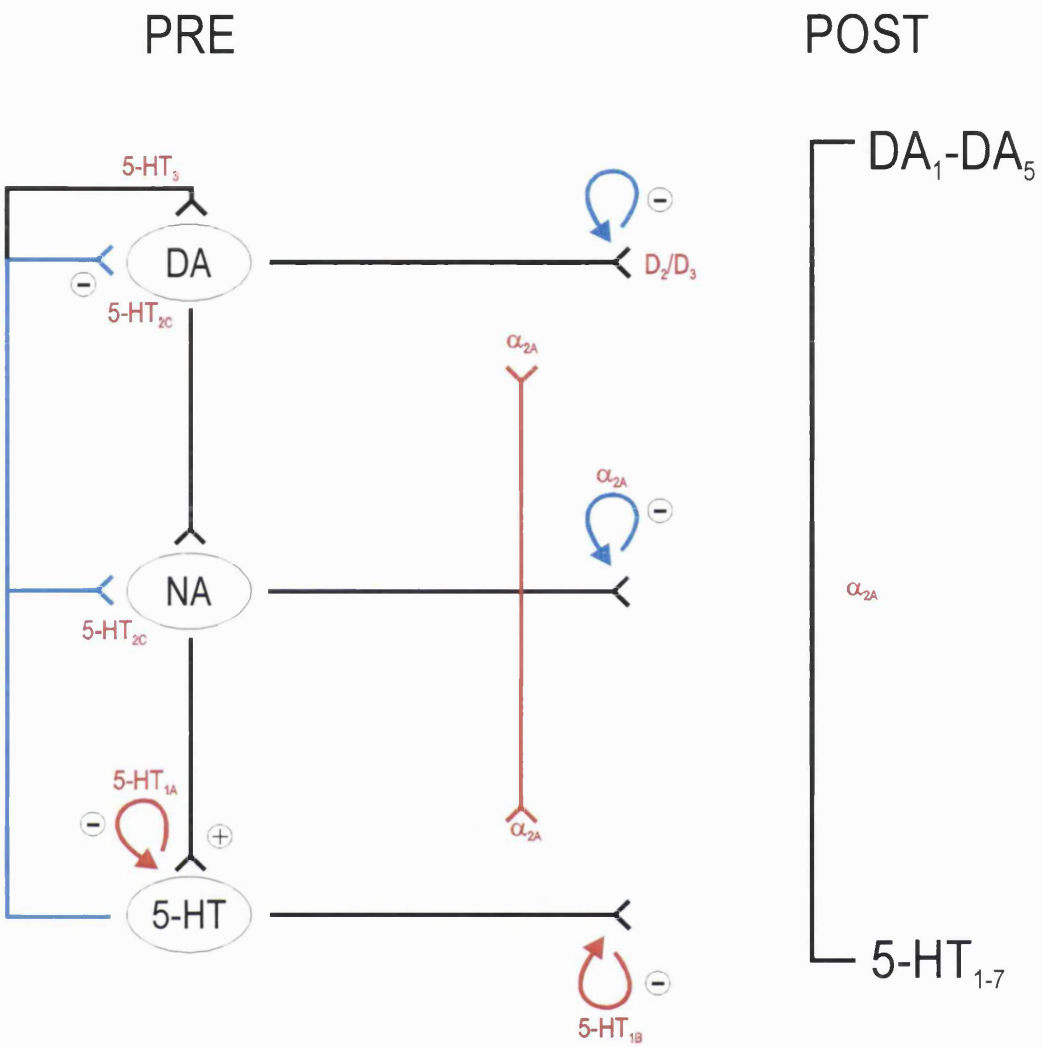


Figure 3.16 Schematic representation of the interrelationship between serotonergic, dopaminergic and noradrenergic transmission in the frontal cortex at the presynaptic level: autoreceptors and heteroreceptors (Adapted from Gobert et al, 1997).

- Dopaminergic neurons bear DA D_2/D_3 (cell body and terminal) autoreceptors and α_2-adrenergic (terminal) heteroreceptors. At the cell body level, they are regulated by a tonic serotonergic input from the dorsal raphe, which involves 5-HT_{2C} receptors (Lejeune et al, 1997). In addition, the LC probably exerts a modulatory role on the VTA via α_1-adrenergic receptors.
- Noradrenergic neurons express α_2-adrenergic (cell body and terminal) autoreceptors. Similar to VTA DAergic neurons, they are controlled by a tonic serotonergic input from the dorsal raphe, which involves 5-HT_{2C} receptors.
- Serotonergic neurons express 5-HT_{1A} (cell body) and 5-HT_{1B} (terminal) autoreceptors and α_2-adrenergic (terminal) heteroreceptors. Noradrenergic neurons also exert a stimulatory influence on the dorsal raphe via α_1-adrenergic receptors.

This is an interesting finding bearing in mind that paroxetine and clomipramine are different classes of ADs. The observation that after subchronic treatment with both clomipramine or paroxetine, extracellular DA was decreased in the frontal cortex suggests that adaptations are occurring within 7 days. A 4-day study using clomipramine performed in this laboratory showed that extracellular DA concentrations were decreased under these conditions too (unpublished observations). This suggests that adaptations can occur after as little as 4 days (Refer section 3.3.1.4).

One explanation for this decrease after 4-7 days treatment with paroxetine or clomipramine is that the 5-HT system is being compromised by these drugs leading to 5-HT being taken up into DA neurons. This is in agreement with a recent study published (Zhou et al, 2002) which suggests 5-HT can be taken into DA neurons when the SERT is not functionally adequate to remove extracellular 5-HT. In addition, these authors suggest that the DA transporter is responsible for 5-HT uptake into DA neurons when SERT is compromised.

This study demonstrates that cross-neuronal uptake exists and serves as a compensatory backup when a specific transporter (such as SERT) is compromised such as following 7 days treatment with paroxetine or clomipramine. The decrease seen in extracellular basal levels of DA suggests a decrease in dopaminergic function as the DA neurons are storing 5-HT as a 'false transmitter', a borrowed transmitter of another system. In this study, a subchronic period of 7-days and not 4-days was chosen as Paul et al (1994) report that the ligand binding properties of the NMDA receptor complex require 7 to 14 days of AD treatment before changes are noticeable. A 21-day chronic dosing regime was also chosen as the same authors report that the effect of imipramine was not fully manifested during 14-days of treatment because a 21-day dosing regime increased the IC_{50} of glycine by 330% of control values. Therefore, this suggests that there may possibly be a further change in NMDA-evoked monoamine efflux measured by microdialysis for a 21 day dosing regimen when compared to the 14 day dosing regime. This study is the first to measure the effects of paroxetine and clomipramine on extracellular DA release in the frontal cortex in both a subchronic (7-day) and a chronic (21-day) time frame.

Although, as previously mentioned, the levels of extracellular DA in the frontal cortex decreased and increased by the same magnitude using both paroxetine and

clomipramine in both the subchronic and chronic studies respectively, the effect of chronic clomipramine and paroxetine on extracellular DOPAC varied. An increase in extracellular DOPAC levels was observed following chronic clomipramine treatment whereas a decrease in extracellular DOPAC levels was observed following chronic paroxetine treatment. This is in agreement with a study by Nomikos et al, (1991) who saw a similar increase in DOPAC levels in the nucleus accumbens and the striatum after a 2 week chronic treatment with desipramine. Further support for the chronic paroxetine findings is provided by, an earlier chronic study by De Montis et al, (1990). Using imipramine (2-3 weeks dosing, twice daily, 10 mg/kg) they reported a reduction in DOPAC levels in the limbic regions (containing the olfactory tubercle, the nucleus accumbens and the septum). These authors suggested that chronic imipramine treatment reduced DOPAC levels as DA in the limbic system is recaptured not only by dopaminergic but also noradrenergic terminals. This is unlikely to occur in the striatum which is more densely innervated by dopaminergic than noradrenergic nerve terminals.

It is unlikely that these changes in DOPAC levels after chronic paroxetine and clomipramine treatment are due to these ADs acting directly on MAO. A recent study by Silver and Youdim (2000), examining the long-term administration (up to 6 weeks) of fluvoxamine, desipramine and saline on MAO-A and MAO-B activities in the frontal cortex and striatum, reported that MAO activity is not altered by chronic TCA or SSRI treatment. In addition, previous studies (Green and Youdim, 1975; Green et al, 1977) showed that a reduction of at least 85% in MAO activity is required to produce a significant elevation in monoamine, NA and 5-HT levels. The changes seen here in basal extracellular DOPAC levels following chronic paroxetine and clomipramine are most probably due to changes in DA synthesis.

3.3.1.4 Adaptation following AD treatment?

The increase in extracellular DA levels following both chronic paroxetine and clomipramine administration could be the result of adaptive changes in serotonergic transmission in the cortex, leading to increased levels of extracellular cortical 5-HT and thereby a direct effect of 5-HT on DA release, most probably mediated by 5-HT_{1B} (Iyer and Bradberry, 1996) or 5-HT_3 receptors (Tanda et al, 1995). Although the mechanism by which 5-HT facilitates extracellular DA release is presently unclear, studies using intracellular recordings in vertebrates indicate that 5-HT affects neuronal activity by

altering K^+ permeability (Joels and Gallagher, 1988). Alternatively, adaptive changes in the dopaminergic regulatory system itself, such as alterations in D_2 receptor density or function, offer another explanation.

It has been suggested that after chronic treatment with antidepressants, the motor stimulant effect of the DA receptor agonist, apomorphine is potentiated, while the hypomotility and inhibition of DA synthesis produced by the small amounts of DA synthesis are prevented (Serra et al, 1979). D'Aquila and colleagues (2000) interpreted these findings by suggesting that chronic AD treatment potentiates DA transmission by activating a population of normally subsensitive DA autoreceptors. Thus, chronic AD treatments potentiate DA transmission possibly via an increased sensitivity of postsynaptic DA receptors (Spyraki and Fibiger 1981) rather than a decreased sensitivity of presynaptic DA receptors.

Although chronic ADs fail to modify the behavioural responses to D_1 receptor stimulation, a decrease in D_1 receptor number and sensitivity has been reported in the prefrontal cortex (Paetsch and Greenshaw, 1992; Gambarana et al, 1995). Another finding consistent with this, is that chronic treatment with a range of ADs, such as fluoxetine, desipramine and tranylcypromine, induced an increase in D_2 receptor mRNA in the nucleus accumbens but not in the striatum (Ainsworth et al, 1998). Additionally, the levels of mRNA coding for D_2 receptors increased both in the nucleus accumbens and in the striatum upon chronic administration of the ADs imipramine and citalopram (Dziedzicka-Wasylewska et al, 1997).

Maj et al (1998) have also reported that chronic treatment with various AD drugs (such as imipramine, amitriptyline, citalopram and mianserin) potentiated the locomotor response to the D_3 receptor agonist 7-OH-DPAT and increased the density of D_3 receptors in the shell of the nucleus accumbens. No studies have yet demonstrated whether chronic paroxetine or clomipramine treatments alter DA receptor numbers or sensitivity but, as previously mentioned, other chronic AD treatments increase the sensitivity to DA receptor stimulation via increases in D_2-like (i.e. D_2 and D_3) receptor function and/or decreased D_1 receptor number and sensitivity. These changes are most prominent in the limbic areas innervated by DA neurones with cell bodies in the VTA or projecting from the VTA.

Dopaminergic pathways are postulated to project from the VTA and SN to the raphe (Ferre and Artigas, 1993), and D_2 receptors have been shown to mediate increased release of 5-HT (Stern et al, 1981; Kalen et al, 1988) in this region. This may lead to DA changes in the raphe which could may regulate raphe 5-HT causing changes in frontal cortex 5-HT and thus DA. It is believed that these adaptive changes occur following subchronic and chronic paroxetine and clomipramine treatment with subsequent modulation of DA levels in the frontal cortex. This supports the idea that paroxetine, a selective SSRI (and clomipramine to a lesser extent), which lack direct effects on the dopaminergic system, exert secondary actions on the dopaminergic system via 5-HT or NA, effects which may be important in the clinic.

For some years evidence has suggested that DA may play a role in the aetiology and possibly treatment of depression. There is a clear difference between the acute and chronic effects of the clinically used ADs, paroxetine (the SSRI) and clomipramine (the TCA), on extracellular DA efflux. In this study acute paroxetine failed to elicit any effect on extracellular DA levels whereas acute clomipramine decreased extracellular DA levels. Additionally, chronic paroxetine and clomipramine treatment increased DA levels. It is evident that acute dosing regimens may be misleading when interpreting antidepressant effects on neurotransmitter release. These changes in extracellular DA would require some sort of biochemical/physiological adaptation for the clinical effect of both paroxetine and clomipramine to take effect. This mechanism may be partly responsible for the latency in effect between onset of AD treatment and clinical effectiveness being achieved.

3.3.2 Effects of NMDA on basal DA and DOPAC efflux in the frontal cortex

Dopamine is thought to play an important role in the associative functions of the prefrontal cortex (Goldman-Rakic, 1987). In the rat, DA projections to the PFC mainly arise from the VTA and innervate primarily the medial layers of the PFC (Fallon, 1989). This area also receives major excitatory (e.g. glutamatergic) inputs from the mediodorsal thalamic nuclei (Groenewegen, 1988) and cortical regions including the hippocampal formation (Jay et al, 1992). This axo-axonal association may be the site for the presynaptic interaction between glutamatergic and DA-ergic terminals within the PFC.

A considerable body of literature indicates that excitatory amino acids are able to facilitate the release of DA (Barbeito et al, 1990; Leviel et al, 1990; Krebs et al, 1991). Krebs and colleagues (1991) speculate that glutamate predominantly potentiates DA release by activating NMDA receptors located presynaptically on DA-ergic afferents. Microdialysis studies have demonstrated that glutamate, AMPA, kainate and NMDA increase the release of DA in the striatum and nucleus accumbens (Imperato et al,1990; Moghaddam et al, 1990; Youngren et al, 1993; see Whitton 1997) whereas NMDA-receptor antagonists have been reported to increase DA turnover in vivo (Wedzony et al, 1993; Hondo et al, 1994).

In the present study, the local application of NMDA (25 and 100 μM), reduced extracellular DA levels in the frontal cortex. Extracellular DOPAC levels were also sharply decreased following NMDA infusion reflecting decreased metabolism of DA within the frontal cortex nerve terminals. This is in agreement with similar findings by Jedema and Moghaddam (1996) who used local application of NMDA 20 and 100 μM. Findings in this current study and the study by Jedema and Moghaddam (1996) using the lower and higher concentrations of NMDA observed there was no difference in the effect on extracellular DA levels for either the lower or higher dose used. Higher concentrations of NMDA (≥ 1mM) have been reported by Westerink et al, (1992) to increase extracellular levels of DA in the striatum. This increase in extracellular DA is associated with behavioural activation and this has been attributed to generalised neuronal excitation (Keefe et al, 1992).

It has also been demonstrated that the increase in the release of PFC DA during stress potentiates the outflow of glutamate (Moghaddam, 1993). The lack of an excitatory effect of NMDA on extracellular DA release in the PFC and the possibility that activation of NMDA receptors in the PFC may decrease the release of DA in this region, is consistent with reports that NMDA-receptor antagonists increase DA turnover in the PFC (Wedzony et al, 1993; Hondo et al, 1994; Nishijima et al, 1994). This suggests that the release of DA in the PFC is under tonic-inhibition by NMDA receptors. Jedema and Moghaddam, (1996) suggest that the efflux of DA in the PFC is may be modulated by non-NMDA receptors. The release of DA in the PFC may also be facilitated by AMPA and kainate receptors.

Another explanation for the decrease in extracellular DA following NMDA infusion could be that the NMDA receptors may indirectly inhibit DA-ergic neurotransmission in the PFC. A number of studies have suggested that GABA interneurons may play a role in the interaction between glutamate and DA neurons in the prefrontal cortex. NMDA receptors may regulate cortical GABA release (Drejer and Honoré, 1987), and $GABA_A$ receptors, which demonstrate a relatively high density in the prefrontal cortex (Bowery et al, 1987), may regulate prefrontal DA release (Santiago et al, 1993). It has also been reported that NMDA (100 μM) increases the efflux of GABA in the PFC (Santiago et al, 1993). Indeed, the same report found that GABA-receptor agonists inhibit, whereas GABA antagonists facilitate the release of DA in the PFC (Santiago et al, 1993) suggesting other neurotransmitters may be involved in regulating extracellular DA levels in the PFC. Taken together, whatever the mechanism these observations suggest a close association between glutamatergic, gabaergic and dopaminergic pathways in the prefrontal cortex.

It is widely accepted that extracellular DA release can be modulated by NMDA receptors (Feenstra et al, 1995; Whitton 1997; Pallotta et al, 1999; Kretschmer, 2000; Kretschmer et al, 2000). However, the relationship between NMDA receptors and DA efflux in the PFC has not been fully investigated. Therefore, I investigated the roles of NMDA receptors on DA receptor neurotransmission induced by the chronic administration of antidepressants.

3.3.3 Effects of NMDA on basal and paroxetine/clomipramine-induced DA and DOPAC efflux in the frontal cortex

NMDA, when infused after a single injection of paroxetine, generated no marked differences between the reduction in PFC DA release evoked by NMDA alone or NMDA + paroxetine treated groups. Similarly, in the case of clomipramine, rats acutely pre-treated with clomipramine displayed decreases in DA release similar in magnitude to those seen after administration of NMDA alone. However, when rats were subchronically (7-days) treated with paroxetine or clomipramine the decrease in extracellular DA following NMDA infusion in the frontal cortex was abolished. In fact, a tendency towards an *increase* in extracellular DA was observed following subchronic paroxetine treatment. Following chronic paroxetine or clomipramine treatment for 21 days, extracellular DA levels increased six and five-fold respectively following acute

NMDA receptor stimulation. Interestingly, no motor stimulation or sterotypical behaviour was seen. It would have been interesting to measure extracellular DA levels in the striatum during this pronounced increase in cortical DA as no behavioural changes were seen when this pronounced increase in DA efflux occurred. It is assumed that as NMDA was infused directly into the frontal cortex, the changes in DA efflux would most probably affect only the region examined. For both subchronic and chronic experiments, extracellular DOPAC efflux was increased following NMDA infusion but during the chronic study, the increase in extracellular DOPAC efflux was greater.

3.3.4 Drug holiday of paroxetine/clomipramine treatment. Any difference in NMDA-induced DA and DOPAC efflux in the frontal cortex?

It can be concluded from the results presented in this study that both chronic paroxetine and clomipramine treatment can cause adaptive changes in as little as 7-days which persist up to 21-days treatment. Using these observations it was decided to establish how long these changes persist after a 'drug holiday' period. A 48 hour drug holiday period and a 14 day drug holiday period were chosen to assess any changes on the NMDA-evoked DA response.

With regard to paroxetine, a 21 day chronic dosing regime was chosen to assess the effects of the drug holiday on NMDA-evoked DA and DOPAC efflux, while for clomipramine, a 14 day dosing regime was chosen. The main reason for this difference was to extend data from a previous study from our laboratory (Pallotta et al, 1999). The effect of a 14-day dosing regime as opposed to a 21-day dosing regime should not affect the drug holiday effects on NMDA-evoked DA efflux. This assumption is based on data from the work of Skolnick et al (1996) who report that adaptation in the radioligand binding properties of NMDA receptors revealed that a statistically significant change in the IC_{50} of glycine to inhibit [^{3}H] 5,7-DCKA binding in the frontal cortex required 10 and 7 days using citalopram and electroconvulsive shock (ECS) respectively. Thus, both the 21 day (in the case of paroxetine) and the 14 day (in the case of clomipramine) dosing regime would be presumed to cause NMDA-evoked changes to the drug holiday study. In order to directly compare paroxetine and clomipramine treatments and their effects on NMDA-evoked DA and DOPAC efflux, it was necessary to investigate the effect of a 14-day chronic clomipramine treatment on NMDA-evoked DA and DOPAC efflux.

After a 14-day chronic treatment with clomipramine, basal extracellular DA levels were increased 2-fold. This is similar to a previous study in this laboratory (Pallotta et al, 1999) in which a 15-day chronic clomipramine treatment increased basal extracellular DA levels dose-dependently. The increase of basal extracellular DA levels was similar to that of the 21 day study (refer section 3.3.1.3). Therefore, it is possible that the changes in basal dialysate DA reflect the fact that clomipramine, within this treatment time, is converted into a metabolite, desmethylclomipramine, which has a higher affinity for the NA transporter than the parent drug leaving excess NA around to mediate increases in extracellular DA in the frontal cortex (Pozzi et al, 1994). However, there was no effect on extracellular DOPAC basal levels.

Following the 14-day clomipramine study, the effect of NMDA on DA efflux was to reverse the sustained decrease seen with acute clomipramine + NMDA, though there was no increase similar to that seen in the 21-day study. These results were similar to previous work performed in this laboratory (Pallotta et al, 1999) who report that NMDA infusion was followed by an increase in dialysate DA efflux which was proportionately greater with the higher dose of 20 mg/kg clomipramine. A reason for the difference between the greater increase seen after the 21-day (10 mg/kg) and the 14-day higher dose (20 mg/kg) of clomipramine may be interpreted from the findings of Skolnicks and colleagues (1996) as a result of the 21 day dosing regime being able to further increase the IC_{50} of glycine to inhibit [^{3}H] 5,7-DCKA binding in the frontal cortex, an effect which is dose dependent. This further change in NMDA receptor binding following the 21 day study may cause the greater increase in DA efflux when the NMDA receptor is stimulated.

After a 21-day treatment, the paroxetine-treated rats with a 48 hour drug holiday showed similar levels of extracellular DA to those animals treated with a similar dose regimen, but without the drug holiday (200% compared to 176% above basal levels). Extracellular DA levels in rats treated with 21 day paroxetine including a 14 day drug holiday, were significantly lower than those values obtained following 21 day drug treatment alone. Nevertheless, extracellular DA concentration in the 21 day treated animals were significantly greater than basal. A similar trend was seen in the clomipramine-treated rats. After a 48-hour drug holiday an augmentation in dialysate DA levels that superceded those values obtained in rats treated with an identical dose regimen but without the drug holiday was seen. As with paroxetine, the clomipramine-

treated rats with a 14-day drug holiday exhibited dialysate DA levels that were significantly greater than basal values. The increase in extracellular DA levels between the paroxetine and clomipramine groups were of similar magnitude.

During the 48 hour drug holiday period, we are confident that both paroxetine and clomipramine were extensively metabolised and excreted from the body. Therefore, over the 48 hour drug-free period we would expect plasma drug levels to fall to pharmacologically negligible levels. A study by Cremers et al, (2000) reported that citalopram plasma levels fell from 0.5 μM to 8.2 nM (the pharmacologically inactive plasma level is 0.01 μM) upon a 2 day washout period. When extracellular DA (and 5-HT) levels were measured after a 48-hour 'drug holiday', these monoamines were still elevated compared to baseline. As mentioned above, it is unlikely, that the persistent effects of chronic paroxetine and clomipramine dosing on extracellular DA (and 5-HT) levels were caused merely by the accumulation and presence of higher plasma levels of paroxetine and clomipramine and their metabolites, as substantial allowance for their clearance was given after the last drug treatment. The mean elimination half life of paroxetine and clomipramine are 8 (Owens et al, 2000) and 6 (Weigmann et al, 2000) hours, respectively, in rats. As mentioned earlier, paroxetine's metabolites (glucuronide and sulphate conjugates; Haddock et al, 1989) are inactive and clomipramine metabolite, desmethylclomipramine is active (Weigmann et al, 2000).

Following chronic treatment with paroxetine for 21 days with 48 hours drug holiday, extracellular DA levels increased transiently upon NMDA receptor stimulation. However, following chronic treatment with clomipramine for 14 days with 48 hours drug holiday, the decrease in extracellular DA levels seen in the acute study was abolished. After the same chronic drug treatments, but with a 14 day 'drug holiday', NMDA receptor stimulation caused a decrease in DA efflux in both paroxetine and clomipramine treated rats. For both 48 hour 'drug holiday' treatment regimes using paroxetine and clomipramine, extracellular DOPAC levels were increased following NMDA infusion. Similar results were found after a 14 day 'drug holiday' in which upon NMDA receptor stimulation, extracellular DOPAC levels increased.

Changes induced by chronic treatment of paroxetine or clomipramine indicate that this protocol reaches an identical functional endpoint: dampening of NMDA receptor function. As explained in section 4.3.5, marked changes are occurring in the NMDA receptors following chronic AD treatment. The exact reason is as yet unknown as to why a robust increase in DA efflux was observed following NMDA infusion into the frontal cortex in rats treated chronically with paroxetine/clomipramine (see section 4.3.3). However, the increase may be due to a change in NMDA receptor subunit composition which is accompanied by a robust reduction in NMDA-evoked increase in $[Ca^{2+}]$ (Brandoli et al, 1998). This effect can readily be mimicked by direct application of NMDA antagonists which have been reported to cause an increase in DA efflux in the frontal cortex (Wedzony et al, 1993; Hondo et al, 1994).

This finding may possibly suggest that a non-NMDA receptor effect may be occurring. For example, both paroxetine and clomipramine have anticholinergic effects (Gareri et al, 2000) and so the involvement of muscarinic receptors could explain the robust increase in DA efflux because muscarinic receptor antagonists increase DA efflux (Jackisch et al, 1992). However, interactions with other receptors [e.g sigma receptors; (Danysz et al, 1997)] are feasible.

3.3.5 Changes in the NMDA receptor following chronic AD treatment?

It is clear that marked adaptive changes in NMDA receptor function occur in a manner whereby NMDA receptors regulate cortical DA efflux (Figure 3.17). This could explain the effect of both chronic paroxetine and clomipramine treatment on basal extracellular DA. Several studies suggest that AD-induced changes in the mesolimbic system depend on the stimulation of NMDA receptors. For example, studies using MK-801 have shown that chronic but not acute treatment prevents the development of behavioural supersensitivity to DA agonists induced by chronic imipramine treatment (D'Aquila et al, 1992; De Montis et al, 1993) and by challenges with ECT (Nimikos et al, 1992; D'Aquila et al, 1997). Nowak et al, (1993) confirm our theory that, after repeated AD treatment, some sort of NMDA adaptation is occurring. These authors find that chronic treatment with imipramine impairs the normal function of the NMDA receptor-associated cation channel in the nominal absence of agonists such as glycine and glutamate. In a recent review, (D'Aquila et al, 2000) it is clear that chronic treatment with ADs influences a variety of neurotransmitter systems (e.g. DA, 5-HT and NA) and induces a great number of adaptive changes in the brain, such as alterations in receptor sensitivity (e.g. increased D_2 sensitivity) or receptor binding properties (e.g. NMDA). These alterations to the receptor proteins induce potentiation of dopaminergic neurotransmission, a conclusion which supports the results generated with both of the ADs investigated in this study.

In addition to behavioural work implicating the involvement of NMDA receptors in the action of ADs (Trullas and Skolnick, 1990; Papp et al, 1994; Layer et al, 1995), there is neurochemical evidence to support the hypothesis that marked and qualitative changes are occuring in the NMDA receptors following chronic AD treatment. For example, in mice it has been shown that chronic AD treatment leads to a decrease in the affinity of {^{3}H}-5,7-DCKA for the NMDA receptor complex associated glycine site (Paul et al, 1994), therefore presumably decreasing excitatory transmission via NMDA receptors *in vivo*. These data provide further evidence for the probable role of the NMDA receptor in the pathology of depression.

In conclusion, adaptive changes appear to occur in the NMDA receptor complex in as little as 7-days following paroxetine or clomipramine administration, an effect which continues for up to 21-days following initiation of treatment. However, these adaptive

changes in NMDA receptor function appear to revert back to those observed for prechronic drug-treatment following a 14-day drug cessation. From this study these experiments suggest that ADs, either acutely or chronically administered, interfere with DA efflux. This study shows the effects of 2 different classes of ADs which both increase DA efflux in the frontal cortex following chronic treatment. This may reflect a potentiation of dopaminergic neurotransmission which may ultimately contribute to the therapeutic effects observed with these ADs, possibly via depression of glutamatergic transmission involving the NMDA receptor. Paroxetine and/or clomipramine may also mediate their clinical effects via an interaction with glutamatergic/dopaminergic neurons and this should be explored further.

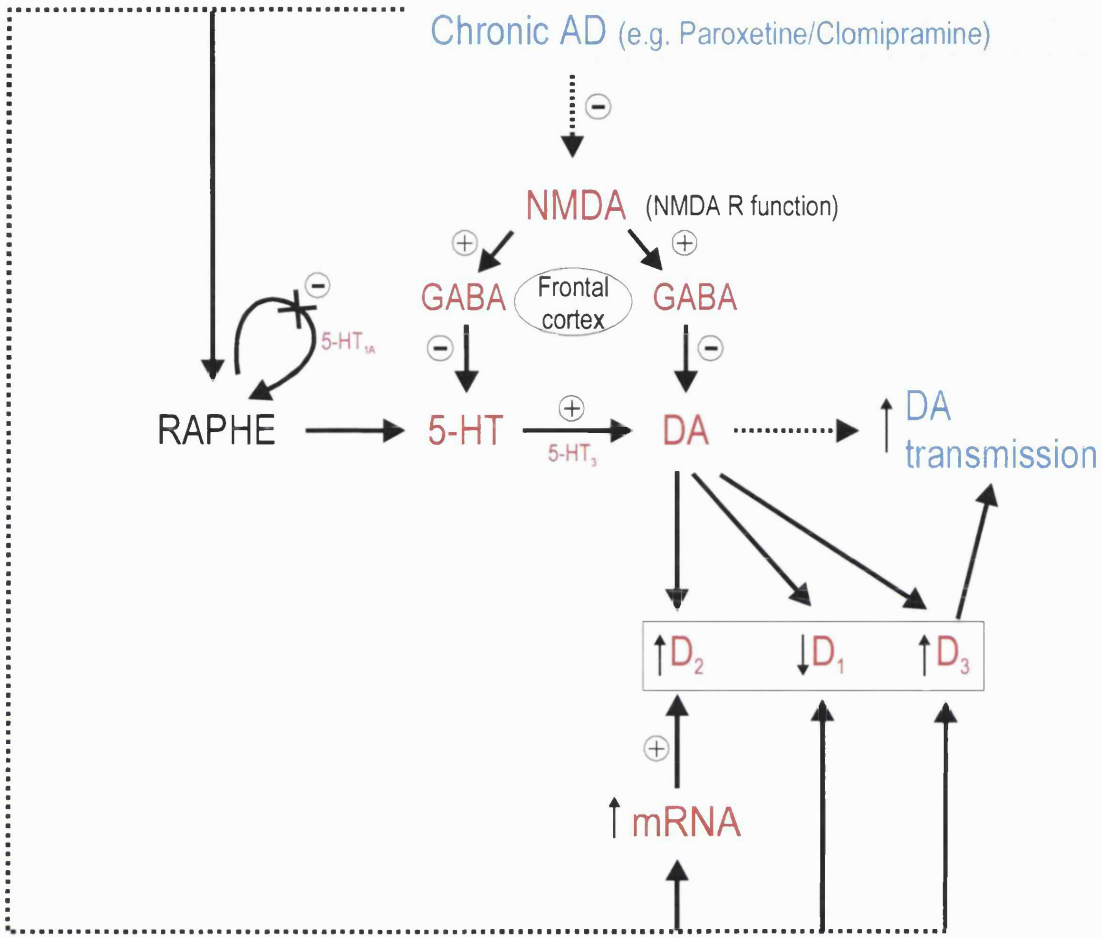


Figure 3.17 Sketch to show the effects of chronic antidepressants on DA transmission.

Chronic ADs (e.g. paroxetine and clomipramine) cause:

- An increase in D_2 receptor mRNA.
- An increase in density of D_3 receptors
- A decrease in D_1 receptor number and sensitivity.
- A functional desensitisation of $5\text{-}HT_{1A}$ autoreceptors, resulting in disinhibition of neuronal firing.
- An increase in 5-HT will cause an increase of DA via $5\text{-}HT_3$ receptors
- A decrease in NMDA receptor function which will cause an increase in 5-HT and DA levels.
- These effects cause an overall **INCREASE IN DA TRANSMISSION**.

Chapter 4

The effect of acute, subchronic and chronic paroxetine and clomipramine treatment on extracellular 5-HT and 5-HIAA efflux in the frontal cortex.

4 The effect of acute and chronic paroxetine and clomipramine treatment on NMDA-evoked extracellular 5-HT and 5-HIAA release in the frontal cortex

4.1 Introduction

It is widely accepted that the neurotransmitter 5-HT plays a crucial role in the aetiology of depression with diminished brain serotonergic activity linked to the manifestation of major depression (Meltzer et al., 1987). Indeed, several classes of antidepressants as well as ECS, lead to a net increase in serotonergic transmission, supporting an important role of 5-HT in antidepressant therapy (Blier and de Montigny, 1994).

As mentioned previously, it has been found that NMDA receptor antagonists can be effective as antidepressants in a rodent behavioural model (Trullas and Skolnick, 1990). It has also been observed that chronic, but not acute treatment with a range of antidepressants including MAOIs, TCAs and SSRIs decreases the sensitivity of NMDA receptors (Paul et al, 1994). We have recently observed that chronic but not acute clomipramine decreases the effect of NMDA receptor activation on serotonergic efflux between the raphe nuclei and the frontal cortex (Pallotta et al, 2001). Collectively, this suggests that NMDA receptor dysfunction may play a role in the pathophysiology of depression. This is supported by the finding that there is a decreased affinity in NMDA receptor binding properties in the frontal cortex of depressed suicide victims (Nowak et al, 1995).

It is possible that interactions between the glutamatergic and serotonergic systems in the brain play a role in the development and potential treatment of depression. As an important role exists for the NMDA receptor in the regulation of 5-HT release in several brain regions (Whitton, et al., 1992ab; 1994b), it is likely that antidepressant related changes in NMDA receptors may alter NMDA-evoked changes in serotonergic transmission. Tao and Auerbach (1996) using microdialysis reported that infusion of NMDA into the dorsal raphe nucleus leads to a concentration-dependent increase in extracellular 5-HT in the same region and this was associated with an increase in extracellular 5-HT in the nucleus accumbens.

In the present study, using in vivo microdialysis in freely moving rats, I investigated whether two clinically used ADs, paroxetine, (a SSRI) and clomipramine, (a TCA) can

alter NMDA receptor mediated 5-HT efflux following acute, subchronic (7-day) and chronic (21-day) treatment and how long these changes persist following cessation of drug treatment ('drug holiday').

The following questions were addressed:

- What are the effects of paroxetine when administered (systemically or locally in the PFC) on 5-HT efflux in the frontal cortex?
- What are the effects of NMDA infusion into the frontal cortex on 5-HT efflux in the same region?
- What are the effects of acute, subchronic and chronic treatment of ADs on NMDA-evoked changes on 5-HT efflux in the frontal cortex?
- If any changes are observed, are they reversed following AD (paroxetine) cessation and what is the time scale?
- Are these changes related specifically to the SSRI, paroxetine, or can we replicate the findings in using a different class of AD, for example the TCA, clomipramine?

4.2 Results

4.2.1 Basal levels of 5-HT and 5-HIAA measured in the frontal cortex

Basal dialysate 5-HT and 5-HIAA values were 19 ± 3 fmoles/10 μL and 3.1 ± 0.2 pmoles/10 μL (n=48). These values are similar to published results from our laboratory (Whitton et al, 1992a; Pallotta et al, 2001).

4.2.2 The effect of acute paroxetine on extracellular levels of 5-HT and 5-HIAA in the frontal cortex

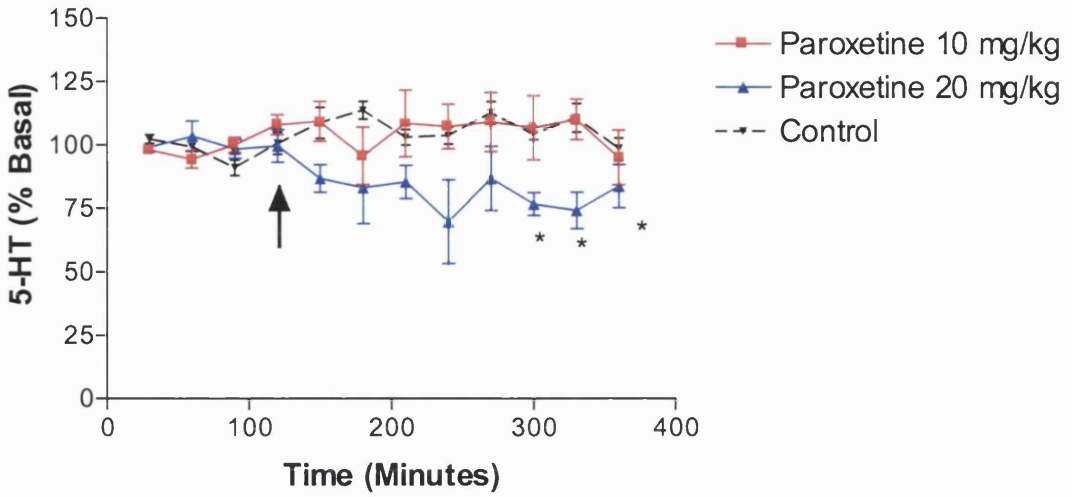
Acute administration of 10mg/kg i.p. paroxetine failed to cause any change in extracellular 5-HT in the frontal cortex. However the higher dose (20 mg/kg) caused a decrease in extracellular 5-HT in the frontal cortex (Figure 4.1A). This decrease in extracellular 5-HT (on average 84% of basal level) was sustained for the duration of the experiment. Two-way repeated ANOVA revealed no significant effects over main factor 'time' [$F(11,99)=1.39$, $p=1.88$] or between treatments of the control and the lower dose of paroxetine [$F(2,9)=5.13$, $p=0.052$].

Paroxetine (10, 20 mg/kg) caused a significant decrease in extracellular 5-HIAA levels over time [$F(11,99)=10.89$, $p<0.05$] and between doses [$F(2,9)=17.41$, $p<0.05$]. The higher dose was slightly more effective by 5% (Figure 4.1B).

Local infusion of paroxetine (100 μM) resulted in a maximal increase in extracellular 5-HT of 283% of basal levels at 150 minutes after the start of infusion (Figure 4.2A). Two-way repeated ANOVA revealed significant effects over time [$F(11,66)=5.36$, $p<0.01$] and between treatment [$F(1,6)=8.04$, $p<0.01$]. This increase of 5-HT was delayed but was sustained until the end of the experiment without returning to basal levels.

Finally, the local infusion of paroxetine did not affect extracellular 5-HIAA levels (Figure 4.2B).

(A)



(B)

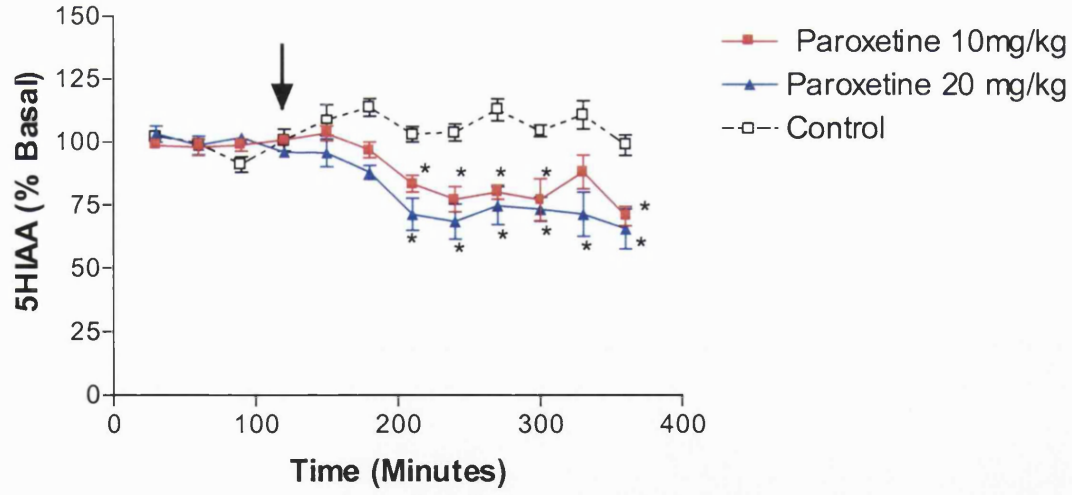
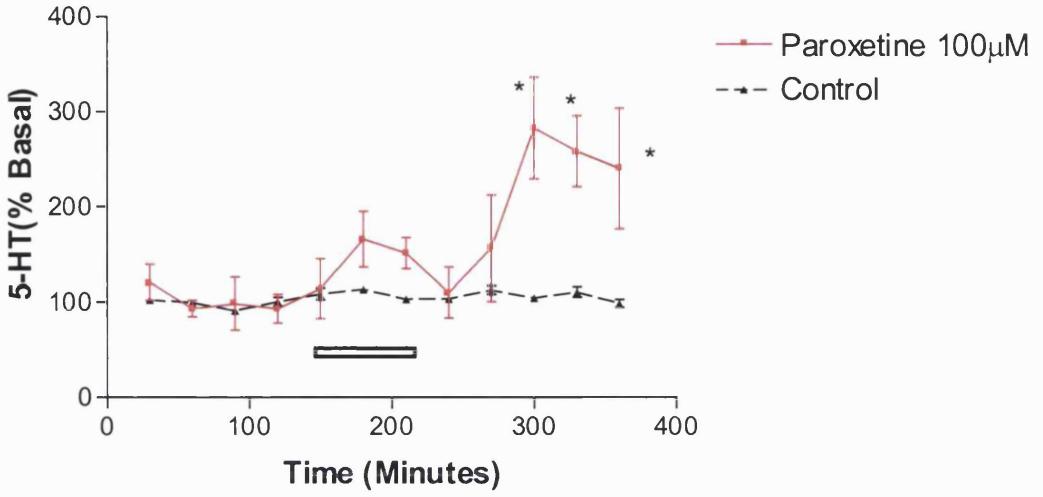


Figure 4.1 The effect of acutely administered paroxetine on extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The arrow indicates the time at which paroxetine (i.p) was administered. Data are the mean ± SEM of six-eight animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

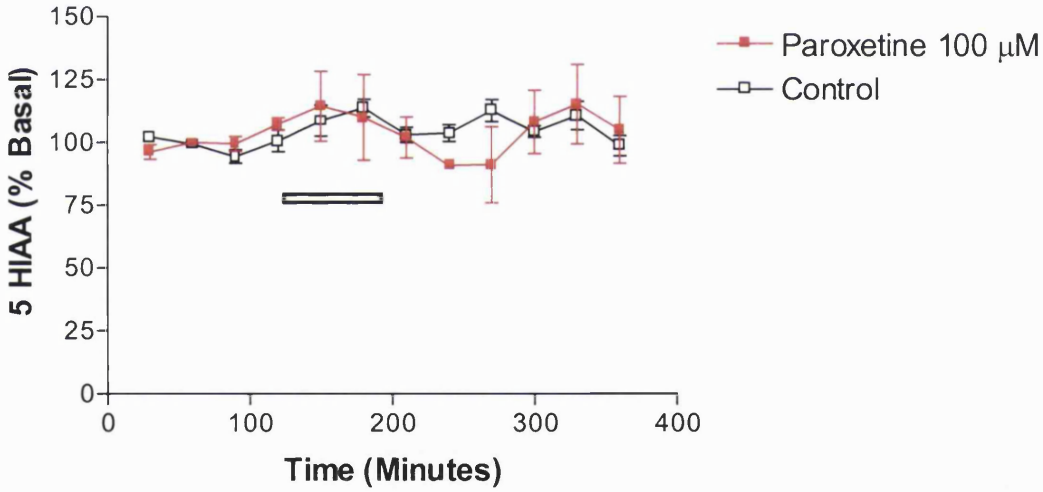


Figure 4.2 The effect of localised paroxetine infusion on extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. Paroxetine was infused into the frontal cortex and extracellular 5-HTmeasured. The solid box indicates the period of paroxetine infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

4.2.3 The effect of acute clomipramine on extracellular levels of 5-HT and 5-HIAA in the frontal cortex

Acute administration of 10 and 20 mg/kg clomipramine caused an apparent decrease in extracellular 5-HT in the frontal cortex that was not significant ($p>0.602$) (Figure 4.3A). Two-way repeated ANOVA revealed no significant effects over main factor 'time' [$F(11,99)=0.6248$, $p=0.99$] or between treatment [$F(2,9)=0.538$, $p=0.602$].

Clomipramine (10, 20 mg/kg) caused a dose-dependent significant decrease in extracellular 5-HIAA levels over time [$F(11,99)=12.20$, $p<0.05$] and between doses [$F(2,9)=31.12$, $p<0.05$] (Figure 4.3B).

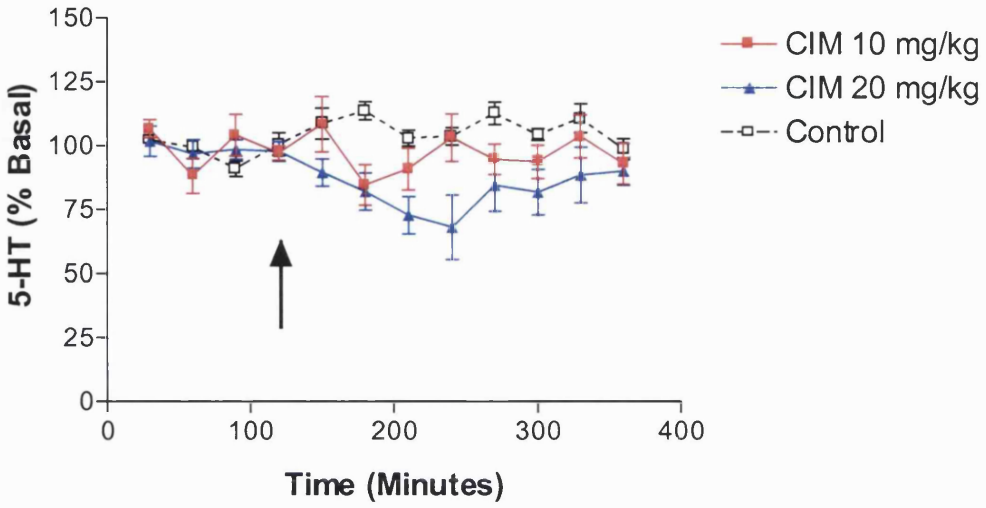
4.2.4 The effect of NMDA infusion into the frontal cortex on extracellular 5-HT and 5-HIAA

Direct, localised infusion of NMDA (100μM) for 30 minutes into the frontal cortex caused a statistically significant decrease of extracellular 5-HT levels ($p<0.01$).

The NMDA (100μM) infusion also caused a rapid, significant [$F(1,6)=10.47$, $p<0.05$] and persistent decline in extracellular 5-HT levels. This significant decrease (57% of basal) [$F(11,66)=4.58$, $p<0.05$] persisted until the end of the experiment (Figure 4.4A).

Figure 4.4B shows that NMDA infused into the cortex did not affect extracellular 5-HIAA levels.

(A)



(B)

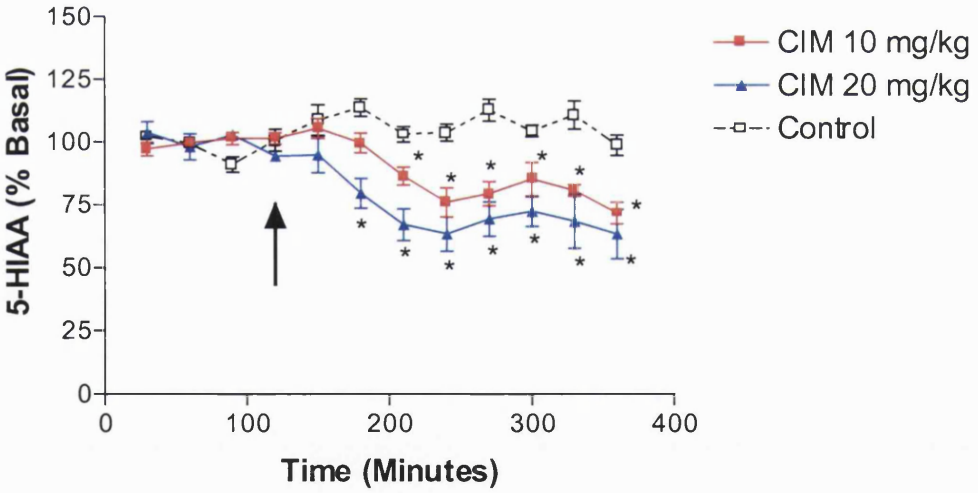
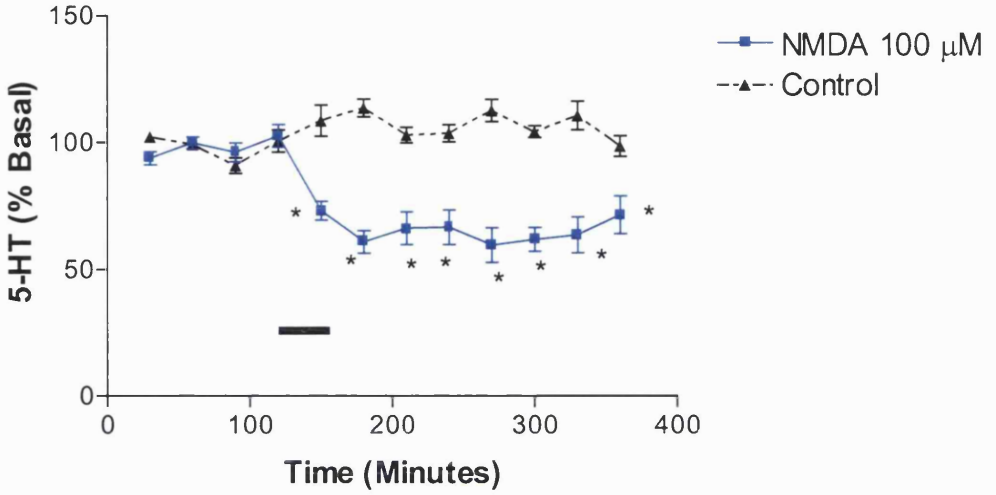


Figure 4.3. The effect of acutely administered clomipramine (CIM) on extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The arrow indicates the time at which clomipramine (i.p) was administered. Data are the mean ± SEM of six-eight animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

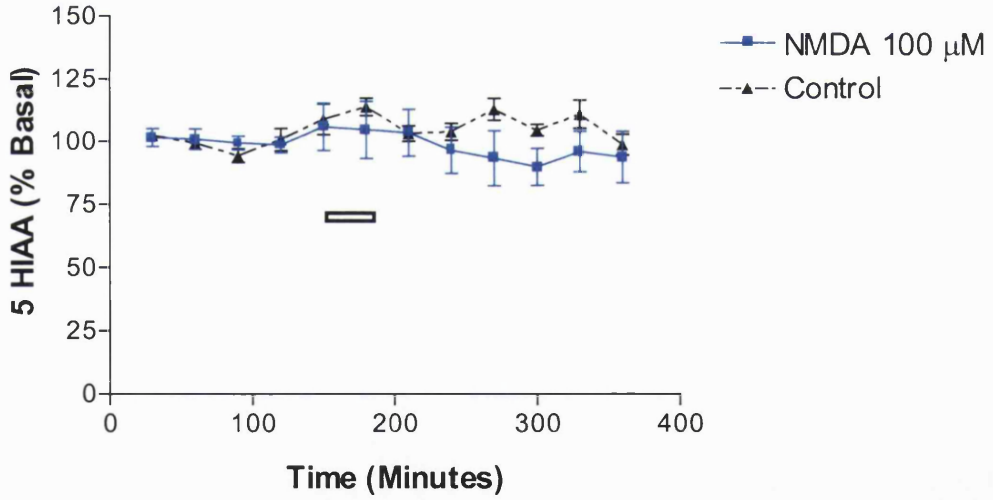


Figure 4.4 The effect of NMDA infusion in the frontal cortex on extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The bar denotes the duration of NMDA infusion. Data are the mean ± SEM of six-eight animals in each group. * indicates significantly different ($p<0.05$) from control.

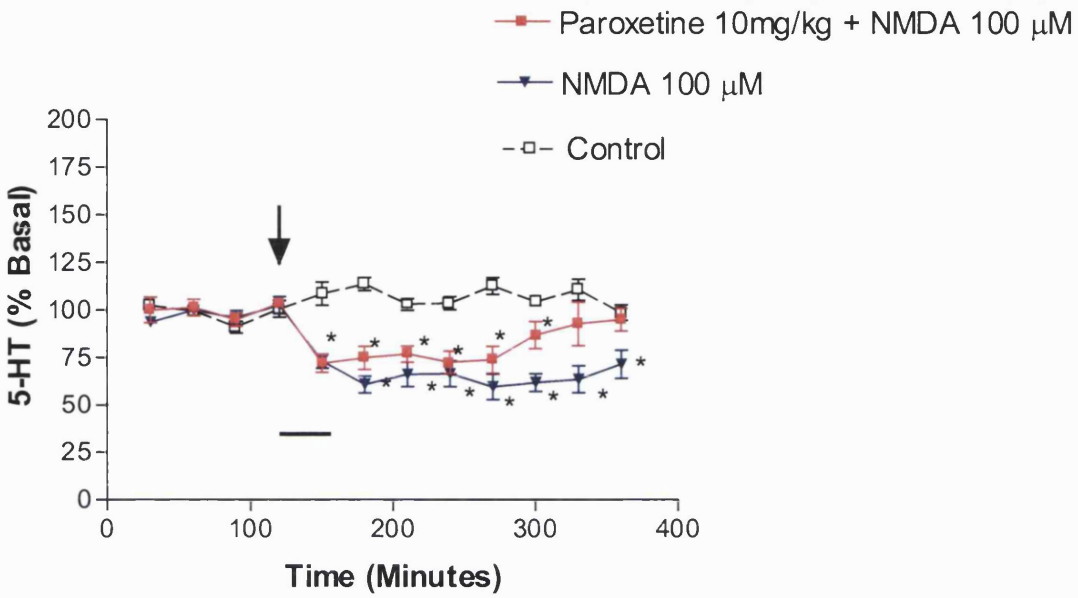
4.2.5 Effect of acute paroxetine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA in the frontal cortex

When animals were given an acute dose of paroxetine (10 mg/kg) systemically prior to 100 μM NMDA infusion into the frontal cortex, there was a rapid, statistically significant decrease in extracellular 5-HT levels (75% of basal) which did not differ from the effect of NMDA alone. Two-way repeated measure ANOVA revealed a significant effect between treatments [$F(2,9)=62.17, p<0.001$] and over main factor 'time' [$F(11,99)=7.68$, $p<0.001$]. However, towards the end of the experiment, extracellular 5-HT levels returned to control basal levels (Figure 4.5A). Although NMDA infusion did not affect extracellular 5-HIAA levels, unlike 5-HT, when animals were pretreated with acute systemic paroxetine there was a significant decrease in extracellular 5-HIAA levels (55% of basal) which lasted for the duration of the experiment. However, the lower dose of paroxetine caused a reduction in extracellular 5-HIAA levels (Figure 4.5B).

4.2.6 Effect of acute clomipramine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA in the frontal cortex

When animals were given an acute dose of clomipramine (10 mg/kg) systemically prior to 100 μM NMDA infusion into the frontal cortex, there was a rapid, statistically significant decrease in extracellular 5-HT levels (64% of basal) which did not differ from the effect of NMDA alone [$F(2,9)=15.99$, $p<0.01$]. This effect was also significant over time [$F(11,99)=7.68, p<0.01$]. This decrease lasted for the duration of the experiment (Figure 4.6A). Although NMDA infusion did not affect extracellular 5-HIAA levels, unlike 5-HT, when animals were pretreated with acute systemic clomipramine there was a significant decrease in extracellular 5-HIAA levels (58% of basal) [$F(2,9)=11.00$, $p<0.01$] which lasted for the duration of the experiment (Figure 4.6B).

(A)



(B)

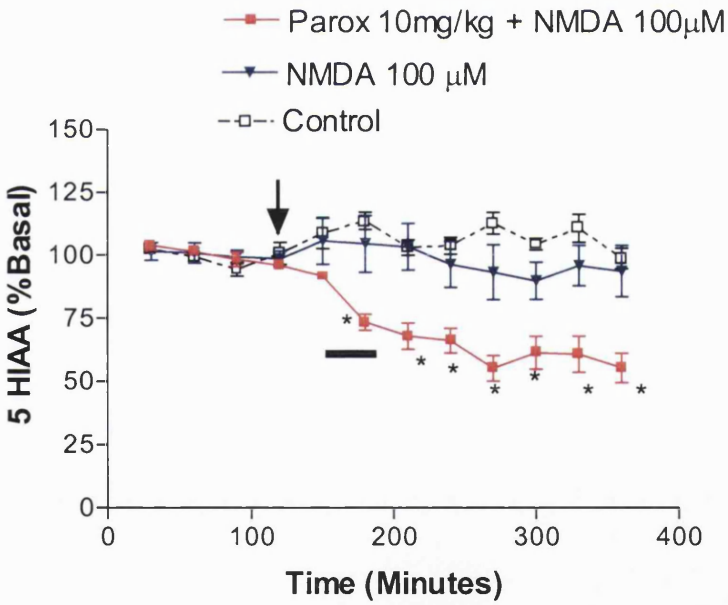
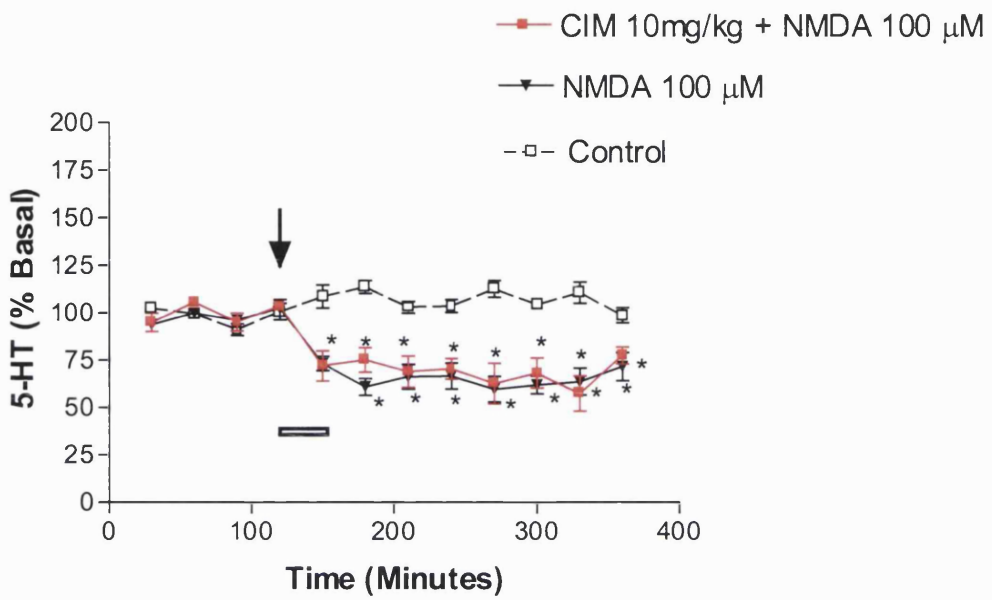


Figure 4.5 Effect of acute paroxetine (Parox) on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. NMDA was infused into the cortex and extracellular 5-HT and 5-HIAA measured. The arrow indicates the time at which paroxetine or vehicle was administered. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

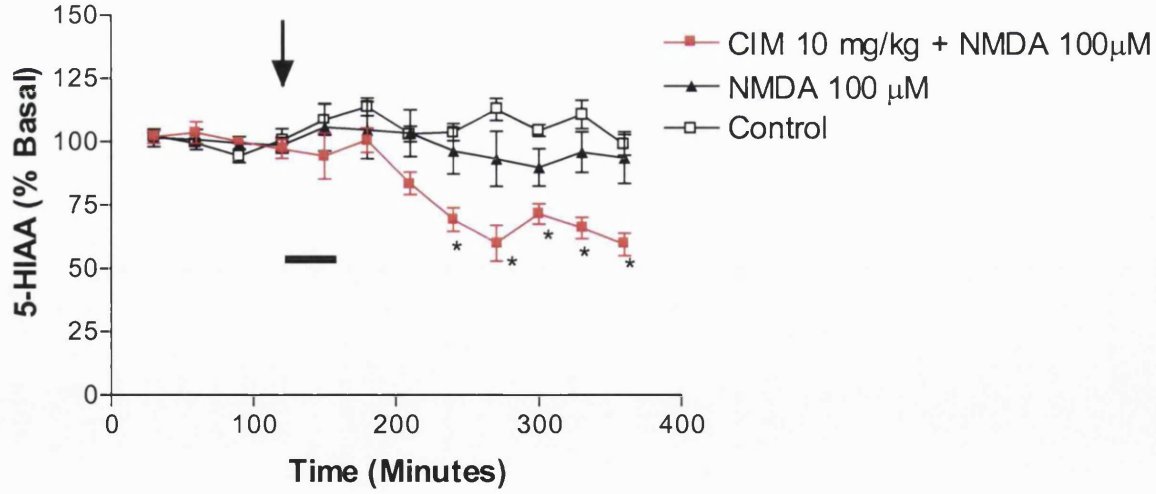


Figure 4.6 Effect of acute clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. NMDA was infused into the cortex and extracellular 5-HT and 5-HIAA measured. The arrow indicates the time at which clomipramine or vehicle was administered. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

4.2.7 Effect of 7-day (sub-chronic) dosing of paroxetine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA levels in the frontal cortex

7-day dosing of paroxetine (10mg/kg) resulted in significant reductions in extracellular 5-HIAA levels while significantly increasing extracellular 5-HT levels.

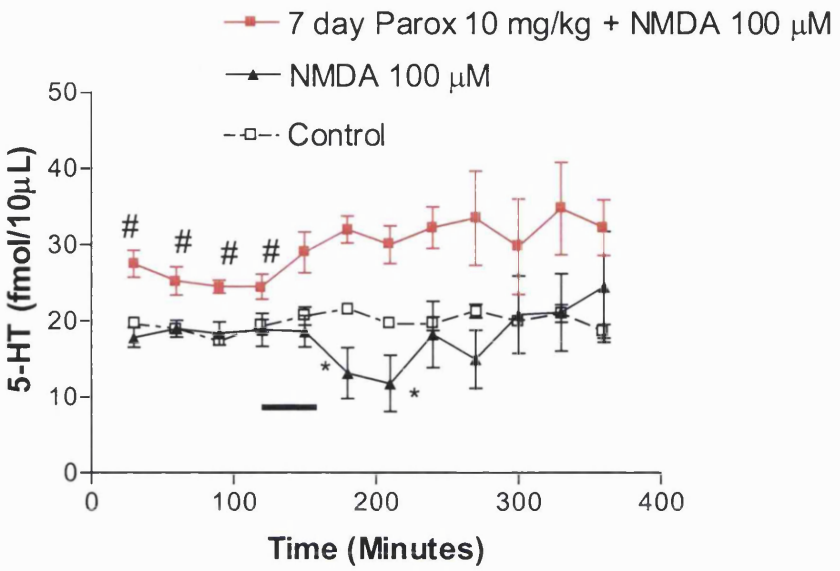
In contrast to the above results, (4.2.2) extracellular 5-HT levels were increased to 125% basal following the 7-day paroxetine treatment (Figure 4.7A) [$F(2,9)=6.27, p<0.05$]. NMDA infusion did not affect the extracellular 5-HT levels (Figure 4.7A) over time [$F(11,99)=1.09$, $p=0.371$]. Extracellular 5-HIAA levels were seen to significantly decrease to 53% of basal levels [$F(2,9)=62.48$, $p<0.05$] following 7-day paroxetine treatment. Here again, NMDA infusion had no effect on extracellullar 5-HIAA levels (Figure 4.7B).

4.2.8 Effect of 21-day (chronic) dosing of paroxetine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA levels in the frontal cortex

After a 21-day chronic treatment with paroxetine, two marked changes were observed in relation to extracellular 5-HT levels. Firstly, extracellular 5-HT levels in this region were 150% (28 fmol/10 μL) above basal values compared with vehicle treated controls (Figure 4.8A). Infusion of 100 μM NMDA evoked a significant change in extracellular 5-HT when compared to NMDA alone [$F(2,6)=16.05$, $p<0.01$]. This was also significant over main factor 'time' [$F(16,96)=24.21$, $p<0.001$]. This was different to the control treatment in which 5-HT was significantly decreased with NMDA infusion, albeit for a short period.

Extracellular 5-HIAA levels were also increased significantly ($p<0.05$) to 134% basal after 21 day repeated administration of paroxetine. Although no change in extracellular 5-HIAA levels was observed when NMDA was infused to saline treated rats, there was a transient significant decrease for 60 minutes in extracellular 5-HIAA levels after which extracellular 5-HIAA levels returned to basal levels (Figure 4.8B).

(A)



(B)

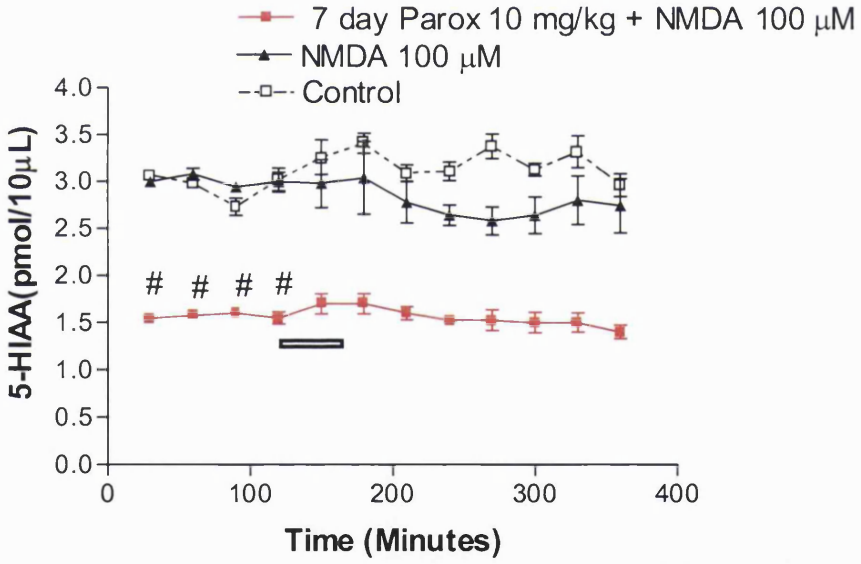
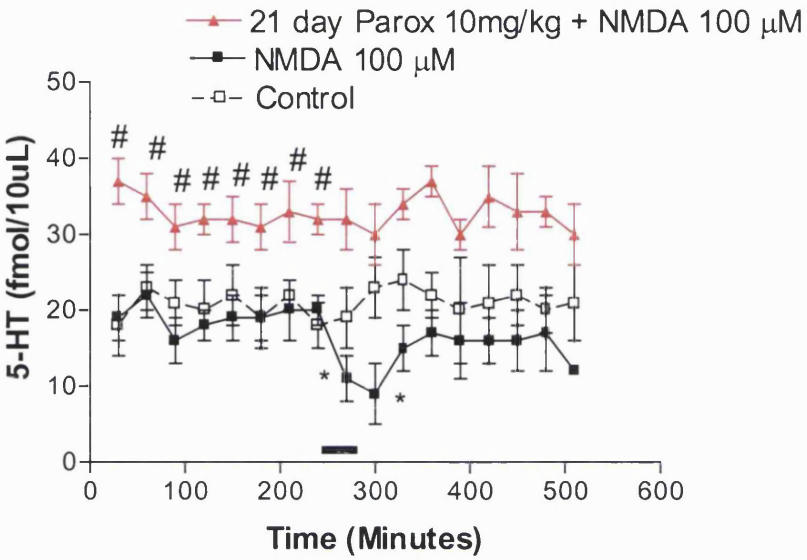


Figure 4.7. Effect of 7-day (sub-chronic) dosing of paroxetine (Parox) on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

(A)



(B)

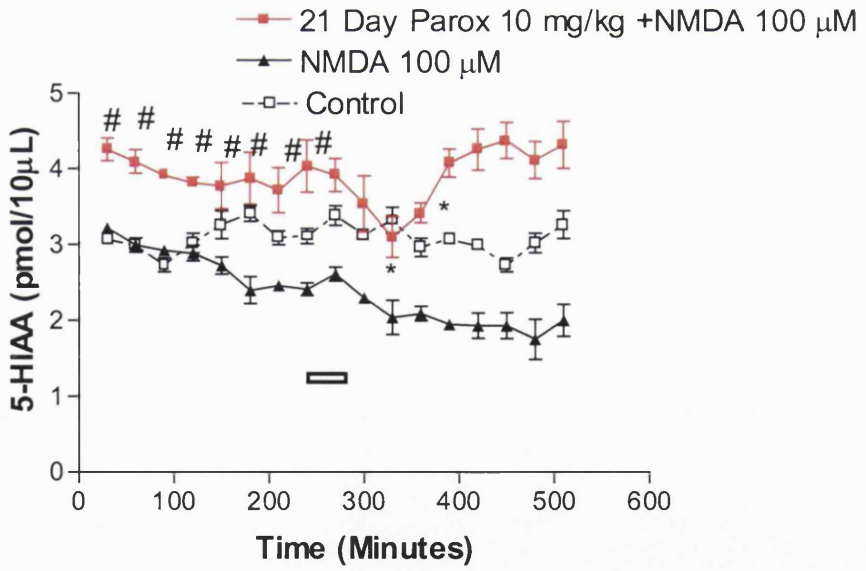


Figure 4.8 Effect of 21-day (chronic) dosing of paroxetine (Parox) on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

4.2.9 Effect of 21-day (chronic) dosing of paroxetine with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA in the frontal cortex

Chronic dosing of 21 days with paroxetine with 48 hours of 'drug holiday' caused an increase in extracellular 5-HT and 5-HIAA levels.

After a 21 day chronic treatment with paroxetine (10 mg/kg) with 48 hours of 'drug holiday', two marked changes were observed in respect to 5-HT levels. Firstly, extracellular 5-HT levels were significantly increased to 165% basal (30fmol/μL) ($p<0.01$). Secondly, upon NMDA infusion, the decrease in 5-HT seen in saline-treated rats was no longer present after chronic administration of paroxetine.Repeated measures two-way ANOVA revealed significant main effects of treatment [$F(2,9)=60.27$, $p<0.001$] but not time [$F(11,99)=0.832$, $p=0.61$]. NMDA infusion here was able to increase extracellular 5-HT levels significantly. However, this increase lasted 60 minutes after which extracellular 5-HT levels returned to basal levels (Figure 4.9A).

The 21-day chronic treatment with paroxetine (10 mg/kg) with 48 hours of 'drug holiday' did not affect extracellular 5-HIAA levels. NMDA infusion had no significant effect on extracellullar 5-HIAA levels, either (Figure 4.9B).

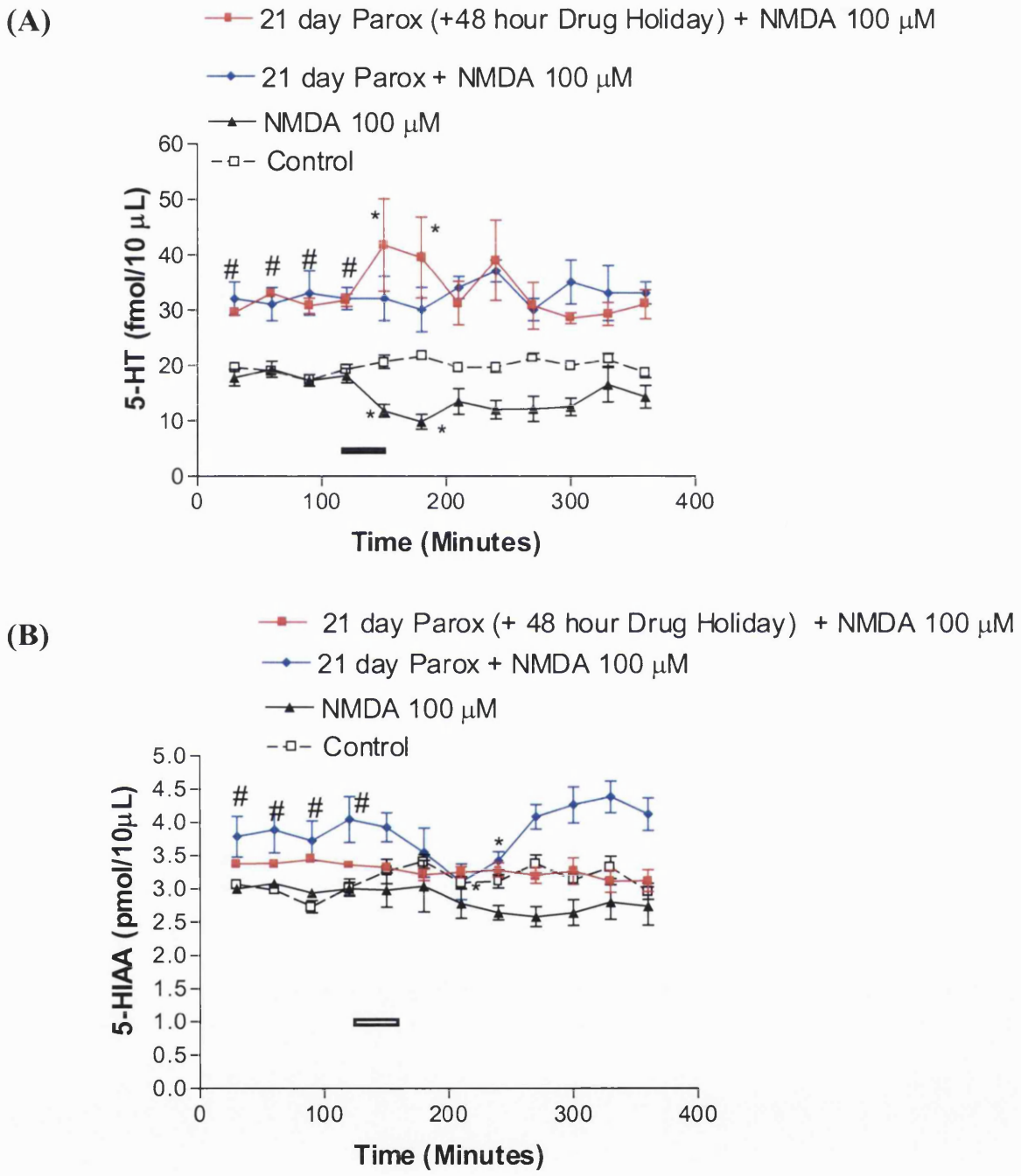


Figure 4.9 Effect of 21-day (chronic) dosing of paroxetine (Parox, 10 mg/kg) with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

4.2.10 Effect of 21-day (chronic) dosing of paroxetine with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in 5-HT and 5-HIAA levels in the frontal cortex

Chronic dosing of 21 days with paroxetine with 14 days of 'drug holiday' increased extracellular 5-HT while not affecting 5-HIAA levels.

After a 21 day chronic treatment with paroxetine (10 mg/kg) with 14 days of 'drug holiday', two marked changes were observed with respect to 5-HT levels. Firstly, extracellular 5-HT levels were significantly increased to 137% basal (22 fmol/μL) [$F(2,9)=6.76$, $p<0.05$]. Secondly, NMDA infusion in these rats caused an immediate, short lasting decrease in extracellular 5-HT levels. This decrease lasted for 90 minutes with a maximal significant decrease of 35% before returning to pre-infusion basal levels (Figure 4.10A).

The 21 day chronic treatment with paroxetine (10 mg/kg) with 14 days of 'drug holiday' did not affect extracellular 5-HIAA levels. NMDA infusion had no effect on extracellullar 5-HIAA levels, either (Figure 4.10B).

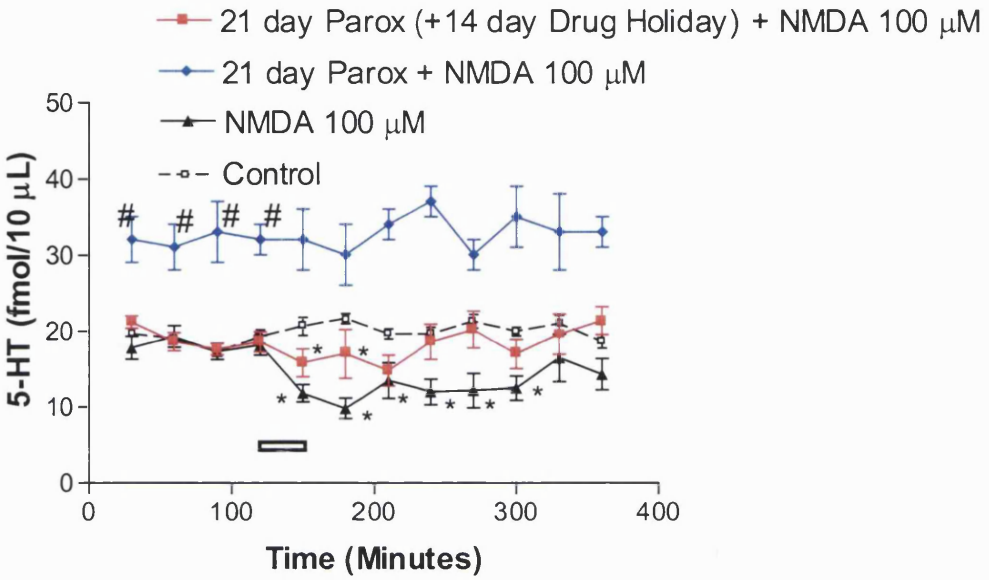
4.2.11 Effect of 7-day (sub-chronic) dosing of clomipramine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA levels in the frontal cortex

7-day dosing of clomipramine (10mg/kg) resulted in significant reductions in extracellular 5-HIAA levels while increasing extracellular 5-HT levels.

Extracellular 5-HT levels were increased to an average of 115 % basal (22fmol/10μL) (average of first samples) following the 7-day clomipramine treatment (Figure 4.11A) [$F(2,9)=1.19$, $p=0.349$]. NMDA infusion to these subchronically-treated rats did not affect the extracellular 5-HT levels over time [$F(11,99)=0.479$, $p<0.912$](Figure 4.8A).

Extracellular 5-HIAA levels decreased drastically to 42% basal (1.4pmol/10μL) [$F(2,9)=84.98$, $p<0.01$] following 7-day clomipramine treatment. Again here, NMDA infusion had no effect on extracellullar 5-HIAA levels [$F(11,99)=1.51$, $p=0.138$] (Figure 4.11B).

(A)



(B)

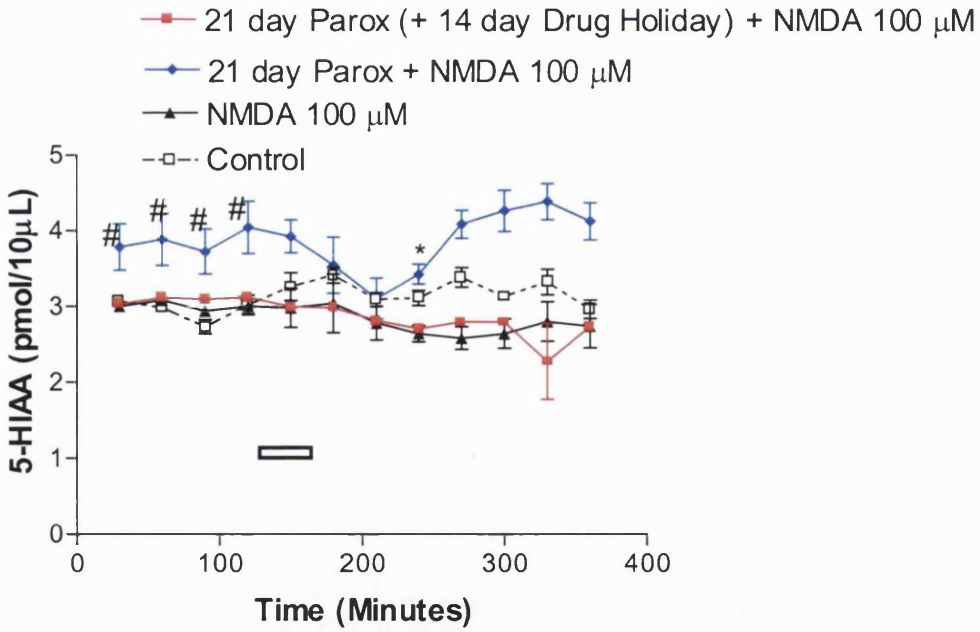
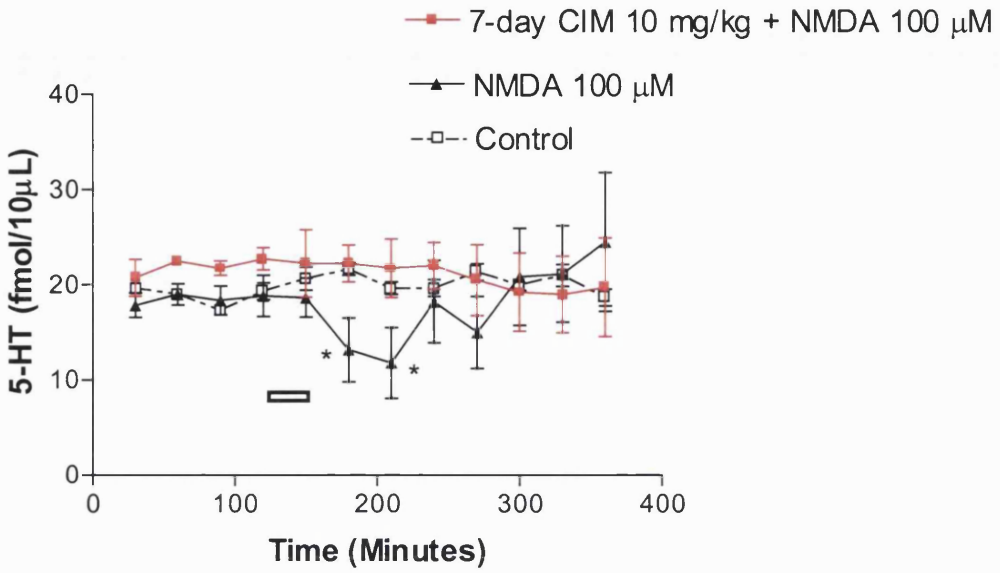


Figure 4.10 Effect of 21-day (chronic) dosing of paroxetine (Parox, 10 mg/kg) with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



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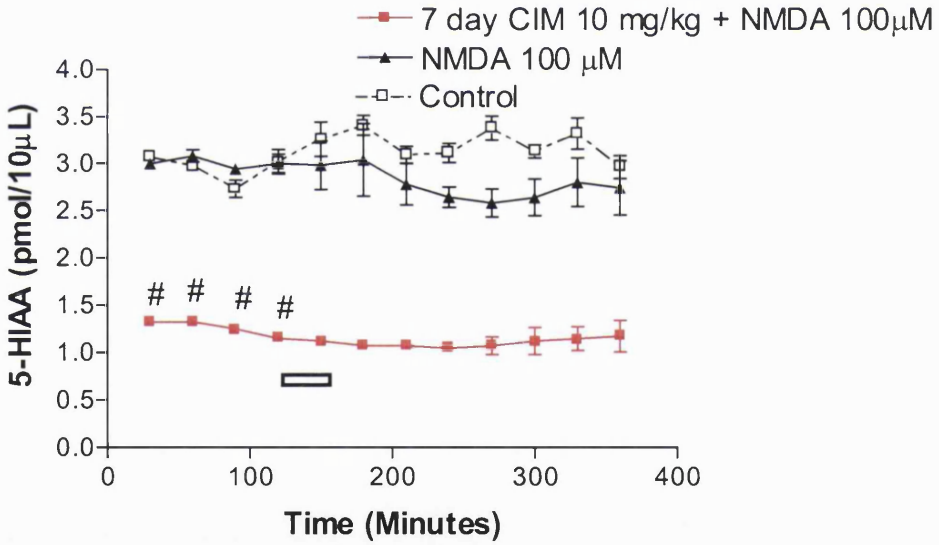


Figure 4.11 Effect of 7-day (sub-chronic) dosing of clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six-eight animals in each group. * indicates significantly different from control ($p<0.05$). # indicates significantly different from treatment basal ($p<0.05$).

4.2.12 Effect of 21-day (chronic) dosing of clomipramine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA levels in the frontal cortex

After a 21-day chronic treatment with clomipramine, two marked changes were observed in relation to extracellular 5-HT levels. Firstly, extracellular 5-HT levels in this region were 147% (32 fmol/10 μL) above basal values compared with vehicle treated controls (Figure 4.12A). Secondly, infusion of NMDA (100 μM) into the frontal cortex caused no significant change in extracellular 5-HT in the frontal cortex (Figure 4.12A). However, there was a significant difference between treatments [$F(2,6)=4.69$, $p<0.05$] and time [$F(16,96)=17.29$, $p<0.001$]. This was different to the control treatment in which 5-HT was significantly decreased with NMDA infusion.

Extracellular 5-HIAA levels increased significantly ($p<0.05$) to 129% basal after 21 day repeated administration of clomipramine. Upon infusion of NMDA into the frontal cortex, extracellular 5-HIAA levels decreased significantly. This maximal decrease of 40% (2.2 pmol/10μL) was achieved 90 minutes after the start of NMDA infusion and lasted the duration of the experiment (Figure 4.12B).

4.2.13 Effect of 14-day (chronic) dosing of clomipramine on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA in the frontal cortex

Chronic dosing of 14 days with clomipramine caused an increase in extracellular 5-HT levels.

After a 14 day chronic treatment with clomipramine (10 mg/kg), two marked changes were observed in respect to 5-HT levels. Firstly, extracellular 5-HT levels were significantly increased to 213% basal (40fmol/μL) [$F(2,9)=159.48$, $p<0.05$]. Secondly, NMDA infusion in these rats was able to prevent the significant decrease in extracellular 5-HT levels in saline-treated rats. NMDA infusion in CIM-treated animals did not cause any change in extracellular 5-HT (Figure 4.13A).

The 14 day chronic treatment with clomipramine (10 mg/kg) did not affect basal extracellular 5-HIAA levels. NMDA infusion had no effect on extracellullar 5-HIAA levels, either (Figure 4.13B).

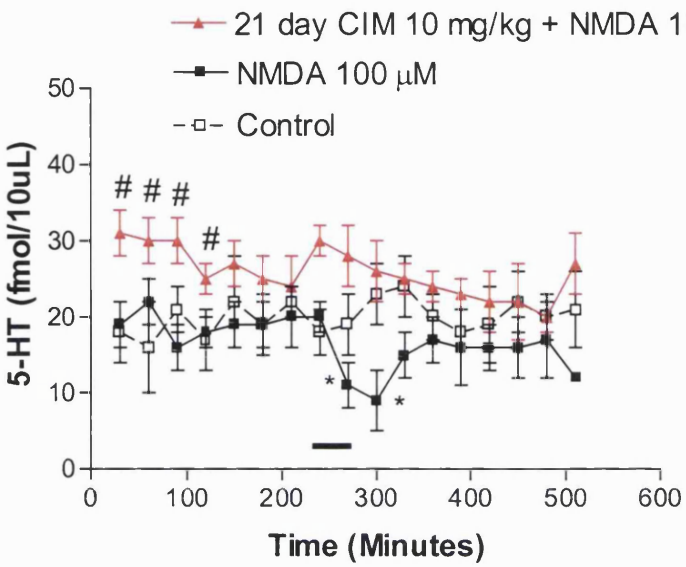
4.2.14 Effect of 14-day (chronic) dosing of clomipramine with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular 5-HT and 5-HIAA in the frontal cortex

Chronic dosing of 14 days with clomipramine with 48 hours of 'drug holiday' caused an increase in extracellular 5-HT and 5-HIAA levels.

After a 14 day chronic treatment with clomipramine (10 mg/kg) with 48 hours of 'drug holiday', two marked changes were observed in respect to 5-HT levels. Firstly, extracellular 5-HT levels were significantly increased to 201% basal (39 fmol/μL) [$F(2,9)=159.48$, $p<0.001$]. Secondly, upon NMDA infusion, the decrease in 5-HT seen in saline-treated rats was no longer present after chronic administration of clomipramine (Figure 4.14A).

The 21 day chronic treatment with clomipramine (10 mg/kg) with 48 hours of 'drug holiday' did not affect basal extracellular 5-HIAA levels. NMDA infusion had no effect on extracellullar 5-HIAA levels, either (Figure 4.14B).

(A)



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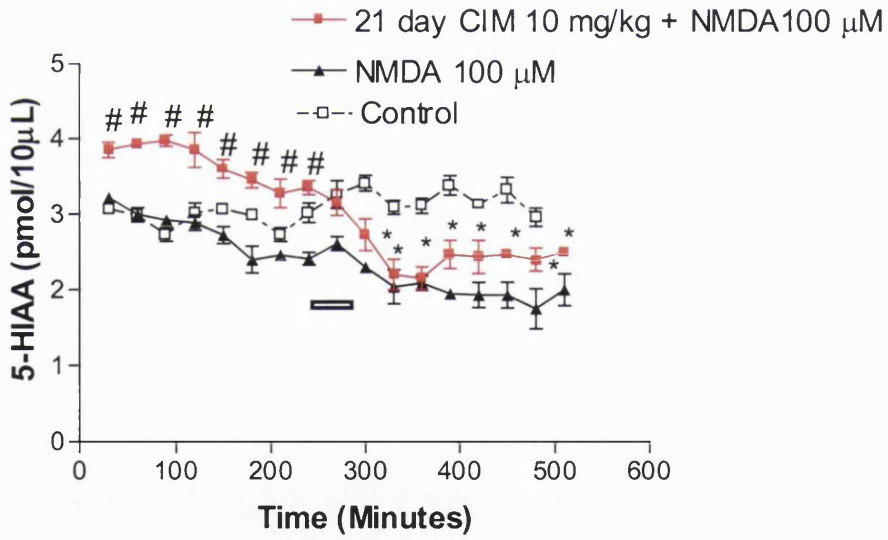
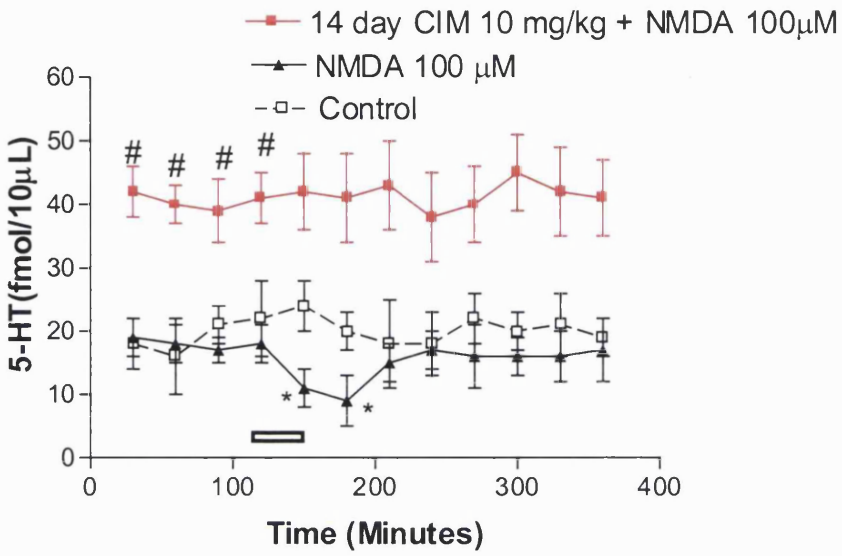


Figure 4.12 Effect of 21-day (chronic) dosing of clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

(A)



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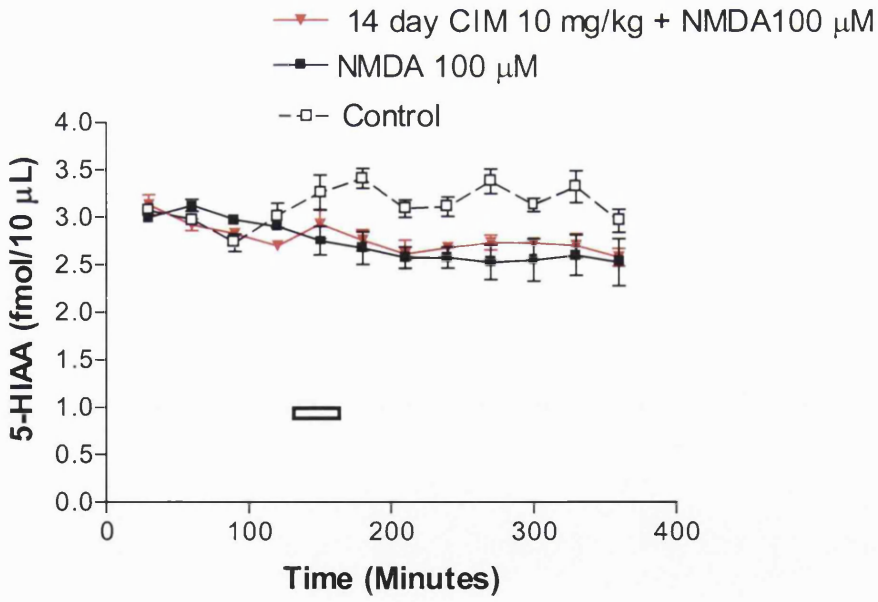
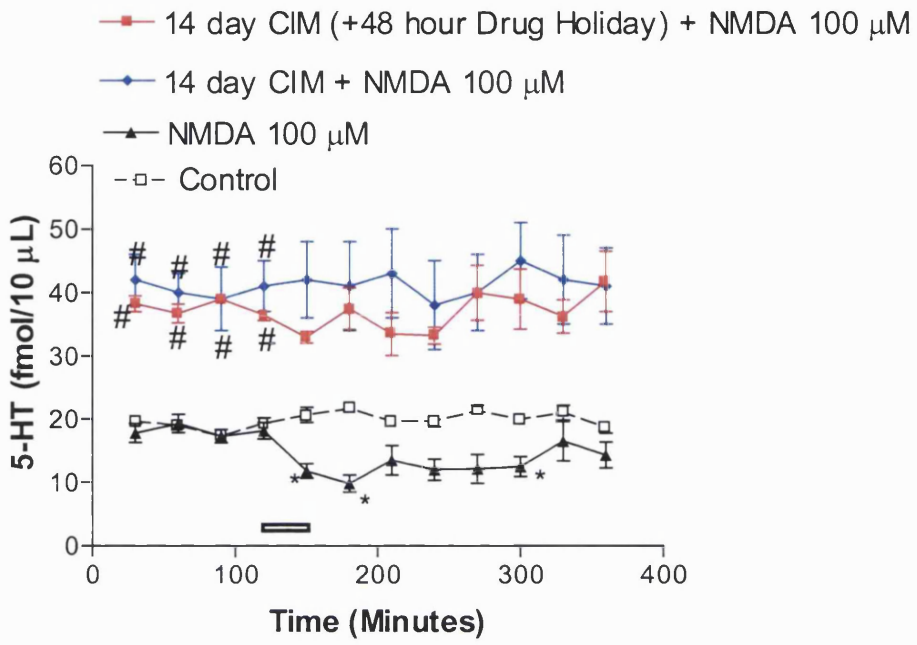


Figure 4.13. Effect of 14-day (chronic) dosing of clomipramine (CIM) on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

(A)



(B)

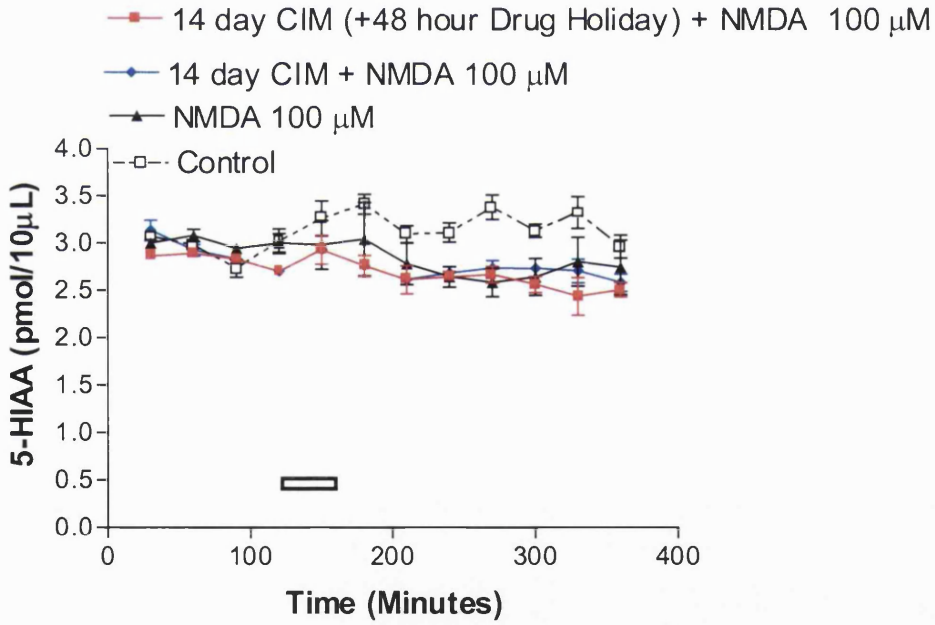


Figure 4.14 Effect of 14-day (chronic) dosing of clomipramine (CIM, 10 mg/kg) with 48 hours 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The solid box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group.* indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

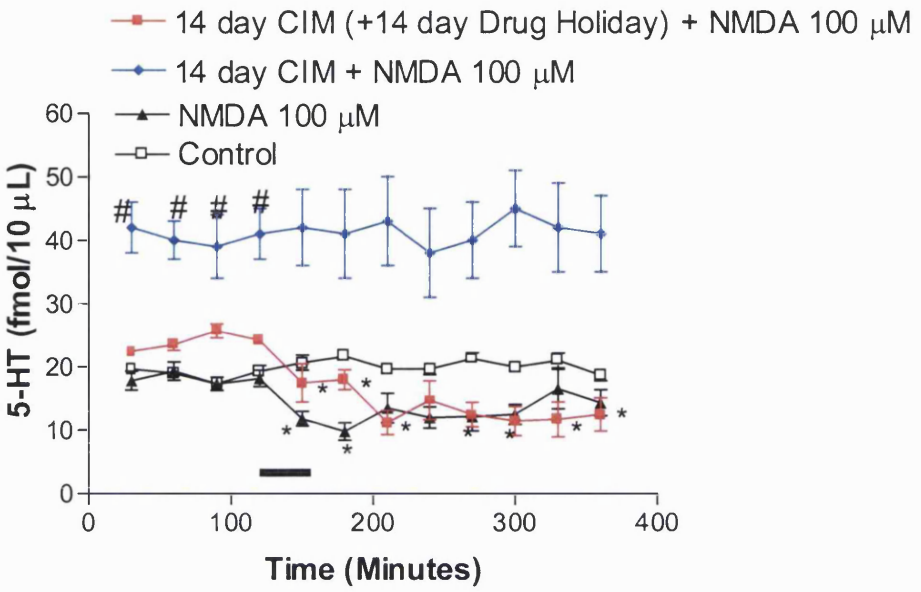
4.2.15 Effect of 14-day (chronic) dosing of clomipramine with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in 5-HT and 5-HIAA levels in the frontal cortex

Chronic dosing of 14 days with clomipramine with 14 days of 'drug holiday' caused an increase in basal extracellular 5-HT and 5-HIAA levels.

After a 14 day chronic treatment with clomipramine (10 mg/kg) with 14 days of 'drug holiday', two marked changes were observed in respect to 5-HT levels. Firstly, basal extracellular 5-HT levels were increased to 126% basal (23 fmol/10μL). Secondly, NMDA infusion in these rats caused an immediate, persistent decrease in extracellular 5-HT levels. This decrease lasted the duration of the experiment with a maximal significant decrease of 48% basal (12 fmol/10μL) which lasted the duration of the experiment (Figure 4.15A). Repeated measure two-way ANOVA revealed significant main effects of treatment [$F(2,9)=11.94$, $p<0.01$] and time [$F(11,99)=7.11$, $p<0.001$].

The 14 day chronic treatment with clomipramine (10 mg/kg) with 14 days of 'drug holiday' did not affect basal extracellular 5-HIAA levels significantly (107% basal). NMDA infusion had no effect on extracellullar 5-HIAA levels, either (Figure 4.15B).

(A)



(B)

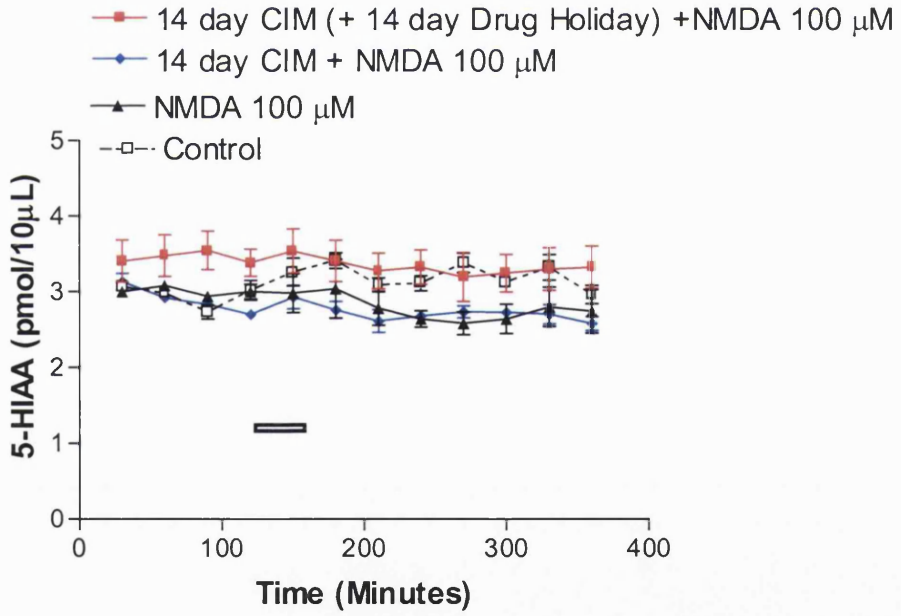


Figure 4.15 Effect of 14-day (chronic) dosing of clomipramine (CIM, 10 mg/kg) with 14 days 'drug holiday' on 100 μM NMDA-evoked changes in extracellular A) 5-HT and B) 5-HIAA in the frontal cortex. The box indicates the period of NMDA infusion. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. # indicates significantly different from treatment basal ($p<0.05$).

Drug Treatment	5-HT	5-HIAA
Paroxetine 10 mg/kg	N/E	↓
Paroxetine 20 mg/kg	↓	↓
Paroxetine 100 μM	↑	N/E
NMDA 100 μM	↓	↓
NMDA 25 μM *	10 % ↓	↓
Paroxetine 10 mg/kg + NMDA 100 μM	↓	↓
Paroxetine 20 mg/kg + * NMDA 100 μM	N/E	↓
Clomipramine 10 mg/kg	↓	↓
Clomipramine 20 mg/kg	↓	↓
Clomipramine10 mg/kg + NMDA 100 μM	↓	↓
7 day Paroxetine 10 mg/kg + NMDA 100 μM	125% Basal N/E	53 % Basal N/E
21 day Paroxetine 10 mg/kg + NMDA 100 μM	150 % Basal N/E	134 % Basal Transient ↓
21 day Paroxetine 10 mg/kg + NMDA 100 μM. Drug Holiday of 48 hours	165% Basal N/E	113 % Basal N/E
21 day Paroxetine 10 mg/kg + NMDA 100 μM. Drug Holiday of 14 days	137% Basal ↓	103 % Basal N/E
7 day Clomipramine 10 mg/kg + + NMDA 100 μM	115 % Basal N/E	42 % Basal N/E
14 day Clomipramine 10 mg/kg + NMDA 100 μM	147 % Basal N/E	129 % Basal ↓
21 day Clomipramine 10 mg/kg + NMDA 100 μM	147 % Basal N/E	129 % Basal ↓
14 day Clomipramine 10 mg/kg + NMDA 100 μM. Drug Holiday of 48 hours	201 % Basal N/E	95 % Basal N/E
14 day Clomipramine 10 mg/kg + NMDA 100 μM. Drug Holiday of 14 days	126 % Basal ↓	107 % Basal N/E

Table 4.1 Summary of chapter 4 results

↑ and ↓ Increase and decrease in efflux respectively.
N/E No effect in efflux.

* Data not shown

4.3 Discussion

4.3.1 The effect of acute, subchronic (7-day) and chronic (21-day) paroxetine and clomipramine treatment on extracellular 5-HT and 5-HIAA changes in the frontal cortex

Clinical depression has been classically linked with a dysfunction of monoaminergic systems within the brain. Evidence from several sources indicates that the disruption of 5-HT mediated neurotransmission in the brain significantly contributes to the pathophysiology of depression (Lopez-Ibor, 1992). Chronic but not acute treatment with SSRIs and TCAs leads to AD effects (Blier and de Montigny, 1994).

There is much experimental evidence that acute treatment with clomipramine, fluvoxamine, sertraline and citalopram markedly raises the extracellular concentrations of 5-HT in the cell body region on 5-HT neurons, with little or no effect on dialysate 5-HT in the frontal cortex (Adell and Artigas, 1991; Invernizzi et al, 1992; Bel and Artigas, 1992). This study confirms these observations in that acute paroxetine administration caused a dose-dependent decrease in both 5-HT and 5-HIAA levels in the frontal cortex, while acute clomipramine had no effect on 5-HT efflux but rather caused a dose-dependent decrease in 5-HIAA efflux. A similar microdialysis experiment by Roberts and colleagues (1998) using the same dose (10 mg/kg i.p.) of paroxetine concurs with these results in that acute paroxetine had no effect on 5-HT efflux in the guinea-pig. However, my observations conflict with those of Malagie and colleagues (2001). These authors report that upon a low dose (1 mg/kg) of paroxetine given acutely, extracellular 5-HT levels increased to 270% of basal levels. The main reason for these differences could be in the different methodologies used. These authors added 1 μM citalopram to their aCSF composition in order to artificially raise extracellular 5-HT levels to a detectable level. The presence of citalopram probably modified the effects of paroxetine on extracellular 5-HT efflux as reported by several authors (Invernizzi et al, 1992; Kreiss et al, 1993). A recent microdialysis study by Hajós-Korcsok et al (2000) reported that, upon administration of acute paroxetine (5 mg/kg s.c.), dialysate 5-HT levels were elevated in the hippocampus, quite different to the results here which demonstrated no change of dialysate 5-HT levels in frontal cortex upon administering paroxetine (10 mg/kg i.p.). This may be due to the higher density of 5-HT receptors in the hippocampus compared to the frontal cortex (Hrdina et al, 1990),

or to the greater density of the inhibitory 5-HT_{1A} autoreceptors in the dorsal raphe, which innervates the frontal cortex, compared to the median raphe which innervates the hippocampus. Ramaiya and colleagues (1997) observed an increase of 400% in extracellular 5-HT levels in the rat striatum after acute paroxetine (10 mg/kg i.p.) treatment. As far as clomipramine is concerned, my data is most similar to that of Adell and Artigas (1991), and to previous studies in this laboratory (Pallotta et al, 2001) which report that after acute administration of clomipramine (10 and 20 mg/kg i.p.) there was no significant change in extracellular 5-HT levels in the frontal cortex. In light of these findings, it is evident that there is regional diversity with regard to the effect of acute paroxetine in different brain regions and this may be due to a differential role of somatodendritic 5-HT_{1A} autoreceptors.

In this study, acute treatment with paroxetine/clomipramine (10 and 20 mg/kg i.p.) caused a dose-dependent decrease in 5-HIAA efflux. During the final preparation of this manuscript, a study by Bymaster and colleagues (2002) report that acute administration of several SSRIs (including paroxetine, fluoxetine, citalopram and sertraline) decreased levels of 5-HIAA in the frontal cortex. The same finding was reported by Adell and Artigas (1991) following acute clomipramine administration: 5-HIAA efflux in the frontal cortex was reduced dose-dependently. These findings are consistent with the idea that blockade of 5-HT uptake by the SSRIs (and clomipramine) results in decreased firing of 5-HT neurons due to autoreceptor activation (Chaput et al, 1986).

However, it is worth addressing what a change in 5-HIAA dialysate actually reflects? For example, the relationship, or the lack thereof, between changes in neurotransmitter and metabolite levels is not often discussed in *in vivo* experiments, as the microdialysis technique has frequently demonstrated a lack of correlation between direction or magnitude of changes in neurotransmitter and metabolite levels (Westerink and Justice, 1991). However, this is not surprising because dialysate metabolite levels, including those of 5-HIAA, are not dependent on the amount of neurotransmitter that is released and taken up again. In addition, 5-HIAA is present in high concentrations in all brain areas with the basal concentration of 5-HIAA in the rat brain being 200-1000 times higher than that of 5-HT, which makes it difficult to measure small changes in the 5-HIAA concentration (Ross and Stenfors, 1997). 5-HIAA is detected in brain areas where there is no 5-HT indicating that 5-HIAA diffuses easily and could originate from distant areas. Thus, these reasons suggest it is highly unlikely that a simple relationship

exists between dialysate concentrations of transmitter and metabolite (Rollema, 1997). Generally speaking, levels of metabolites are not always reliable parameters to estimate actual transmitter release in a specific brain region.

4.3.1.2 Different routes of administration: Local infusion

Adell and Artigas (1991) suggested that antidepressants have different effects on dialysate 5-HT release depending on the route of administration. This finding supports these results that the local infusion of paroxetine into the frontal cortex increased extracellular 5-HT levels whilst systemic administration had no significant effect. From these results, it is clearly evident that there is a peak delayed increase in extracellular 5-HT release upon local paroxetine infusion. Therefore, the increase of dialysate 5-HT in the frontal cortex after local application of paroxetine is most probably the result of the *in vivo* blockade of the 5-HT reuptake, since no promotion of 5-HT release by the drug has been observed (Baker et al., 1977). An increase in extracellular 5-HT upon local infusion was also expected as it is assumed that paroxetine would by-pass the effects of an increase in raphe 5-HT which under systemic administration would decrease the firing rate and consequently the amount of dialysate 5-HT in the frontal cortex. Raphe nuclei are enriched in 5-HT_{1A} receptors (Pazos and Palacios, 1985), and the affinity of 5-HT for them is in the low nanomolar range (Pedigo et al., 1981). Therefore, the assumed increase in extracellular 5-HT in raphe nuclei after systemic paroxetine administration could activate 5-HT_{1A} receptors located in cell bodies and dendrites, which in turn would lead to an inhibition of cell firing (Scuvee-Moreau and Dresse, 1979). This would then reduce or even inhibit the release of 5-HT in the frontal cortex. Several studies have reported different effects in terminal areas by systemically administered SSRIs (e.g. Fuller, 1994). This is generally attributed to a divergent involvement of release-controlling somatodendritic 5-HT_{1A} autoreceptors in the raphe nuclei (Kreiss and Lucki, 1994). The 300% increase of extracellular 5-HT above basal levels following local infusion of paroxetine in our study contrasts with the lack of effect following acute, systemic paroxetine administration which suggests a strong involvement of somatodendritic 5-HT_{1A} receptor-mediated feedback. Thus, upon local infusion there was a 3-hour lag between the onset of drug perfusion and the maximal increase in dialysate 5-HT. As paroxetine is highly lipophilic molecule and tends to accumulate in the tissue (Ramaiya et al., 1997), it can be retained in the glial cells after diffusing out of the probe and so its pharmacological effect can be delayed. A similar

phenomenom was observed with local infusion of clomipramine, which is also lipophilic (Adell and Artigas, 1991).

Adell and Artigas (1991) also report a decrease in extracellular 5-HIAA levels in the frontal cortex following clomipramine dosing which supports these findings. A similar decrease was observed when clomipramine was administered through the probe in the raphe and the resultant extracellular 5-HIAA levels were measured in the frontal cortex. In this study, paroxetine infused into the frontal cortex, which differed in effect from that seen following systemic administration only, caused a transient decrease in extracellular 5-HIAA levels. This difference could be due to several reasons. Firstly, paroxetine has a higher affinity than clomipramine for the 5-HT transporter (K_is= 0.05 and 0.47 nM respectively, Owens et al, 1997). Secondly, there were methodological differences. Adell and Artigas (1991) use a lower dose of 10 and 40 μM clomipramine and the clomipramine was infused directly into the raphe, which as mentioned before, has a much higher density of 5-HT_{1A} autoreceptors. Overall dialysate extracellular 5-HIAA levels tend to decrease following paroxetine treatment. Since 5-HIAA is formed partly after reuptake of 5-HT (Fuller and Wong, 1977), decreases in 5-HIAA are possibly derived from the reduced reuptake of 5-HT after paroxetine treatment.

4.3.1.3 At the synapse

Many microdialysis experiments have demonstrated that a single exposure to SSRIs, such as paroxetine, leads to an increase in extracellular 5-HT levels near the cell bodies and dendrites of serotonergic neurones in the raphe nuclei compared to brain regions innervated by serotonergic nerve terminals such as the frontal cortex (e.g. citalopram: Invernizzi et al, 1992; fluoxetine: Malagie et al, 1995; fluvoxamine: Bel and Artigas, 1992; paroxetine: Gartside et al, 1995). SSRIs increase 5-HT levels in the vicinity of serotonergic neurones by blocking the 5-HT carrier (Fuller, 1994). It is interesting to note that the administration of TCAs such as clomipramine and imipramine result in large increases in extracellular 5-HT levels in terminal regions including the frontal cortex only when doses of 10 mg/kg or higher are used (Bel and Artigas, 1996). In addition, 5-HT_{1A} autoreceptors on 5-HT cell bodies and 5-HT_{1B} receptors on axon terminals become increasingly activated. Thus when 5-HT_{1A} autoreceptors are stimulated, a reduction in the firing rate of 5-HT neurones occurs (Sprouse and Aghajanian, 1987) as well as a reduction in the release of extracellular 5-HT (Bosker et

al, 1996) and a reduction of 5-HT synthesis (Hutson et al, 1989). When 5-HT_{1B} presynaptic receptors are stimulated, 5-HT release is also decreased (Limberger et al, 1991). The existence of a neuronal feedback loop between projection areas and raphe nuclei has been hypothesised by Blier et al (1987). The raphe nuclei are known to be enriched with 5-HT_{1A} autoreceptors displaying a high affinity for 5-HT in the low nanomolar range (Pazos and Palacious, 1985, Pedigo et al, 1981). Bel and Artigas (1992) observed that fluvoxamine, an antidepressant devoid of noradrenergic activity *in vivo* (Jordan et al, 1994; see Stanford, 1996), preferentially increased both raphe and frontal cortex 5-HT levels, but the increase in the raphe was several-fold that in the frontal cortex (Bel and Artigas, 1992). However, in a microdialysis study performed by Malagie and colleagues (2000), a single dose of paroxetine induced similar increases in extracellular 5-HT in both the frontal cortex and raphe nuclei. This was an unusual finding which the authors were unable to explain.

4.3.1.4 Duration of treatment

7-day sub-chronic and 21-day chronic paroxetine and clomipramine treatment were observed to increase extracellular 5-HT in the frontal cortex. Interestingly, both paroxetine and clomipramine 7-day and 21-day dosing regimens gave similar relative changes in extracellular 5-HT and 5-HIAA levels.

These findings of an increase in extracellular 5-HT levels agree with previous work conducted both in our laboratory by Pallotta et al (2001) and also in the work of Gur et al (1999b) who report a 2-fold increase in extracellular 5-HT levels in the frontal cortex after chronic administration of 10 mg/kg clomipramine for 14 days (Pallotta et al, 2001) and 28 days (Gur et al, 1999b). Although the literature does not report any study measuring the effect of repeated paroxetine treatment in the frontal cortex, Hajós-Korcsok et al, (2000) reported that basal extracellular 5-HT levels were increased 2-fold in the hippocampus upon 14-day chronic treatment with paroxetine (5 mg/kg s/c twice daily). Chronic imipramine has also been shown to initiate larger increases in terminal regions, with lower doses than those used in acute studies (Bel and Artigas 1993, 1996). A study of the effects of chronic fluvoxamine at a dose of 1 mg/kg for 14 days showed increases in frontal cortex extracellular 5-HT. These studies suggest that chronic AD treatment raises serotonergic levels in the frontal cortex and which may partially contribute towards its therapeutic effect.

The 7-day paroxetine treatment decreased the extracellular 5-HIAA levels while the 21-day paroxetine treatment slightly increased extracellular 5-HIAA levels. There were contrasting effects between the 7-day and 21-day paroxetine and clomipramine treatment with regard to extracellular 5-HIAA levels. Basal extracellular 5-HIAA levels are decreased by 60% following the 14-day paroxetine treatment (Hajós-Korcsok et al, 2000). The decrease in extracellular 5-HIAA levels following chronic paroxetine treatment is in contrast to these findings. In this study, extracellular 5-HIAA levels were measured from the frontal cortex while Hajós-Korcsok and colleagues (2000) measured levels in the hippocampus. Once again it is evident that the same drug has different actions in different brain regions. This is not surprising due to different innervating pathways. The modest increase in extracellular 5-HIAA levels seen in this study is in agreement with a study by Nomikos et al (1991) who saw a similar increase in 5-HIAA levels in the nucleus accumbens and the striatum after a 2 week chronic treatment with desipramine. Imipramine, the TCA, when given acutely by these authors reduced dialysate 5-HIAA levels in both the raphe and the frontal cortex, with the effects more marked in the latter brain region (Bel and Artigas, 1996). However, when the drug (4 mg/kg) was given over a 2-week period using minipumps, the dialysate extracellular 5-HIAA levels in both the raphe and the frontal cortex did not differ from control. ADs clearly affect extracellular 5-HIAA levels differently whether given acutely or chronically, whether pulse injection or continuous infusion and are region specific.

There are no other studies in animals that report the effect of chronic paroxetine treatment on extracellular 5-HIAA levels. However, a clinical study in patients by Lundmark et al, (1994) concludes that paroxetine reduces 5-HIAA levels from the CSF in depressed patients after 3 weeks of treatment. This decrease in CSF 5-HIAA was expected as preclinical work with paroxetine indicated a selective serotonin neuronal reuptake inhibiting effect *in vitro*. This is different to my 21 day study but agrees with my 7-day study. Interestingly, a recent clinical study by Bäckman and colleagues (2000) report that the reported reduction in CSF 5-HIAA after AD treatment does not persist during long-term treatment. These authors also report that 5-HIAA seemed to return to a higher level when the drug treatment continued. Unfortunately, these authors were unable to fully interpret or evaluate the validity of their findings. The only hypothesis that could be suggested to explain the variance of 5-HIAA levels after a 7-day and 21-day treatment with paroxetine or clomipramine is the role of kynurenines in the action of these ADs (see Maroni, 1999; Stone, 2001). Any pharmacological manipulation

which alters the amount of 5-HT in neurons (as observed with repeated paroxetine and clomipramine treatment in this study) will have a secondary effect on the activity of synthetic enzymes of the kynurenine cycle which may in turn affect 5-HIAA levels. Thus paroxetine and clomipramine may have changed the tryptophan levels and/or 5-HIAA concentrations varied due to a change in activity and concentration of the components of the kynurenine pathway.

In conclusion, it is evident that there is a difference in outcome in terms of extracellular 5-HT levels during acute and chronic dosing regimens. In recent years, amongst the monoamines associated with the monoamine hypothesis of depression the case for a pivotal place for 5-HT, largely as a result of recent drug developments, appear to have been pre-eminently strengthened. This increase in extracellular 5-HT levels observed after paroxetine and clomipramine treatment may contribute to the therapeutic effects of these drugs in the clinic.

4.3.1.5 Adaptation/Genetic regulation?

Chronic treatment therefore reverses the decrease in frontal cortex extracellular 5-HT levels observed following the ADs used in this study (acute paroxetine and clomipramine treatment), suggesting that some form of adaptation within the system is occurring. The data suggests that paroxetine and clomipramine enhance the 5-HT function in the frontal cortex after chronic treatment. Prolonged administration of paroxetine may induce adaptive changes either in a serotonergic nerve terminal in the cortical region or in the raphe nuclei region. Extracellular unitary recordings and {^{3}H}-paroxetine binding assays by Piñeyro et al (1994) have shown that repeated administration of paroxetine results in adaptive changes in the form of down-regulation of the 5-HT transporter. More recently, Benmansour and colleagues (1999) report that after a 21-day paroxetine (10 mg/kg) treatment the SERT is downregulated. In addition Lesch et al (1993) found decreased 5-HT transporter mRNA levels in the raphe nuclei following long term AD administration to rats. This suggests ADs regulate the 5-HT transporter at the level of gene expression. This evidence would confirm the theory that adaptive changes (e.g. desensitisation of 5-HT_{1A} autoreceptors) must occur before the clinical benefit of antidepressants can be seen (Artigas et al, 1996, Blier and de Montigny, 1994).

Another example of an adaptive process occurring was suggested by Edwards et al (1991). These authors showed that the induction of learned helplessness in rats was associated with up-regulation of 5-HT_{1B} receptors in the cortex and hippocampus while Neumaier et al (1997) found increased levels of 5-HT_{1B} receptor mRNA specifically in dorsal raphe of learned helplessness rats, with no changes in the frontal cortex or hippocampus. These results implied a specific increase in 5-HT_{1B} autoreceptors as they are synthesised in the cell bodies of serotonergic neurones in the raphe nucleus and then transported to the nerve terminal areas. Alternatively, 5-HT_{1B} receptors are synthesised in the cell bodies of non-serotonergic neurones in brain regions including the hippocampus. It is worth noting, clomipramine has been shown to alter presynaptic 5-HT_{1B} receptor sensitivity in rat hypothalamus and 5-HT_{1A} hippocampus (Newman et al, 2000). Thus this suggests that these effects are highly region-specific.

The increase in frontal cortex extracellular 5-HT levels initiated by antidepressants (e.g. paroxetine and clomipramine) may be potentiated by co-administration with a 5-HT_{1A} autoreceptor antagonist (Artigas et al, 1994, Blier and Bergeron, 1995). Anatomical data indicate that the frontal cortex is innervated by 5-HT neurones of the dorsal raphe nucleus in an exclusive manner (Imai et al, 1986). The effect of co-administrating a 5-HT_{1A} antagonist is analagous to the desensitisation of raphe 5-HT_{1A} autoreceptors by chronic administration with SSRIs (Blier and de Montgny, 1994). Pindolol prevents the autoinhibition of dorsal raphe serotonergic neurones produced by SSRIs and TCAs and therefore potentiates their effects in various brain areas (Artigas et al, 1994; Romero et al, 1996). The process of blocking 5-HT_{1A} autoreceptors is thought to contribute towards the therapeutic effect of pindolol as an adjunct to AD therapy (Artigas et al, 1994; Blier et al, 1997). A study performed by Romero and Artigas (1997) using the co-administration of paroxetine/ clomipramine in addition to other ADs such as citalopram, fluoxetine and duloxetine and the 5-HT_{1A} antagonist WAY 100635 support the hypothesis that selective 5-HT_{1A} autoreceptor antagonists may augment the clinical effects of SSRIs by increasing serotonergic transmission. This effect takes place preferentially in forebrain areas with selective innervation from the dorsal raphe nucleus (e.g. cortex). These findings and others (Gartside et al, 1995; Romero et al, 1996; Romero and Artigas, 1997; Millan et al, 1998; Cryan et al, 1999) indicate that the effects of ADs can be potentiated by the 5-HT_{1A} autoreceptor blockade and therefore emphasises the role of this receptor in the mechanism of action of ADs.

4.3.2 The effects of NMDA infusion on basal 5-HT and 5-HIAA efflux in the frontal cortex

The discharge of 5-HT neurons may be regulated under physiological conditions by EAA inputs to the raphe (Levine and Jacobs, 1992). Evidence from *in vitro* studies of cultured raphe neurons indicates that NMDA can stimulate 5-HT release (Becquet et al , 1993). The role of NMDA receptors in regulating transmission in ascending serotonergic pathways is evidently complex since Lejeune and colleagues (1994) observed that NMDA receptor antagonists increase serotonergic transmission to the striatum but did not, however, observe any effect of NMDA alone on firing of DRN neurons. Research investigating the role of NMDA on 5-HT transmission has been reviewed in a number of CNS regions including the raphe and the hippocampus and have included the effects of infusing NMDA into the raphe and measuring the resultant change in 5-HT extracellular release in the frontal cortex (Tao and Auerbach, 1996; Pallota et al, 1998). The latter authors have suggested that 5-HT release in the raphe nuclei is dependent on neuronal discharge, and regulated by autoreceptors and GABA as well as EAAs. This finding has been supported by previous work (Nishikawa and Scaton, 1985; Bosker et al, 1994).

NMDA receptor antagonists and tetradoxin (TTX) used in the studies of Becquet et al (1990, 1993) suggest that NMDA-induced changes in extracelluar 5-HT release are due to direct excitatory effects on 5-HT cell bodies and indirect inhibition of 5-HT release in some terminal sites. For this reason, microdialysis measurements in the raphe, as opposed to the frontal cortex, have been favoured as localised effects of receptor ligands can be examined more extensively to test this hypothesis.

In this study, infusion of NMDA (100 μM) into the frontal cortex caused a statistically-significant decrease in extracellular 5-HT efflux. Little change in extracellular 5-HIAA levels was seen following NMDA infusion. This is in agreement with a previous study in our laboratory by Pallotta et al (1998) and is further supported by the findings of Tao and Auerbach (1996) who reported that when NMDA is infused into the frontal cortex, nucleus accumbens or hippocampus 5-HT levels are found to decrease in these forebrain sites. However, the maximal decrease in extracellular 5-HT in the frontal cortex obtained in this study was slightly greater compared to that found by the authors using the same dose of NMDA (100 μM) as ourselves. This could be due to methodological

differences in which the authors used ketamine as their anaesthetic which could have possibly retarded the NMDA receptors. The dose of NMDA used in our study may seem high relative to effective concentrations determined from *in vitro* binding studies. However, it is important to note that NMDA was infused at a slow rate (0.8 μL/min) via a short segment of dialysis membrane that represents a significant barrier to diffusion. Thus concentrations of low molecular weight substances, such as NMDA, fall sharply outside the probe and are close to zero at distances between 1.0 and 1.5 mm from the dialysis probe (Dykstra et al, 1992).

Previous work in this laboratory involving local infusion of NMDA into the raphe have shown that NMDA has a biphasic effect on extracellular 5-HT release in the raphe and frontal cortex (Pallotta et al, 1998; 2001). At lower concentrations of NMDA (25 μM), infusion leads to a marked transient decrease in extracellular 5-HT levels followed by a prolonged increase in terminal frontal cortex 5-HT efflux. It can be seen that the role of NMDA receptors in regulating transmission in ascending serotonergic pathways is evidently complex since Lejeune et al (1994) observed that NMDA receptor antagonists increased serotonergic transmission to the striatum but did not elicit any effect of NMDA alone on DRN neurones. The actions of NMDA on 5-HT release are blocked by the selective NMDA receptor antagonist 2-aminophosphonopentanoic acid (D-AP5) indicating the receptor specificity in our experiments (Pallotta et al, 1998). Presumably, localised infusion of lower doses of NMDA into the raphe would not activate all 5-HT neurones with projections to the specific forebrain sites where our dialysis probe would be implanted. At higher doses of NMDA, there could have been a more robust stimulation of the 5-HT neurones with forebrain projections. The data suggest that the degree of NMDA receptor activation results in dramatically different outcomes with regard to serotonergic transmission to the frontal cortex and there appears to be a differential role for the 5-HT_{1A} receptor in regulating these effects. It could be possible due to different NMDA receptor subtype populations. Each subtype will be localised to a particular region/pathway and each subtype will have a different affinity for the agonist: it depends on what is a 'high dose' of NMDA and what is a 'low dose' as to whether one subtype or another is activated.

4.3.3 The effects of acute, subchronic and chronic paroxetine and clomipramine treatment on basal and NMDA receptor activation on serotonergic transmission in the frontal cortex

As mentioned previously, it has been found that NMDA receptor antagonists are as effective as antidepressants in a rodent behavioural model (Trullas and Skolnick, 1990). It has been shown that chronic, but not acute treatment with a range of antidepressants including SSRIs decreases the sensitivity of NMDA receptors (Paul et al, 1994). This suggests that NMDA receptor dysfunction may play a role in the pathophysiology of depression. This is supported by the finding that NMDA receptors are abnormal in the frontal cortex of depressed suicide victims (Nowak et al, 1995), and suggests that interactions between the glutamatergic and serotonergic systems in the brain may play a pivotal role in the development and possibly treatment of depression.

In the present study, when NMDA infusion was proceeded by a single injection of either paroxetine or clomipramine no marked differences between NMDA or NMDA + paroxetine treated groups were observed as regards to extracellular 5-HT efflux. However, following subchronic (7-day) and chronic (21-day) treatment of paroxetine or clomipramine, the decreases seen in extracellular 5-HT levels were abolished. For both experiments, there was no change in extracellular 5-HIAA levels. It is therefore clear that repeated paroxetine or clomipramine treatment causes marked and qualitative adaptive changes to the NMDA receptors regulating extracellular 5-HT release. Notably, these effects are seen in as little as 7-days of repeated drug administration. Of course, the converse to this hypothesis may also be true, in that monoamines could be modulating the release of glutamate. However, there seems to be substantial evidence from other laboratories in support of my own observations, favouring modulation of monoamine release by NMDA (Feenstra et al, 1995; Jedema and Moghaddam, 1996; Tao and Auerbach, 1996; see Whitton, 1997).

4.3.4 Drug Holiday of paroxetine/clomipramine treatment. Any difference in NMDA-induced 5-HT and 5-HIAA efflux in the frontal cortex?

Similar to the findings of section 3.3.4, it can be concluded that both chronic paroxetine and clomipramine treatment can cause adaptive changes in as little as 7-days, persisting up to 21-days treatment. A 48 hour and a 14 day drug holiday period were chosen to

assess any changes on the NMDA-evoked 5-HT response (refer to section 3.3.4 for the reasons behind validity of this strategy).

After a 14-day chronic treatment with clomipramine, basal extracellular 5-HT levels were increased 2-fold. This is similar to a previous study in this laboratory (Pallotta et al, 2001) in which a 15-day chronic clomipramine treatment increased basal extracellular 5-HT levels dose-dependently. The magnitude of increase of these basal extracellular 5-HT levels was similar to that of the 21 day study (refer to section 4.3.1). However, there was no effect on extracellular 5-HIAA basal levels.

Following the 14-day clomipramine treatment, the effect of NMDA on 5-HT efflux was to prevent the sustained decrease seen with acute clomipramine + NMDA. This finding is similar to a study in this laboratory (Pallotta et al, 2001) in which animals treated repeatedly (15 day) with clomipramine (10 or 20 mg/kg) greatly attenuated or abolished the effect of NMDA infusion (25 μM or 100 μM respectively) into the raphe.

The drug holiday study yielded very interesting results. After a 21-day treatment, the paroxetine-treated rats with a 48 hour drug holiday showed similar levels of extracellular 5-HT to those animals treated with a similar dosing regimen, but without the drug holiday. Additionally, the 14 day clomipramine treated rats with a 48 hour drug holiday showed a modest increase (0.6 fold) in extracellular 5-HT levels compared to the 14 day clomipramine treatment which showed a 2 fold increase in extracellular 5-HT levels.

Interestingly, after a 48 hour drug holiday, the paroxetine and clomipramine were extensively metabolised and excreted from the body (see 3.3.4). Recently, a study by Weignmann and colleagues (2000) report that after 12 hours following five oral doses of clomipramine (20 mg/kg) treatment, plasma drug levels fell below the pharmacologically active plasma levels. The therapeutic plasma levels for clomipramine in the blood is 40-80 ng/mL. The plasma levels after a 12 hour drug washout period was 20 ng/mL. In this study, it can be therefore assumed that the clomipramine and paroxetine drug levels fall below the pharmacologically active plasma levels after a 48 hour washout period. This observation is inconsistent with the notion that depressed patients' mood is improved during AD drug treatment and when withdrawn prematurely from their treatment, patients relapse rapidly. These observations provide further

evidence that the aetiology of depression is more complex than simply 5-HT (and DA) neuronal activity and therefore increasing extracellular efflux of 5-HT (and DA) may be insufficient in explaining the mechanism of action behind ADs clinical effectiveness.

Following chronic paroxetine/clomipramine treatment with a 14 day drug holiday, extracellular 5-HT levels were still greater than controls and this suggests that some adaptive process has led to this increase. Although as mentioned earlier (section 4.3.4), paroxetine and clomipramine plasma levels must have been negligible after a 14 day drug holiday, the SERT may still have been blocked by these ADs. This is the first study of its kind which measures the extracellular monamine (5-HT) levels after a drug holiday (washout period).

Similar to the chronic paroxetine and clomipramine treatment, the 48 hour drug holiday dosing regime was able to abolish the decrease in extracellular 5-HT levels seen after acute paroxetine/clomipramine treatment following NMDA infusion. On the other hand, when NMDA was infused to rats treated chronically with paroxetine/clomipramine with a 14 day drug holiday, there was a decrease in 5-HT efflux similar to the acute study. There were no changes in the extracellular 5-HIAA levels in either of the studies (refer to section 4.3.1.1).

4.3.5 A change in NMDA receptor subunit composition?

The potential role of NMDA receptors in AD treatment was first proposed by Trullas and Skolnick (1990) who observed that antagonists of NMDA receptor function exerted an AD like action in the forced swim test. Subsequently, a number of compounds, acting at different sites on the NMDA receptor complex, have shown AD-like activity (Maj et al, 1992ab; Layer et al, 1995). Papp and Moryl (1993, 1994) found that chronic treatment with both competitive and non-competitive antagonists to be as effective as imipramine in a rodent behavioural model of depression. In mice it has been shown that chronic antidepressant treatment leads to a decrease in the affinity of (^{3}H)-5, 7- dichlorokinurenic acid for the NMDA receptor complex associated glycine site in the frontal cortex (Paul et al, 1994), which could lead to a decrease in excitatory transmission via NMDA receptors *in vivo*. This chronic AD-induced adaptive change in the ligand binding properties of the NMDA receptor complex persist for 5-10 days after the cessation of treatment (Paul et al, 1994) and this may explain why after a 14 day

drug holiday the effects of chronic paroxetine/clomipramine treatment on 5-HT efflux in the frontal cortex were diminished when the NMDA receptor was stimulated.

The effect of ADs on the NMDA receptor complex are regionally specific, as imipramine's action on NMDA receptors only manifests in membranes prepared from cortex and not hippocampus or striatum (Nowak et al, 1993). A study by Porter and Greenamyre (1995) used quantitative autoradiography to study the regional binding characteristics of a number of NMDA receptor antagonists including amantadine and MK-801. These authors reported regional variations in the pharmacology of NMDA receptor channel blockers, suggesting that the pharmacological properties of NMDA receptors are region-specific.

More recently, Boyer et al (1998) reported more widespread decreases in NMDA subunit mRNA throughout the rat brain following AD treatment (imipramine and citalopram). To date, this study is the only of its kind which determines the effects of chronic AD treatment on the expression of NMDA receptor subunit mRNA in the rat brain. Both the drugs used in the study were observed to have altered the levels of mRNA encoding the ξ subunit in several brain regions including the cortex, thalamus, striatum and the cerebellum while having no significant effect in the hippocampus. The mouse ξ subunit is thought to be analogous to the NR1 subunit while the ε1 and ε2 subunits to be analogous to NR2A and NR2B subunits respectively. The authors found that imipramine produced widespread reductions in levels of ε2 mRNA subunits in the cortex, hippocampus and amydala while citalopram had a different effect on the subunits. Citalopram reduced ε1 mRNA levels in the same areas. Ongoing research by our colleagues (Hutson et al, personal communication) reveal that NR1 protein levels in the frontal cortex, hippocampus but not the amygdala are decreased upon chronic AD (clomipramine and roboxetine) treatment. The greatest effect was seen in the frontal cortex. We are currently extending these studies using paroxetine. These 3 brain regions were chosen as they are reported to be vital in the processing of emotions and are implicated in depression (Marano, 1999). Thus the studies undertaken by Boyer et al (1998) and Hutson et al (2001 unpublished results) are in agreement with our data suggesting that chronic AD treatment produces region-specific changes in NMDA receptor function most probably altering NMDA receptor subunit composition. These different subunit compositions in different brain regions dictate the physiological and the pharmacological differences of regional NMDA receptors and may influence the

therapeutic actions of ADs. A parallel study by Riva et al (1997) using acute and chronic dosing with antipsychotic drugs (such as haloperidol and clozapine) showed significant differences in the composition of NMDA receptor subunits following treatment. Therefore, variability in NMDA receptor subunit composition and the adaptive changes exerted on these subunits by pharmaceutical agents may not apply to depression but to a wide range of neurological disorders.

This data suggest an association between repeated AD treatment with paroxetine/clomipramine and the regulation of serotonergic transmission by NMDA receptors in the frontal cortex (Figure 4.16). The observation that acute paroxetine and clomipramine treatment had no significant effect on NMDA-evoked changes in extracellular 5-HT levels indicates that the effects of chronic treatment may be significant in the therapeutic action of paroxetine, clomipramine and most likely other ADs.

In addition to the NMDA receptor population, other neuronal components, e.g. the 5-HT carrier and 5-HT_{1B} receptors, may have also undergone adaptive changes. Findings by our colleagues (Hutson et al, unpublished results) that clomipramine and roboxetine decrease NR1 NMDA subunit would support these interpretations.

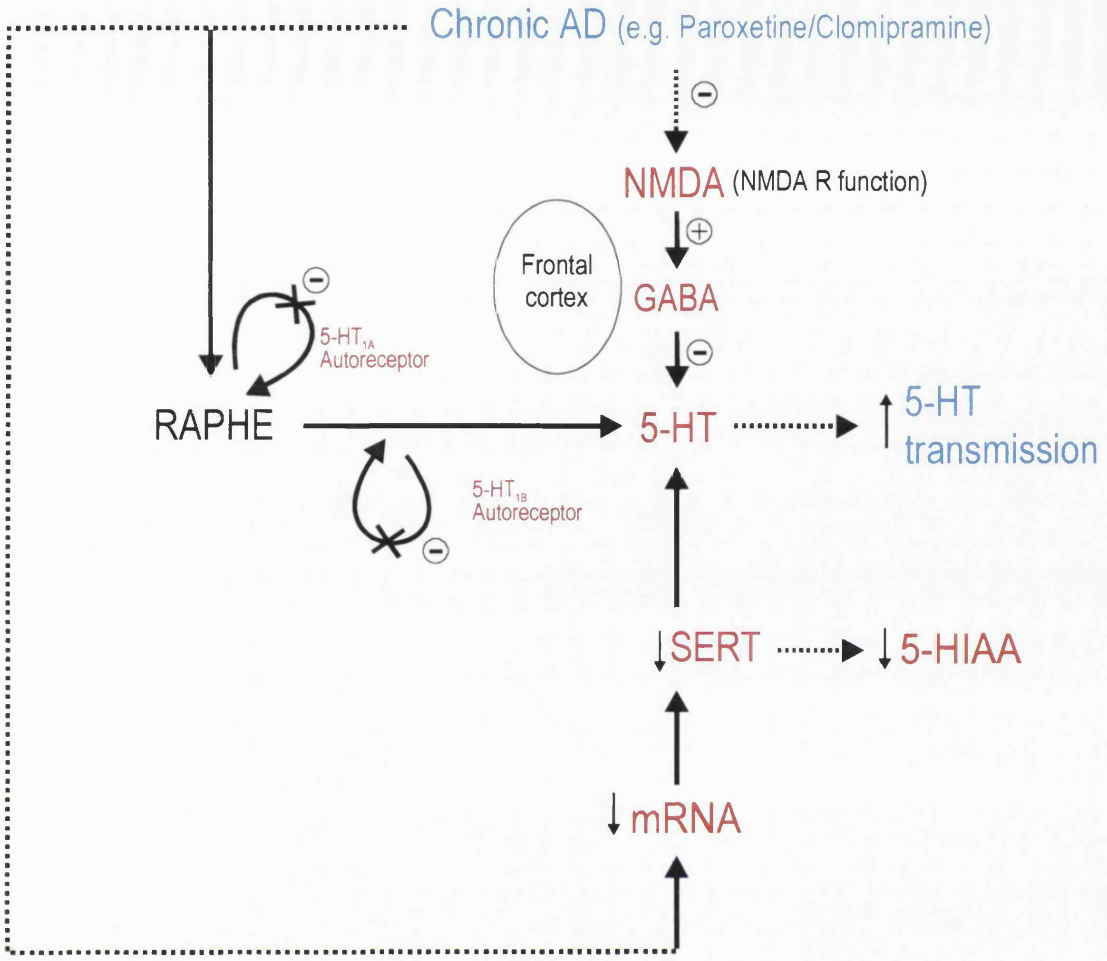


Figure 4.16 Sketch to show the effects of chronic antidepressants on 5-HT transmission.

Chronic ADs (e.g. paroxetine and clomipramine) cause:

- A functional desensitisation of both 5-HT_{1A} and 5-HT_{1B} autoreceptors, resulting in disinhibition of neuronal firing and a reduction in feedback inhibition of transmitter release from the terminals.
- A decrease in SERT mRNA which leads to decrease 5-HIAA levels.
- A decrease in NMDA receptor function which will cause an increase in 5-HT levels.
- These effects cause an overall **INCREASE IN 5-HT TRANSMISSION**.

Chapter 5

The effects of co-administration of paroxetine with NMDA antagonists on DA, DOPAC, 5-HT and 5-HIAA efflux in the frontal cortex

5 The effects of co-administration of paroxetine with NMDA antagonists

5.1. Introduction

5.1.1 NMDA antagonsits

Several NMDA antagonists have previously been shown to display some antidepressant properties in both animal models of depression (Papp and Moryl, 1994; 1996) and in the clinic (e.g. amantadine: see Huber et al, 1999).

5.1.1.1 Competitive antagonists

Competitive antagonists act on the GLU recognition site, an example of which is R-E-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116). CGP40116 is the synthetic biologically active enantiomer of CGP 37849, a competitive NMDA antagonist with improved bioavailability (Wlaz et al, 1999). Thus new NMDA antagonists that have increased affinity for the NMDA receptor and improved brain tissue penetration represent the next generation of NMDA antagonists.

Competitive NMDA antagonists have several behavioural effects. When given systemically or by local injection into the striatum or nucleus accumbens, they cause psychomotor stimulating effects (Schmidt, 1994).

A range of doses of CGP40116 given i.p. have been used in rats ranging from 0.5 mg/kg (Bienkowski et al, 1997) to 5 mg/kg (Wedzony et al, 1996; Wlaz et al, 1999; Fisher et al, 1998; Fisher and Starr, 2000; Paschoa et al, 2000;). Behavioural studies performed in this laboratory have shown that the higher doses (5 mg/kg) of CGP40116 dramatically reduce the exploratory behaviour of rats (Eradiri at University of London, personal communication). Based on these observations, it was decided to use a dose of 1 mg/kg in this study. The use of a higher dose might not be pharmacologically specific in its action and it would be difficult to relate any observations purely to the antagonistic action of CGP40116.

5.1.1.2 Non-competitive antagonists

These antagonists act on sites other than the GLU recognition site. These compounds can be further divided into channel blockers (e.g. amantadine and budipine), polyamine site antagonists (e.g. ifenprodil) and GLY site antagonists (e.g. HA-966). These compounds are known to exhibit strong psychomotor stimulating effects and to elicit rewarding effects.

5.1.1.2a Ion channel blockers - amantadine and budipine

Compounds that act on the MK-801 binding site on the NMDA receptor-gated ion channel are referred to as ion channel blockers. One such compound, amantadine, a 1-amino-adamantane derivative was introduced in 1963 and has been used successfully in a variety of neurological, psychiatric, and other clinical disorders. Not only does amantadine improve the classical symptoms of Parkinsonism, such as tremors and bradykinesia, but it also exerts a positive effect on the feeling of well-being and cognitive functions in Parkinsonian patients. Amantadine also alleviates the impairment of motor function induced by neuroleptic drugs (see Huber et al, 1999).

There is only limited data on the use of amantadine as an antidepressant. However, clinical evidence, suggests that amantadine might be useful to treat depressive symptoms (see Huber et al, 1999). There are no serious adverse effects associated with amantadine therapy. However, there are some reports of minor adverse effects such as nervousness, drowsiness, attention deficit and insomnia (see Huber et al, 1999). Upon discontinuation of the drug, these effects disappear rapidly.

After a review of the literature (Kornhuber et al, 1995; Dansyz et al, 1997; Fisher et al, 1998; 1999; Huber et al, 1999,), and based on studies carried out in our department (P.S. Whitton), it was decided that a dose of 40 mg/kg amantadine given i.p. was appropriate for this study. Kornhuber and colleagues (1995) report that at behaviourally active doses in the rat (doses between 23 and 92 mg/kg) and under therapeutic conditions in man, extracellular amantadine concentrations were found to be in the low micromolar range (between 6 and 21 μM). With these concentrations, amantadine

probably interacts with the phencyclidine (PCP) binding site of the NMDA receptor as shown by binding studies by these authors (K_i= 10 μM).

Jackisch et al (1994) reported that budipine binds with a weak affinity to NMDA receptors and inhibits NMDA-induced ACh release. This has to lead to the hypothesis that it can also reduce the excitability of glutamatergic projection neurones. 1-*tert*-butyl-4,4-diphenylpiperidine (budipine) was first synthesised in the 1970s and recognised as an effective antiParkinsonian drug shortly afterwards. Budipine's mechanism of action has not yet been fully elucidated. Initial observations by Menge and Brand (1982) that budipine reverses the cataleptic state induced by neuroleptics suggested that budipine might act upon the dopaminergic system. However, receptor binding studies failed to demonstrate specific binding of budipine to DA receptors (Przuntek and Stasch, 1985). Furthermore, budipine does not affect KCl-induced DA release (Jackisch et al, 1993). In contrast, a recent *in vitro* study reported that budipine inhibits synaptosomal DA uptake and enhances spontaneous DA release from rabbit caudate nucleus slices (Jackisch et al, 1993). This observation suggests that a DA-releasing action of budipine contributes significantly to the antiParkinsonian action of budipine *in vivo.*

Budipine possesses antimuscarinic properties. It inhibits ACh-induced contractions of isolated guinea pig ileum in a dose-dependent way, yielding a pA_2 value of 6.68 (Klockgether et al, 1996). However, it has generally been agreed that budipine's antimuscarinic action, in itself, cannot account for its therapeutic effects (Iizuka, 1985; Xinde, 1985). In searching for an alternative explanation, investigators have turned their attention to a possible interaction of budipine with NMDA receptors. Budipine binds with relatively low affinity to NMDA receptors (K_i= 12 μM, Kornhuber et al, 1995). In addition a recent microdialysis study by Klockgether et al (1996) reports that budipine mainly acts by blocking muscarinic and NMDA transmission. Facilitation of dopaminergic transmission does not appear to contribute to its *in vivo* action.

On the basis of evidence in the literature (Biggs et al, 1998; Fisher et al, 1999 Klockgether et al, 1996), it was decided that a dose of 10 mg/kg budipine was appropriate for this study. According to Zech et al (1985), low micromolar concentrations (10 μM) of budipine will be present in the CNS of the rat after administering systemic doses in the range of 5-20 mg/kg. This concentration represents the upper limit of budipine that can be reached in the human brain after therapeutic

doses. At this concentration it can be assumed that budipine is able to bind to the PCP binding site of the NMDA receptor in the frontal cortex (K_i= 12μM, Kornhuber et al, 1995).

5.1.1.2b Polyamine site antagonists - ifenprodil

Initially, ifenprodil was developed as a commercial antihypertensive agent and was found to possess potent activity at several brain receptors, including α_1 adrenergic receptors and NMDA receptors (Karbon et al, 1990; Chenard et al, 1991). Ifenprodil was reported to be structurally unique as an NMDA antagonist. The molecular cloning and expression of NMDA receptor subunits in *xenopus oocytes* demonstrated that the affinity of NR1A/NR2A receptors for ifenprodil (IC_{50}=146 μM) was 400-fold lower than that of NR1A/NR2B receptors (IC_{50}=0.34 μM) indicating that ifenprodil was more selective for receptors containing the NR2B subunit compared to those containing the NR2A subunit (Williams, 1993).

A review of the literature showed that 0.9 mg/kg i.p. in the rat is the ID_{50} value which antagonises the stimulatory effect of intrastriatally dialysed NMDA on rat striatal release (Carter et al, 1988), therefore this dose was used in this study.

5.1.2 Is it possible to produce a rapid AD response?

Whatever AD is used to treat clinical depression, there is in general a minimum 3-week delay or 'therapeutic lag' before a clear therapeutic effect is obtained. The introduction of ADs which can be given at a therapeutic dose from the start of treatment, and which reach steady-state levels within a few days, has not shortened the delayed onset of action of ADs. It has been suggested that co-treatment with the 5-HT_{1A} antagonist pindolol with selected ADs may accelerate their action so reducing therapeutic lag (Blier and de Montigny, 1994; Artigas et al, 1996). Consequently, the lag in response to AD treatment is most likely explained by adaptive changes that gradually develop secondary to the acute effects of the AD. Increased efficacy or reduced latency, perhaps from a new class of ADs would be an ultimate goal. It is worth exploring NMDA antagonists because they have a promising pharmacological and possibly, therapeutic profile. Functional antagonists that act on the NMDA receptor complex [e.g. AP-7 (Watkins and Olverman, 1987), the competitive NMDA receptor antagonist; ACPC, a

glycine partial agonist and MK-801, the use dependent channel blocker (Wong et al, 1986)] are all as effective as TCAs in preclinical tests that predict AD activity.

5.1.3 The role of NMDA receptors in AD action

Trullas and Skolnick (1990) suggested that NMDA receptors may be involved in behavioural deficits induced by inescapable stress. Hence, these authors deduced that the NMDA receptor could be involved in the pathophysiology of depression and that substances that reduce transmission at these receptors may represent a new class of antidepressants.

Additionally, it is well established that *in vivo* monoamine release is partially regulated by glutamatergic receptors in several brain regions (Becquet et al, 1990; Whitton et al, 1992; 1994a; 1994b). These findings are consistent with the hypothesis that the NMDA receptor complex may play a role in antidepressant action and that the NMDA receptor complex is implicated in the pathophysiology of depression as has been mentioned in the previous chapters.

In the present study the effects of acute paroxetine treatment on monoamine efflux was investigated when it is co-administered with the clinically used NMDA antagonists amantadine and budipine and the polyamine site antagonist, ifenprodil as well as the competitive antagonist, CGP 40116.

Three classes of NMDA antagonists were used to address the following questions in this study:

- What are the effects of the acute co-administration of paroxetine and the clinically used weak NMDA antagonists, amantadine and budipine on DA and 5-HT efflux and their metabolites, DOPAC and 5-HIAA respectively?
- Are the same effects achieved if paroxetine and the competitive NMDA antagonist, CGP 40116 or the polyamine site antagonist, ifenprodil were co-administered?

5.2 Results

5.2.1 The effect of CGP40116 on paroxetine-induced changes in the extracellular levels of DA, DOPAC, 5-HT and 5-HIAA in the frontal cortex

Acute administration of CGP40116 alone caused a decrease in extracellular levels of DA and DOPAC while having no effect on 5-HT and 5-HIAA levels.

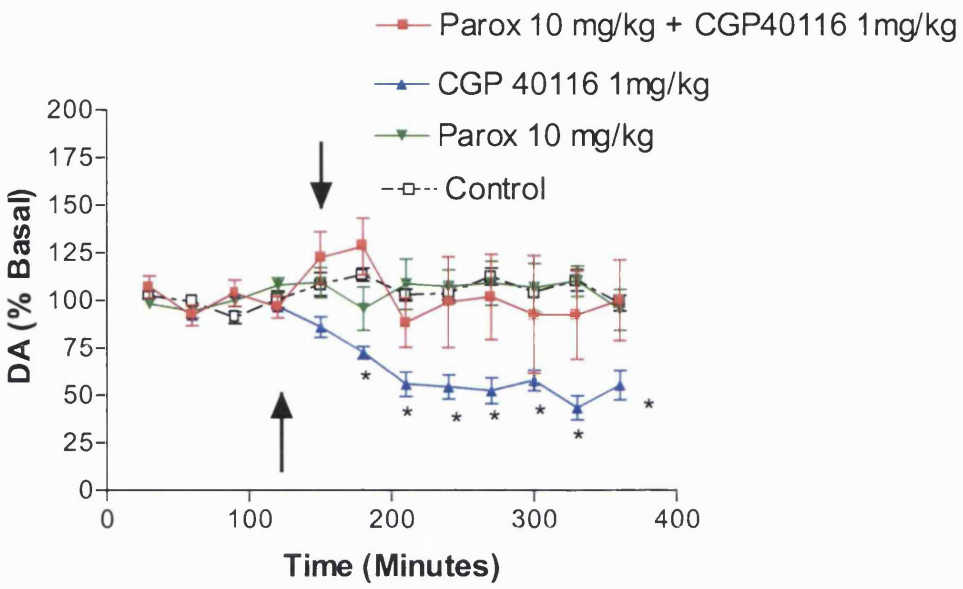
CGP40116 when given alone caused extracellular DA levels to fall rapidly to 45 % of basal by 180 minutes post injection ($p<0.01$) (Figure 5.1A). These low levels remained for the duration of the experiment. This clear downward trend was observed immediately following CGP40116 administration. Interestingly, the combination of CGP40116 and paroxetine was able to prevent the decrease in extracellular DA seen when CGP40116 was given alone (Figure 5.1A). Two-way repeated ANOVA revealed significant differences over time [$F(11,132)=2.97$, $p<0.01$] and between drug treatments [$F(3,12)=7.60$, $p<0.01$].

CGP40116 alone decreased DOPAC efflux [$F(3,12)=8.62$, $p<0.05$] while the combination of CGP40116 and paroxetine did not have any effect on extracellular DOPAC levels (Figure 5.1B).

CGP40116 given alone, caused an apparent decrease in 5-HT efflux to 73% of basal levels was not significant ($p>0.05$). Interestingly, the combination of CGP40116 and paroxetine caused a rapid and significant increase in extracellular 5-HT levels (191% basal levels) [$F(3,12)=40.90$, $p<0.01$] that lasted and remained significant for the duration of the experiment [$F(11,132)=4.51$, $p<0.05$] (Figure 5.2A).

Finally, neither CGP40116 alone nor the combination of CGP40116 and paroxetine had any effect on extracellular 5-HIAA levels (Figure 5.2B).

A)



(B)

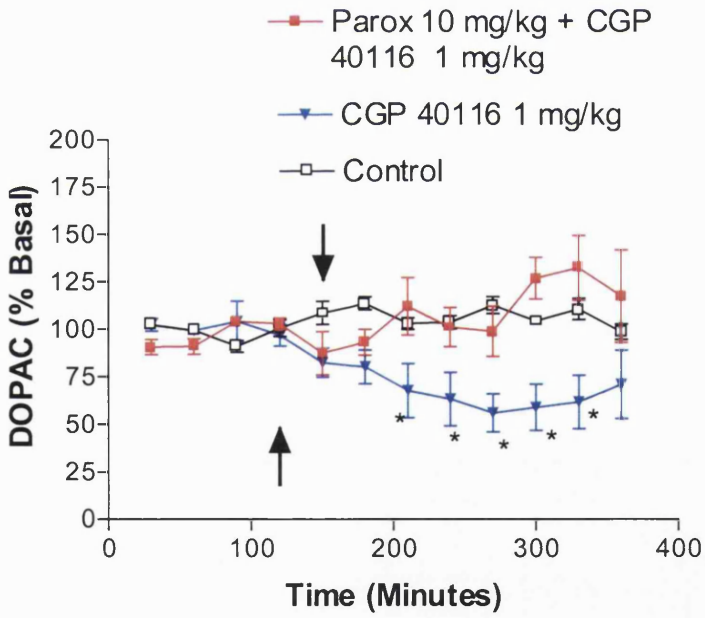
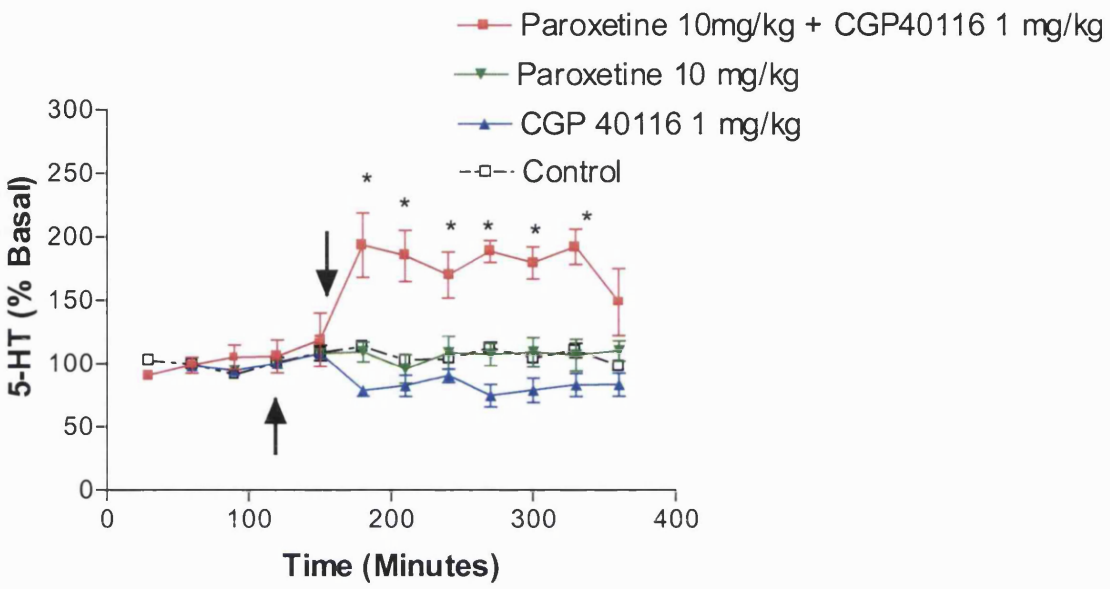


Figure 5.1. The effect of CGP-40116 on paroxetine(Parox)-induced changes in the extracellular levels of A) DA and B) DOPAC in the frontal cortex. The first arrow indicates the time of the i.p injection of CGP40116 and the second is the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

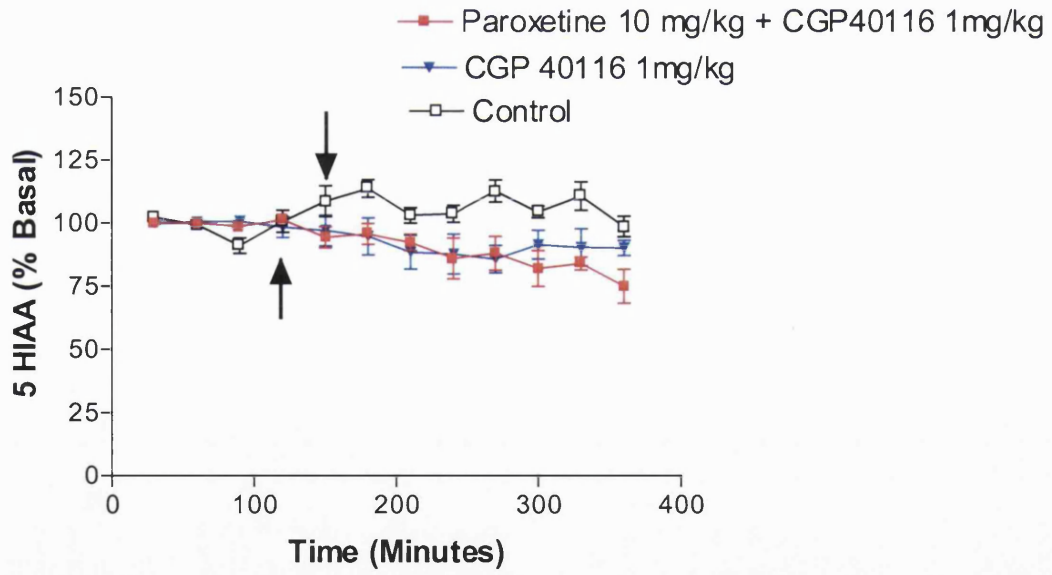


Figure 5.2. The effect of CGP-40116 on paroxetine(Parox)-induced changes in the extracellular levels of A) 5-HT and B) 5-HIAA in the frontal cortex. The first arrow indicates the time of the i.p injection of CGP40116 and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

5.2.2 The effect of amantadine on paroxetine-induced changes in the extracellular levels of DA, DOPAC, 5-HT and 5-HIAA in the frontal cortex

Acute administration of amantadine (40 mg/kg) significantly increased DA efflux in the frontal cortex. However, the same amantadine dose had no significant effect on DOPAC, 5-HT and 5-HIAA efflux.

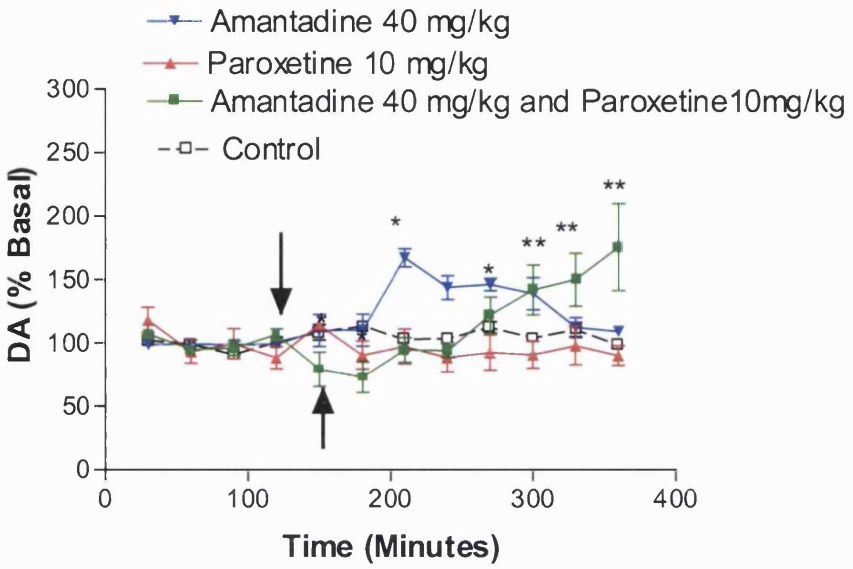
The effect of acute amantadine on extracellular DA was rapid in onset. A statistically significant increase in DA efflux was seen using cluster one way ANOVA over 3 time points (210-270 minutes) ($p<0.05$). When paroxetine (10 mg/kg) was given in conjunction with amantadine, a delayed statistically significant increase after 120 minutes post paroxetine injection (270 minutes) was observed that lasted until the end of the experiment. Two-way repeated ANOVA revealed significant differences over main factor 'time' [$F(11,132)=32$, $p<0.01$] and between treatments [$F(3,12)=22.6$, $p<0.01$]. The maximal increase in extracellular DA was observed at the last time point after injection which was 200% above basal levels (Figure 5.3A).

Paroxetine when given in conjunction with amantadine did not have any effect on extracellular DOPAC in the frontal cortex (Figure 5.3B).

Unlike the effect of acute amantadine on DA efflux, amantadine did not cause any change on extracellular 5-HT. However, when paroxetine was combined with amantadine a profound statistically significant increase in extracellular 5-HT was clearly evident [$F(3,12)=11.32$, $p<0.01$]. This significant increase was immediate and peaked at 230% of basal and remained at this level for the duration of the experiment [$F(11,132)=4.90$, $p<0.01$] (Figure 5.4A).

The effect of acute amantadine as well as the combination of paroxetine and amantadine failed to have a significant effect on extracellular 5-HIAA (Figure 5.4B).

(A)



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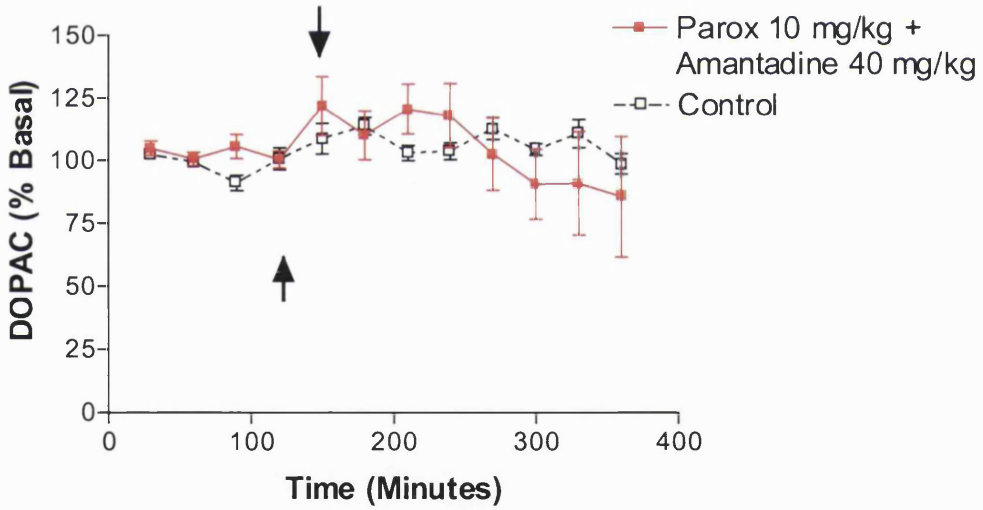
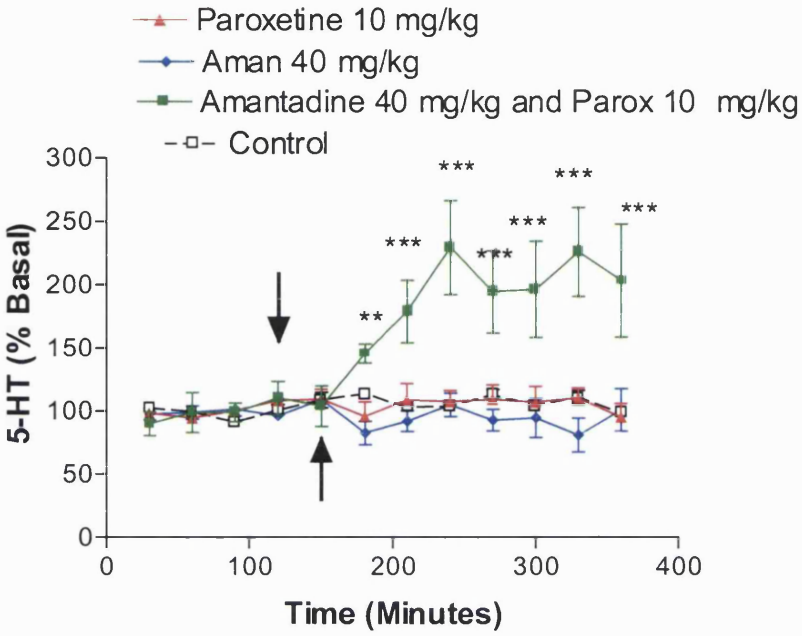


Figure 5.3. The effect of amantadine on paroxetine(parox)-induced changes in the extracellular levels of A) DA and B) DOPAC in the frontal cortex. The first arrow indicates the time of the i.p injection of amantadine and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group.* indicates significantly different ($p<0.05$); ** ($p<0.01$) from control.

(A)



(B)

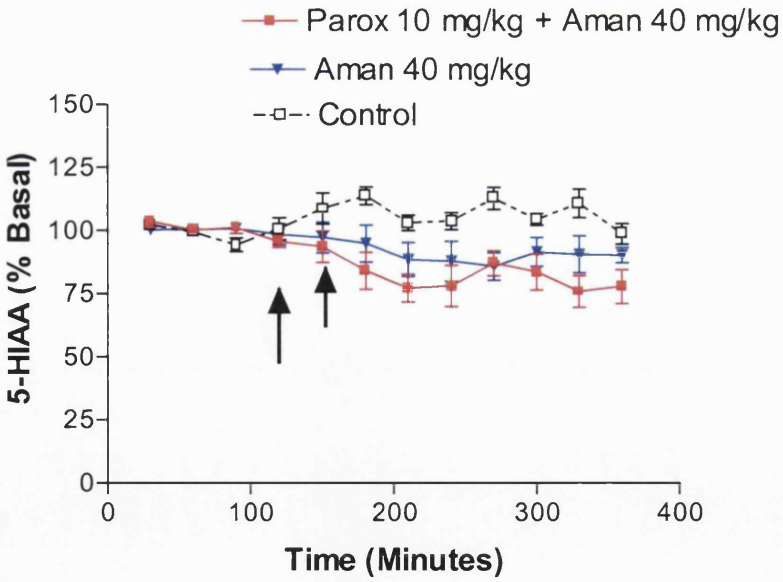


Figure 5.4 The effect of amantadine (Aman) on paroxetine(parox)-induced changes in the extracellular levels of A) 5-HT and B) 5-HIAA in the frontal cortex. The first arrow indicates the time of the i.p injection of amantadine and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group.** indicates significantly different ($p<0.05$); *** ($p<0.001$) from control.

5.2.3 The effect of budipine on paroxetine-induced changes in the extracellular levels of DA, DOPAC, 5-HT and 5-HIAA in the frontal cortex

Budipine, when given alone was observed to cause a delayed decrease in extracellular DA while having no effect on extracellular DOPAC, 5-HT and 5-HIAA.

Acute budipine administration caused a delayed decrease in extracellular DA levels. DA levels fell to 45% of basal at 300 minutes (180 minutes post budipine injection) and this lasted until the end of the experiment.

However, the combination of budipine and paroxetine had no significant effect on DA efflux [$F(3,12)=1.92$, $p=0.181$] with the maximum levels of DA being 150% of basal at 210 minutes (Figure 5.5A).

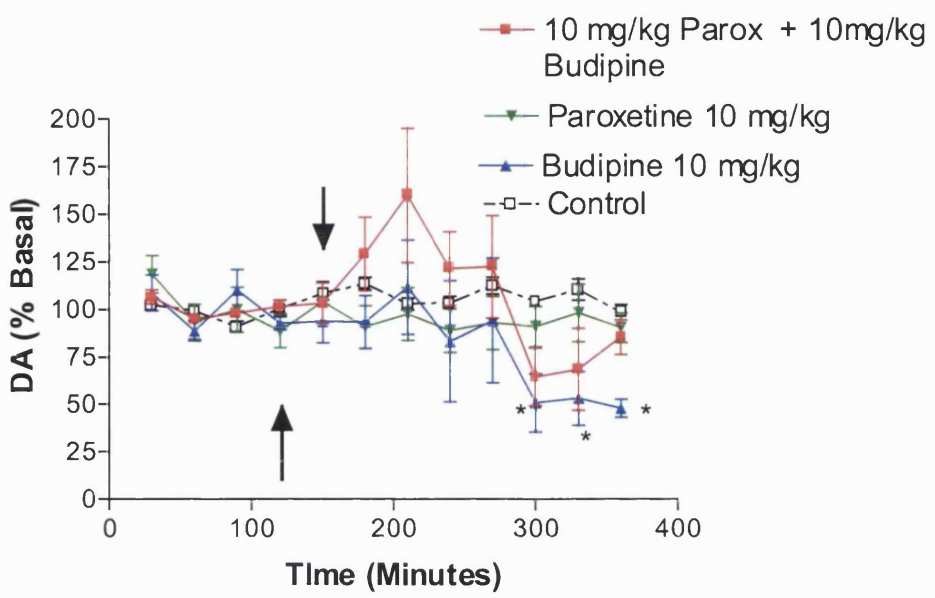
The apparent decrease seen for the last 3 time points (270-360 minutes) for the combination of paroxetine and budipine in DA efflux (65% basal) was not significant ($p>0.05$) (Figure 5.5A).

Budipine alone had no effect on DOPAC efflux. DOPAC levels only slightly rose towards the end of the experiment at 300 minutes with a maximal increase of 125% of basal levels (not significant, $p>0.05$). Similarly, when budipine was combined with paroxetine, there was no effect on DOPAC efflux (Figure 5.5B).

Although budipine alone did not have any effect on extracellular 5-HT levels when administered alone, the combination of budipine and paroxetine produced a statistically significant increase in extracellular 5-HT levels over time [$F(11,132)=2.19$, $p<0.05$] and between treatments [$F(3,12)=8.63$, $p<0.01$] A significant maximal increase in extracellular 5-HT was observed (170% of basal). This increase in extracellular 5-HT was rapid in onset following budipine administration and remained constant for the duration of the experiment (Figure 5.6A).

Neither budipine alone or the combination of budipine and paroxetine were able to cause any change to the extracellular 5-HIAA levels (Figure 5.6B).

(A)



(B)

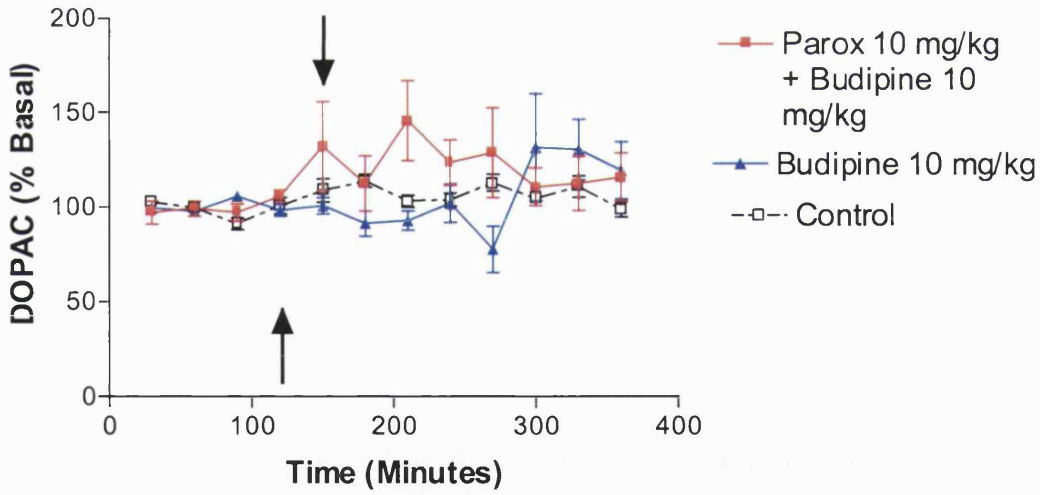
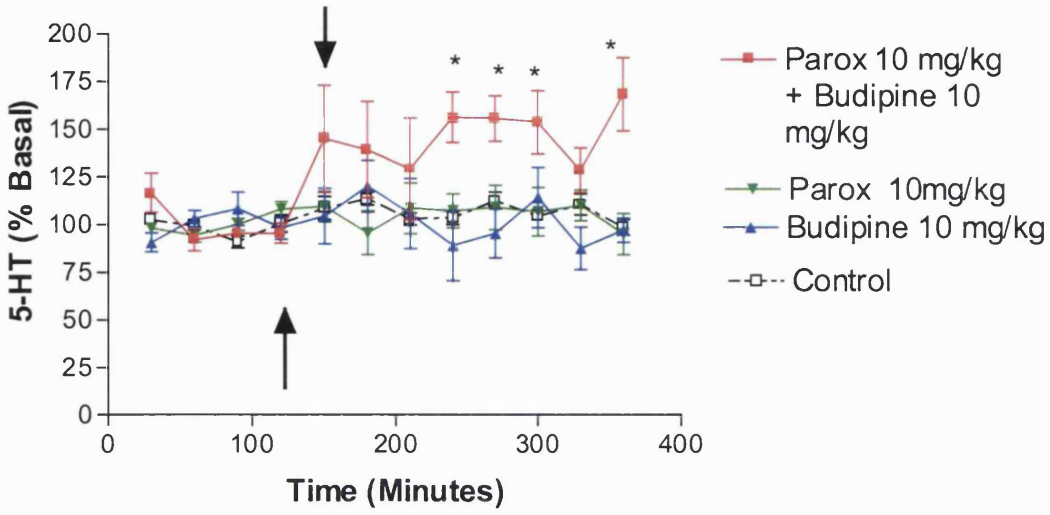


Figure 5.5 The effect of budipine on paroxetine(Parox)-induced changes in the extracellular levels of A) DA and B) DOPAC in the frontal cortex. The first arrow indicates the time of the i.p injection of budipine and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

(A)



(B)

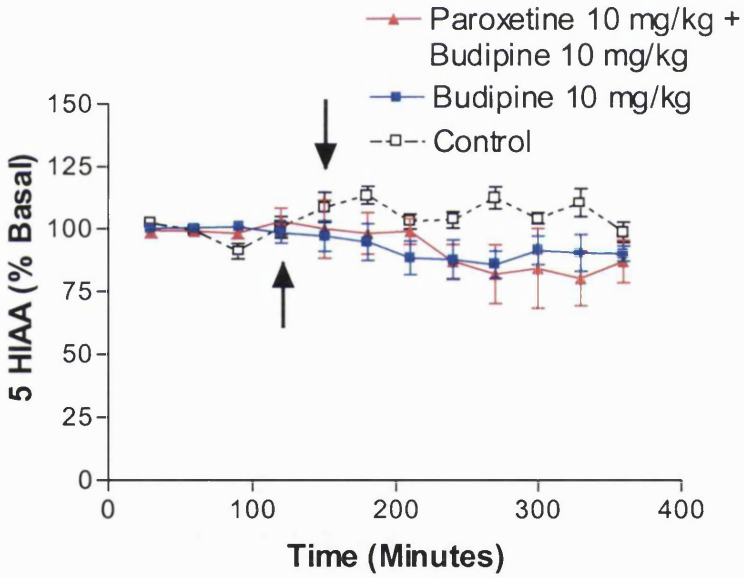


Figure 5.6. The effect of budipine on paroxetine(Parox)-induced changes in the extracellular levels of A) 5-HT and B) 5-HIAA in the frontal cortex. The first arrow indicates the time of the i.p injection of budipine and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

5.2.4 The effect of ifenprodil on paroxetine-induced changes in the extracellular levels of DA, DOPAC, 5-HT and 5-HIAA in the frontal cortex

Acute ifenprodil given alone did not seem to have any effect on extracellular DA, DOPAC, 5-HT and 5-HIAA.

Ifenprodil alone failed to cause any change in extracellular DA levels. The combination of ifenprodil and paroxetine similarly had no effect on DA efflux (Figure 5.7A).

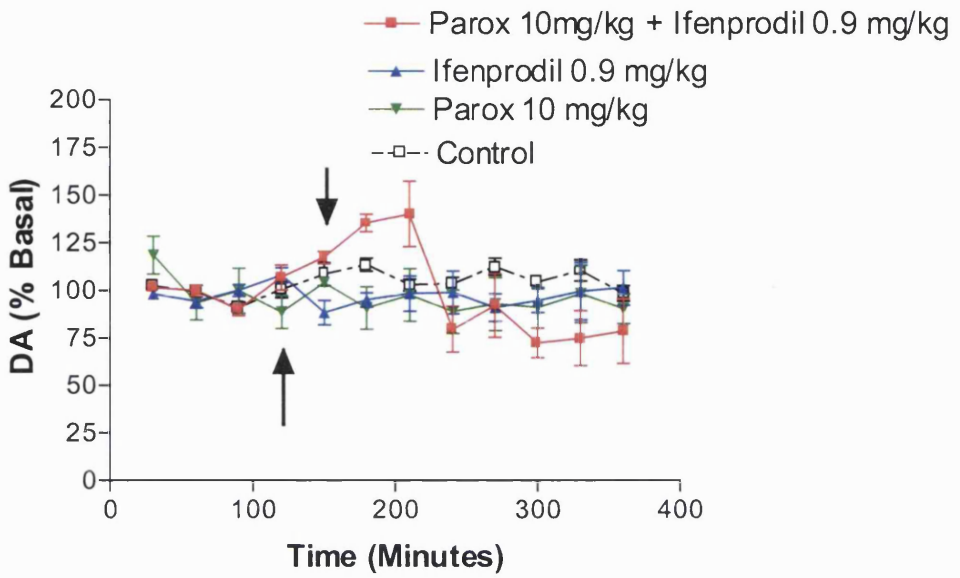
The apparent immediate, transient increase in extracellular DA to 133% of basal levels. followed by an immediate decrease in DA levels to 75% of basal which persisted for the duration of the experiment was not significant as revealed by cubic regression between treatments [$F(3,12)=0.813$, $p=0.511$] but were significant over time [$F(11,132)=2.33$, $p<0.05$].

The combination of ifenprodil and paroxetine caused a delayed effect in decreasing extracellular DOPAC levels. This significant decrease (68% of basal) was only seen at 270 minutes which lasted till the end of the experiment (Figure 5.7B).

As above, ifenprodil did not seem to have any effect on extracellular 5-HT levels when given alone, but interestingly the combination of ifenprodil and paroxetine caused an immediate significant increase in extracellular 5-HT (maximal levels 191% of basal levels) [$F(3,12)=5.30$, $p<0.05$]. This increase, significant over time [$F(11,132)=4.8$, $p<0.05$], persisted for 240 minutes before levels rapidly returned to basal levels (Figure 5.8A).

Ifenprodil alone nor the combination of ifenprodil + paroxetine did not have any effect on 5-HIAA efflux (Figure 5.8B).

(A)



(B)

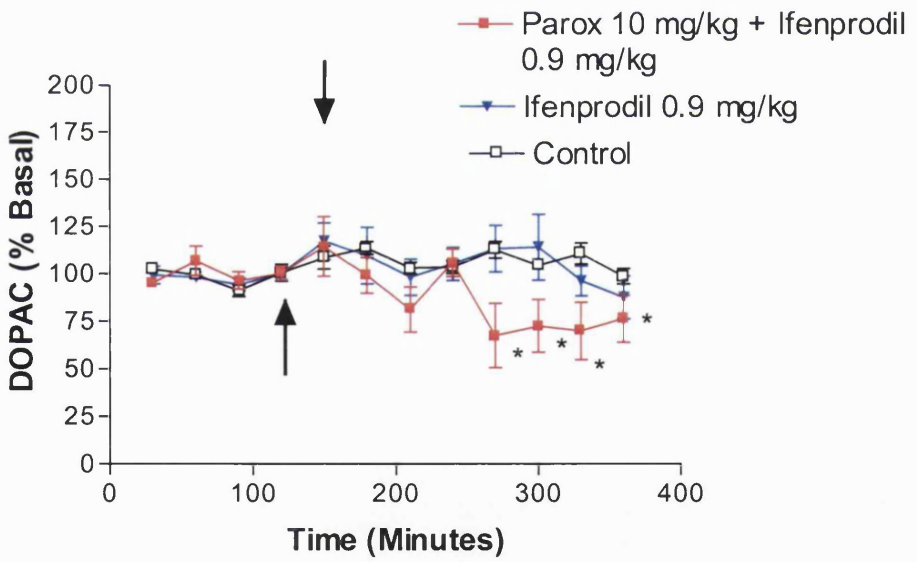


Figure 5.7. The effect of ifenprodil on paroxetine(Parox)-induced changes in the extracellular levels of A) DA and B) DOPAC in the frontal cortex. The first arrow indicates the time of the i.p injection of ifenprodil and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control.

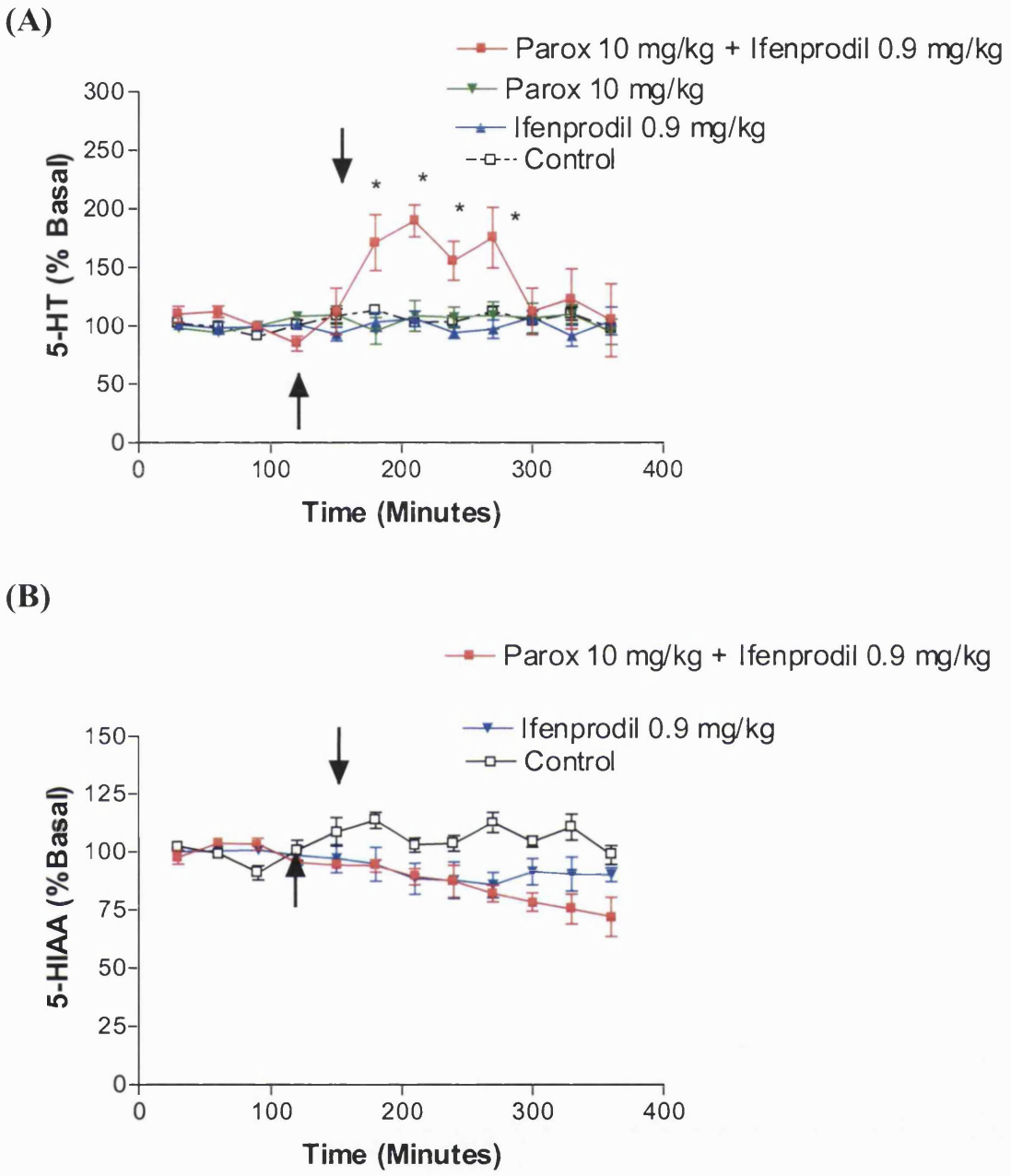


Figure 5.8. The effect of ifenprodil on paroxetine(Parox)-induced changes in the extracellular levels of A) 5-HT and B) 5-HIAA in the frontal cortex. The first arrow indicates the time of the i.p injection of ifenprodil and the second the time of the paroxetine injection. Data are the mean ± SEM of six animals in each group.* indicates significantly different ($p<0.05$) from control.

5.2.5 The effect of 7-day (sub-chronic) dosing of paroxetine on amantadine induced changes in the extracellular levels of DA, DOPAC, 5-HT and 5-HIAA in the frontal cortex.

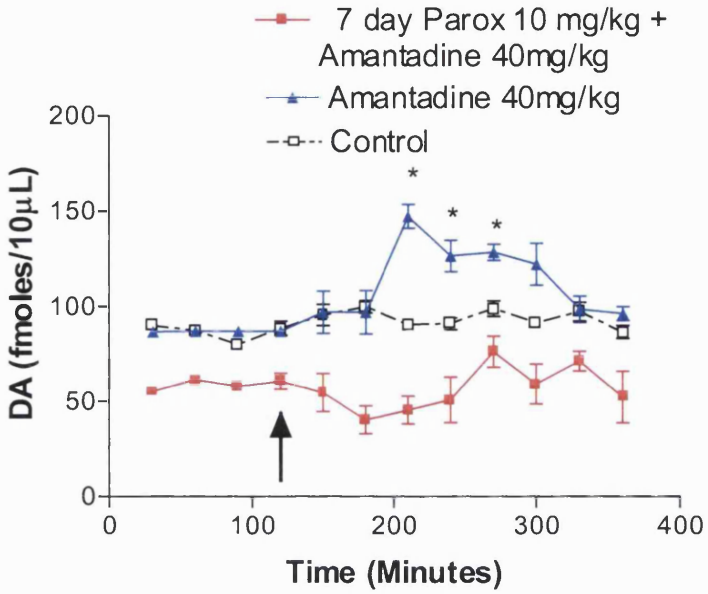
After a 7-day sub-chronic treatment with paroxetine, extracellular DA levels were reduced significantly to 67% basal [$F(11,99)=5.10$, $p<0.01$]. Amantadine failed to cause any change to extracellular DA levels in these rats. However, amantadine when given acutely to drug-free treated rats was able to affect a significant increase in extracellular DA levels (Figure 5.9A).

Extracellular DOPAC levels were observed to decrease drastically to 47% basal following 7-day paroxetine treatment. The administration of amantadine to these rats did not have any effect on extracellular DOPAC levels (Figure 5.9B).

Extracellular 5-HT levels were significantly increased to 125 % basal [$F(2,9)=54.40$, $p<0.05$] following the 7-day paroxetine treatment but here amantadine administration alone and in paroxetine-treated rats did not have any effect on extracellular 5-HT levels over time [$F(11,99)=0.556$, $p=0.86$] (Figure 5.10A).

Extracellular 5-HIAA levels were seen to drastically decrease to 53% basal following 7-day paroxetine treatment. Amantadine administration to this group of rats did not affect extracellular 5-HIAA levels (Figure 5.10B).

(A)



(B)

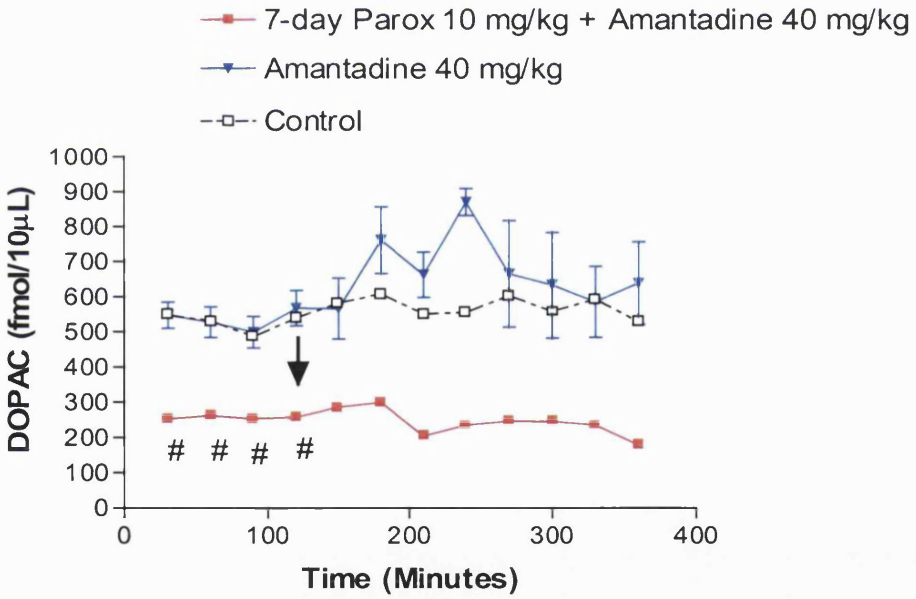
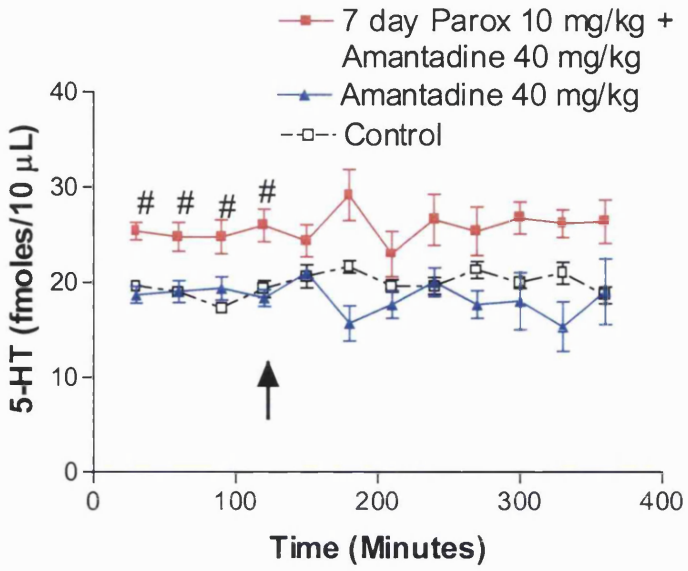


Figure 5.9. The effect of 7-day (sub-chronic) dosing of paroxetine (Parox) on amantadine induced changes in the extracellular levels of A) DA and B) DOPAC in the frontal cortex. The arrow indicates the time of the i.p injection of amantadine injection. Data are the mean ± SEM of six animals in each group. * indicates significantly different ($p<0.05$) from control. . # indicates significantly different from treatment basal ($p<0.05$).

(A)



(B)

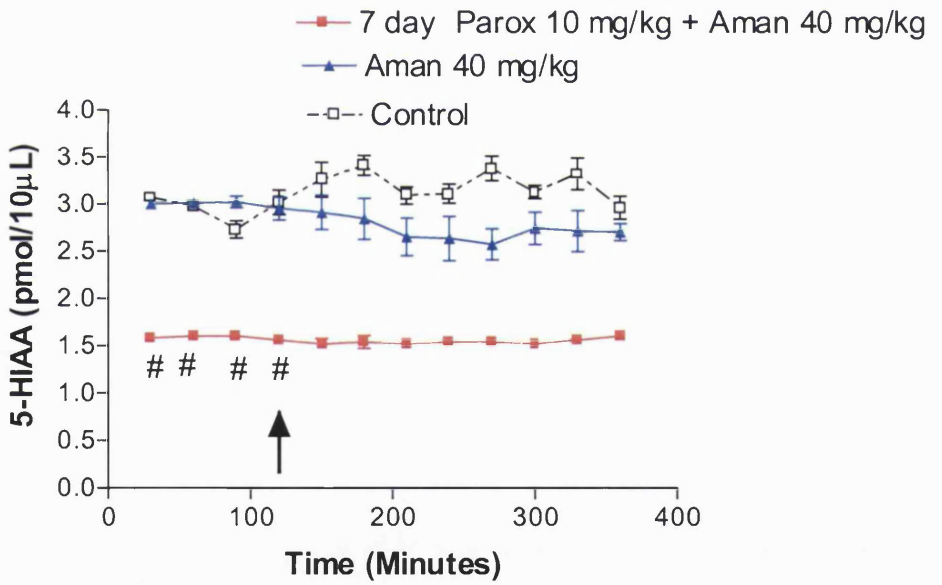


Figure 5.10. The effect of 7-day (sub-chronic) dosing of paroxetine (Parox) on amantadine (Aman) induced changes in the extracellular levels of A) 5-HT and B) 5-HIAA in the frontal cortex. The arrow indicates the time of the i.p amantadine injection. Data are the mean ± SEM of six animals in each group. # indicates significantly different from treatment basal ($p<0.05$).

Drug	**DA**	**DOPAC**	**5-HT**	**5-HIAA**
CGP40116 1mg/kg	↓	↓	N/E	↓
CGP40116 1mg/kg + Paroxetine 10mg/kg	N/E	N/E	↑	↓
Amantadine 40 mg/kg	↑	↑	N/E	↓
Amantadine 40 mg/kg + Paroxetine 10 mg/kg	↑↑	N/E	↑↑	↓
Budipine 10 mg/kg	Transient ↑	↓ then ↑	Transient ↑	N/E
Budipine 10 mg/kg + Paroxetine 10 mg/kg	Delayed ↓	↑	↑	N/E
Ifenprodil 0.9 mg/kg	N/E	N/E	N/E	↓
Ifenprodil 0.9 mg/kg + Paroxetine 10 mg/kg	↑ then ↓	↓	Transient ↑	↓
Amantadine 40 mg/kg + 7 day Paroxetine 10 mg/kg	Transient ↑	N/E	N/E	N/E

Table 5.1 Summary of chapter 5 results

There is considerable variation between different laboratories in the effects of agonists and antagonists at NMDA receptors on DA efflux (see: Whitton, 1997). Microdialysis studies have demonstrated that glutamate, AMPA, kainate and NMDA increase the efflux of DA in the striatum and nucleus accumbens (Imperato et al, 1990; Moghaddam et al, 1990; Youngren et al, 1993; see Whitton, 1997). Yet, paradoxically, NMDA-receptor antagonists have been reported to increase DA release in the prefrontal cortex (Wedzony et al, 1993; Hondo et al, 1994).

In this study, both the local application of NMDA (100 μM) and systemic CGP 40116 (1 mg/kg) reduced DA efflux in the frontal cortex. The lack of an excitatory effect of CGP 40116 on DA efflux in the frontal cortex conflicts with reports that NMDA-receptor antagonists increase DA release in the PFC (Wedzony et al, 1993; Hondo et al, 1994; Nishijima et al, 1994). The explanation for this lack of an excitatory effect of CGP 40116 is currently unknown. However it is reasonable to speculate that this could be due to modulation of DA efflux by non-NMDA receptors such as AMPA and kainate which may be mediated by CGP 40116.

5.3 Discussion

Trullas and Skolnick (1990) have shown that the administration of a competitive antagonist, AP-5, a use-dependent channel blocker (MK-801) and a glycine partial antagonist (ACPC) to mice reduced immobility in the forced swim test. In addition, a small clinical trial (Berman et al, 2000) recently reported that i.v. infusion of ketamine, a use-dependent NMDA channel blocker, produced short-lasting psychotomimetic effects (hours) and long-lasting AD action (days). The above findings suggest that blockade of NMDA receptors could be a useful clinical adjunct in the treatment of depression. Having said that, as most of the NMDA antagonists have significant drawbacks such as poor CNS penetration or hallucinogenic side-effects, they are not favoured for use in the clinic. On the contrary, amantadine and budipine are examples of weak non-competitive NMDA antagonists that are widely used clinically.

The question addressed in this study is whether these non-competitive antagonists accelerate the neurochemical changes and would possibly reduce the clinical lag associated with more potent ADs. This is a crucial issue, as the literature reports (Blier et al, 2000) it would be impossible to obtain such an early onset of action (e.g. by bypassing the neurobiological events which would lead to the return of the euthymic mood).

CGP 40116, a competitive antagonist at the NMDA receptor, caused a decrease in extracellular DA levels when given alone but had no effect on extracellular 5-HT levels in the frontal cortex. Therefore, it could be predicted, CGP 40116 when given alone would not have AD properties. However, a very interesting study by Papp and Moryl (1994), using CGP 40116 as an example of a competitive NMDA antagonist, showed that a 5-week chronic treatment (25 mg/kg p.o., twice daily) was able to reverse chronic mild stress-induced anhedonia with the same effect as chronic imipramine in terms of time course and magnitude. This study confirmed the results of previous studies that suggested that NMDA receptor antagonists may exhibit AD properties (Trullas and Skolnick, 1990; Maj et al, 1992ab). In this present study, the co-administration of acute paroxetine with CGP 40116, another competitive NMDA antagonist, increased extracellular 5-HT levels similar to those seen after chronic paroxetine treatment (Refer to Figure 4.8, Chapter 4). Again, it can be seen that co-administration of acute

paroxetine with an NMDA antagonist increases extracellular 5-HT levels in the frontal cortex.

Amantadine is believed to interact with the MK-801 binding site in the NMDA receptor channel, thus blocking the flow of Na^+, K^+ and Ca^{2+} ions through the receptor. In this present study, acute amantadine administration increased DA efflux in the frontal cortex. These results were in agreement to the observations by Spanagel et al (1994) who used both amantadine and memantine (another clinically used weak NMDA antagonist), and observed increases in extracellular DA in the frontal cortex. On the other hand, acute amantadine treatment did not alter extracellular 5-HT efflux in the same brain region. Amantadine has been shown to have low affinity for the NMDA receptor and has been shown to displace radiolabelled MK-801 from postmortem human Parkinsonian brain slices at doses which were physiologically-relevant (Kornhuber et al, 1991). It was originally believed that these drugs elicited a direct dopaminergic effect by facilitating presynaptic release of DA. However, this proposal has been disputed due to failure of other investigators to demonstrate direct stimulation of DA receptors (Mercuri et al, 1991; Jackisch et al, 1992). In addition, it is believed that amantadine reduces DA uptake into nerve cells and glia. This would allow DA to act on postsynaptic receptors for longer, without being degraded by catabolic enzymes such as MAO, although this action requires high concentrations of drug (Gianutos et al, 1985).

In contrast, a previous study in this laboratory, has shown another NMDA receptor antagonist, MK-801, to increase extracellular 5-HT levels in the hippocampus and striatum (Whitton et al, 1992a). Other investigators also report an increase in extracellular 5-HT levels seen with MK-801 in the nucleus accumbens (Yan et al, 1997) and the raphe (Callado et al, 2000). In addition, there is evidence that amantadine affects the serotonergic system by blocking 5-HT uptake into synaptosomes from rat forebrain, and releases 5-HT directly from rat brain synaptosomes (see Herblin, 1972). Tanaka et al, (1973) studied the effect of amantadine on the metabolism of 5-HT in the rat brain and they found that 1 hour post amantadine treatment (100 mg/kg), MAO activity, and in turn 5-HIAA levels, were reduced in the rat whole brain. In addition to amantadine's possible effect on the dopaminergic and serotonergic systems, amantadine may also possess noradrenergic properties. For example, amantadine but not memantine enhances the hind limb flexor reflex in spinal rats in a manner similar to enhancers of

noradrenergic transmission (Maj et al, 1974). In addition, amantadine attenuates reserpine-induced hypothermia which is also indicative of noradrenergic properties (Moryl et al, 1993). Thus it is worth noting that amantadine has actions at sites other than the NMDA receptor which might contribute to its synergistic effect in the when combined with paroxetine.

As reported in the previous chapters, acute paroxetine treatment alone does not have any effect on extracellular DA efflux and causes a dose-dependent decrease in extracellular 5-HT efflux in the frontal cortex. Interestingly, when amantadine was administered 30 minutes prior to paroxetine treatment, the effect on 5-HT efflux differed to that seen when paroxetine was used alone. In fact, the levels of extracellular 5-HT were similar to those seen after chronic treatment of paroxetine (Refer to Figure 4.8, Chapter 4). In addition, extracellular DA efflux increased towards the end of the experiment but not to the same extent as 5-HT levels. The slow increase in DA and 5-HT efflux strongly suggests that the paroxetine and amantadine combination are causing this effect due to uptake blockade and not by acting as releasing agents, such as fenfluramine (Gundlah et al, 1997). In the microdialysis study by Gundlah et al (1997), to establish the criteria to differentiate monoamine reuptake inhibitors from releasing agents, flenfluramine produced a much larger rapid increase in 5-HT efflux (20 fold) that was transient. In this study, the slow increase in both DA and 5-HT efflux suggests that uptake blockade is taking place.

This study is the first to report the effects of co-administration of amantadine with paroxetine. Results from this laboratory using clomipramine (Owen and Whitton, unpublished observation) indicate that when amantadine is given prior to clomipramine treatment, raised levels of extracellular 5-HT levels are seen.

The other important question considered in this study was whether it is possible to accelerate the neurochemical changes in the brain and hence obtain a rapid AD response with this co-treatment regime. The above results may suggest that the use of a clinically tolerated NMDA receptor antagonist may produce a more rapid therapeutic effect as it is widely assumed that a sustained increase in synaptic monoamine levels in brain regions plays a crucial role in the mechanism of action of ADs. There are three points of clinical evidence that support the notion that a rapid or immediate AD effect are feasible with this co-administration regime.

First, although controversial, the results of Benca et al (1992) indicated a one-night sleep deprivation produces a marked AD effect the following day in at least 50% of patients. Secondly, ECS often produces a marked AD effect in the first week of treatment (Rich et al, 1984; Rodger et al, 1994; Segman et al, 1995). Thirdly, patients in remission who have responded well to AD treatment after having undergone acute tryptophan depletion relapse within 5 hours, in most cases with the symptomology being identical to that presented before the AD regimen was initiated. Subsequently, when the normal AD diet is restored, the AD response returns within 24-48 hours (Delgado et al, 1990). The above three points of clinical evidence indicate that a depressive state can be rapidly reversed within 24 hours.

Budipine, another clinically used weak NMDA ion channel antagonist caused a delayed decrease in extracellular DA levels in the frontal cortex while having no effect on extracellular 5-HT levels. Based on the results of Przuntek and Stasch (1985) who suggest that budipine's pharmacological profile results from the blockade of synaptic uptake of DA and subsequent degradation of the monoamine, it was assumed an increase in extracellular DA following budipine administration would be observed. Budipine binds with a weak affinity to the NMDA receptor (K_i= 12 μM, Kornhuber et al, 1995) and inhibits NMDA-induced ACh release (Jackisch et al, 1994). This observation led to the hypothesis that, in addition to its mild indirect dopaminomimetic effects, it can reduce the excitability of glutamatergic projection neurones (Albin et al, 1989). Fisher et al (1998) have shown that NMDA antagonists including amantadine and budipine, showed marked activation of AADC activity, an enzyme important in monoamine biosynthesis, in the striatum and the substantia nigra. This increased enzyme activity may help potentiate the antiParkinsonian actions of L-DOPA in the clinic, which is decarboxylated to DA by this enzyme. In addition, Fisher and Starr (2000) concluded that glutamate exerts a physiological influence on DA biosynthesis by tonically suppressing AADC activity.

As with my results with amantadine, following the co-administration of acute paroxetine and budipine, extracellular 5-HT levels were increased to similar levels as those seen after chronic paroxetine treatment (Refer to Figure 4.8, Chapter 4). On the other hand, there was no increase in extracellular DA levels but the reduction of extracellular DA levels seen upon budipine administration was attenuated. It can be suggested that marked increases in extracellular 5-HT in the frontal cortex with

amantadine or budipine co-administered with paroxetine may lead to a more rapid therapeutic effect due to the accelerated neurochemical changes in 5-HT efflux in the frontal cortex.

The polyamine site antagonist, ifenprodil, exhibits marked cytoprotective activity in animal models of focal ischaemia (Gotti er al, 1988) and is already in clinical use (Otomo et al, 1985). This suggests that the interaction of ifenprodil with the NMDA receptor is the most likely mechanism responsible for its cytoprotective properties. Ifenprodil also alters cerebral blood flow (Delage et al, 1985) via an interaction with α-adrenoceptors (Honda and Sakai, 1985). There is no evidence of any AD activity of ifenprodil but it was decided to test whether the co-administration of this clinically used NMDA antagonist, which acts on a different site to amantadine/budipine and CGP 40116, has any effect on DA or 5-HT efflux.

In this study, administration of ifenprodil alone did not have any effect on DA or 5-HT efflux. On the other hand, when acute paroxetine was co-administered with ifenprodil, there was an increase in extracellular 5-HT levels similar to those observed following chronic paroxetine treatment (Refer to Figure 4.8, Chapter 4). However, these increases were different to the other NMDA antagonists tested as the increase was only transient.

Williams (1993) has shown ifenprodil to be highly selective for the NMDA NR2B subunit and this may be significant in explaining why ifenprodil's co-administration with paroxetine only caused a transient increase in extracellular 5-HT. As yet, there is still no consensus as to where ifenprodil binds to the NMDA receptor. Initially, Carter et al (1990) reported that ifenprodil binds to the same binding site as spermine, which is the 'polyamine site'. However, recent evidence by Kew and Kemp (1998) suggests that ifenprodil and spermine act at discrete sites with an allosteric interaction. Grimwood et al (2000) report that there are differences in the allosteric interactions between the ifenprodil site, the polyamine and glutamate sites on the NMDA receptor complex. The authors suggest that these differences were due to the presence of different subunit combinations in different ratios, i.e. NR1 to NR2 subunits or the NR1 splice varient(s). Despite the inconclusive location of the ifenprodil binding site, it can again be seen that the co-administration of acute paroxetine with a potentially allosteric NMDA antagonist increases extracellular 5-HT levels in the frontal cortex and this may have some clinical use.

In Chapter 3 it was reported that a 7-day treatment with paroxetine abolishes the effect of NMDA infusion on DA and 5-HT efflux. It was also reported that 7-day paroxetine treatment decreases and increases extracellular DA and 5-HT basal levels respectively. Hence, it was suggested that after 7-day treatment, adaptive changes are taking place at the NMDA receptor level. From the above results, it was decided to see whether there would be an even greater potentiating effect on extracellular 5-HT efflux with the co-administration of amantadine to rats treated with 7-day paroxetine. Unlike the acute co-administration of paroxetine and amantadine, 7-day paroxetine treatment failed to further potentiate the effects of this coadministration. Presently, no suggestions can be made for these observations other than that 7-day paroxetine treatment altered the binding properties of the NMDA receptor which had an effect on amantadine binding to the PCP site.

These data suggest that NMDA antagonists accelerate functional changes in the regulation of 5-HT transmission by NMDA receptors. Thus, NMDA antagonists when co-administered with paroxetine increase extracellular 5-HT levels. As 5-HT is thought to play a major role in the aetiology of depression, these results suggest ways of improving the clinical efficacy and latency of ADs. The co-administration of amantadine and paroxetine was the only drug combination that increased both extracellular DA and 5-HT levels in the frontal cortex. However, there is a drawback to this study as single doses of paroxetine (10 mg/kg) and NMDA antagonists (1 mg/kg CGP 40116, 40 mg/kg amantadine, 10 mg/kg budipine, 0.9 mg/kg ifenprodil) were used to produce these accelerated neurochemical changes. In view of this, during the preparation of this manuscript, a report by Rogoz et al (2002) investigated the possibility of synergistic interactions between three ADs at different doses [imipramine (5 and 10 mg/kg), venlafaxine (10 or 20 mg/kg) and fluoxetine (5 or 10 mg/kg)] with three non-competitive antagonists at different doses [amantadine (10 or 20 mg/kg), memantine (2.5 or 5 mg/kg) and neramexane (5 or 10 mg/kg)]. These authors report that most combinations resulted in synergistic AD-like effects in the FST. These additive effects were only seen when the drugs were co-administered. For example, fluoxetine when given alone was inactive while showing a positive effect when co-administered with the NMDA antagonists. Thus, it could be that the combination of traditional ADs and NMDA receptor antagonists may produce increased efficacy and reduced latency when given in the clinic.

As mentioned previously, various neurotransmitters have been proposed to be involved in the role of clinical depressive syndromes. It has been shown that the mechanism of ADs action may be related to effects on 5-HT, DA, NA and NMDA antagonism. From chapter 3, it was observed that chronic paroxetine and clomipramine treatment increase basal DA levels and it is suggested that AD action involves an increase in synaptic DA levels as well. Amantadine's combination with paroxetine not only increases 5-HT levels but also DA levels. This could be due to the fact that amantadine appears to share actions with a variety of medications that are used to treat depression. It may have actions similar to those of amphetamines, as both amantadine and amphetamine are reported (for review, see Huber et al, 1999) to be capable of releasing DA and NA from extragranular stores in central neurons, resulting in increased DA and NA activity (although amantadine is 25-50 fold less potent than amphetamines). In addition, amantadine may inhibit DA and NA reuptake as many ADs do, and may also act in a similar fashion to SSRIs such as paroxetine used in this study. Amantadine also has weak, non-competitive NMDA-antagonistic properties, which have been proved to exert AD effects. It should be mentioned that, of the NMDA receptor antagonists used in this study, amantadine is the least selective given that at therapeutic doses it also binds to the σ_1 site (Danysz et al, 1997). Thus, amantadine, with its many pharmacological properties, may in fact be used as a potential AD in the clinic when administered with paroxetine as it does not merely work by a single mechanism of action, but targets several mechanisms thought to be of importance in depressive disorders. Therefore, the actions of amantadine at sites other than NMDA receptors may have contributed to its greater activity when combined with paroxetine in this study. Clinical studies will be needed to assess the clinical usefulness of the co-administration of paroxetine and amantadine.

Chapter 6

Concluding Remarks

6.1 Concluding Remarks

The monoamine theory of depression has dominated research and treatment of depression for over 30 years. The neurotransmitters 5-hydroxytryptamine (5-HT) and noradrenaline (NA) are believed to play a crucial role. The role of dopamine (DA) is less clear but evidence suggests that it plays some role in the pathogenesis of depression (Willner, 1983).

Recently, it has been found that N-methyl-D-aspartate (NMDA) receptor antagonists can give a positive result in a preclinical screen for ADs (Trullas and Skolnick, 1990). It has also been observed that chronic, but not acute treatment with a range of antidepressants from different generic groups, including selective serotonin reuptake inhibitors (SSRI's) decreases the binding affinity of NMDA receptors (Paul et al, 1994). This data suggests that some dysfunction in NMDA receptor-mediated transmission could play a role in the pathophysiology of depression. As an important role exists for the NMDA receptor in the regulation of monoamine release in several brain regions (Whitton, 1997), it is feasible that antidepressant related changes in NMDA receptors affect monoaminergic transmission.

In light of this background experimental evidence for the involvement of NMDA receptors in depression, four main aims of this study were addressed.

- Do ADs of different classes e.g. SSRI and TCA, have similar effects on extracellular DA/5-HT efflux in the frontal cortex when given acutely and chronically?
- What are the effects of acute, subchronic (7-days) and chronic (21-days) treatment on local NMDA-evoked monoamine efflux in the frontal cortex?
- Are these changes reversed following AD cessation?
- What are the effects of polypharmacy studies involving the administration of NMDA antagonists as adjuncts to the SSRI, paroxetine?

In Chapter 3, the effects of acute, subchronic (7 days) and chronic (21 days) systemic administration of the SSRI, paroxetine and the TCA, clomipramine, using *in vivo* microdialysis, on DA efflux were investigated. This chapter also included studies

looking at the effect of local NMDA infusion on NMDA-evoked DA efflux and the effects of the NMDA-evoked DA changes following AD cessation.

Acute injection of paroxetine did not affect DA efflux while clomipramine administration caused a decrease in DA efflux. On the other hand, the results of this study indicate that paroxetine and clomipramine exert similar effects on basal DA efflux in the frontal cortex. When administered subchronically they caused a decrease in DA efflux but increased DA efflux when administered chronically. These drugs also have similar effects on NMDA-evoked DA efflux. They decrease DA efflux when given acutely, prevent the decrease when given subchronically and increase DA efflux when given chronically. These changes in NMDA-evoked DA efflux lasted for at least 48 hours after drug cessation and were comparable to pretreatment basal levels after 14 days of drug cessation. From these findings, it can be inferred that adaptive changes occur in NMDA receptor function during treatment with an AD which may alter NMDA-evoked changes in dopaminergic transmission.

In Chapter 4, the effects of acute, subchronic and chronic systemic administration of paroxetine and clomipramine on 5-HT efflux were investigated. I have also conducted experiments which have addressed the effects of local NMDA infusion on NMDA-evoked 5-HT efflux and also the effects of these changes following AD cessation.

The results of this study indicate that paroxetine and clomipramine exert similar effects on basal 5-HT efflux in the frontal cortex whether administered acutely, subchronically or chronically. Acute i.p. injection of paroxetine and clomipramine had no effect on 5-HT efflux while both subchronic (7 days) and chronic (21 days) administration of the drugs caused a dose-dependent increase in extracellular 5-HT concentration. Both drugs had similar effects on NMDA-evoked 5-HT efflux which lasted for at least 48 hours after drug cessation and were comparable to pretreatment basal levels after 14 days of drug cessation.

The data in Chapters 3 and 4, also showed that subchronic and chronic treatment with paroxetine and clomipramine abolishes or reverses the effect of NMDA infusion on DA and 5-HT efflux. In Chapter 5, it was therefore decided to investigate the effects of a range of NMDA antagonists on paroxetine-induced DA/5-HT efflux. All NMDA antagonists used in this study increased paroxetine-induced extracellular 5-HT levels.

However, amantadine was the only NMDA antagonist to increase DA efflux in the frontal cortex. This is the first study to demonstrate that NMDA antagonists potentiate the effect of terminal 5-HT efflux. It appears that NMDA antagonists accelerate the effects of ADs on 5-HT efflux and this further reinforces the potential involvement of the NMDA receptor in the aetiology of depression.

Although this study concentrated on the effects of glutamate antagonists that displayed affinity at the NMDA subtype, ligands which bind to the AMPA subtype could also enhance the effects of ADs. Beattie et al (2000) and Ehlers (2000) showed that the application of NMDA triggers internalisation of the AMPA receptors. If such a system of regulating AMPA subtype availability is in place, it may act alongside NMDA receptors in regulating AD-induced transmitter release.

6.2 Other areas of interest and future directions

Preclinical studies have indicated that ligands that display antagonistic actions at the NMDA receptor may possess AD properties. Moreover, chronic administration of ADs has been shown to alter both the mRNA levels encoding NMDA receptor subunits and radioligand binding to these receptors in certain brain regions including the frontal cortex. It is hypothesised that AD treatments converge to produce an identical functional endpoint: a region- specific dampening of NMDA receptor function (see Skolnick, 1999 for review). Recent research has shown that that NR1 protein levels in the frontal cortex and hippocampus are decreased following chronic AD (clomipramine and roboxetine) treatment (Hutson et al, personal communication). We are currently extending these studies using paroxetine. We are also looking at the effects of chronic AD treatment on NR1 and NR2 mRNA levels using molecular biological techniques such as PCR. This molecular biology approach will help us to examine whether our data shows a change in mRNA NMDA subunit composition following chronic AD treatment.

During the past decade, research into depression has been extended beyond the monoaminergic synapse. The study of intracellular signalling pathways especially those involving the neurotrophic factor BDNF are now being explored. NMDA antagonists and BDNF have been shown to be protective against neuronal insults, presumably by dampening NMDA receptor function (Choi, 1988; Kornhuber and Weller, 1997). Given

the coexistence of AMPA and NMDA receptors at many central synapses (He et al, 1998) and the role of AMPA receptors in NMDA receptor activation, further experiments will provide an insight into whether there is a connection between BDNF formation and NMDA receptor function dampening in AD action. These results suggest that co-administration of paroxetine (and other ADs) with NMDA antagonists should be further investigated in order to determine the influence of BDNF on the processes I have observed.

The present study demonstrates that chronic AD treatments can modify NMDA receptor function in the frontal cortex. These present findings suggest an associated role between repeated AD treatment with paroxetine and clomipramine on the regulation of serotonergic and dopaminergic transmission by NMDA receptors within the frontal cortex. This study underlies the importance of giving a chronic treatment regimen with ADs before making assumptions as to the functional roles of different transmitter systems in the mechanism of AD treatment since the results of the acute treatment study are quite different from that of chronic treatment. It can be concluded that the potentiation of serotonergic and dopaminergic neurotransmission induced by chronic AD (paroxetine and clomipramine) treatments may contribute to their therapeutic effects. Whether any single neurotransmitter system will be ascribed a central role as the final common pathway in the aetiology of depression is unclear. A dysfunction in the interaction between glutamatergic/serotonergic/dopaminergic transmission may be significant in the aetiology of this disease. The primary targets for AD actions at the monoamine synapse may only be the beginning.

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Appendix

Appendix

I. Rat weights

Rat weights were monitored daily to ensure that paroxetine and clomipramine were not having any detrimental effects on rat health in accordance with the Home Office guidelines.

I.I The effect of chronic paroxetine and clomipramine dosing on body weight over 21 days

Over a period of 21 days, saline-treated (control) rats were seen to steadily gain weight at a rate of 4g/day. At the start of monitoring, the average weight of rats was176g and these saline-treated rats increased in weight to 250g (Figure I). Rats of an identical starting weight but treated with clomipramine for 21 days were also observed to gain weight, but less rapidly than controls. During the 21 day dosing regime, these rats gained weight at an overall rate of approximately 2g/day. These clomipramine-treated rats weighed an average of 223g at the end of the experiment, which was significantly lower than that of saline-treated (control) rats. Similar to the clomipramine-treated rats, rats treated with paroxetine for 21 days also increased in weight less rapidly than controls at an average of 2g/day but these rats ceased gaining any weight from day 11 till day 19. At day 19, these rats had an average weight of 215g. Interestingly on days 20 and 21 these rats gained weight to 223g eventually weighing the same as the clomipramine-treated rats.

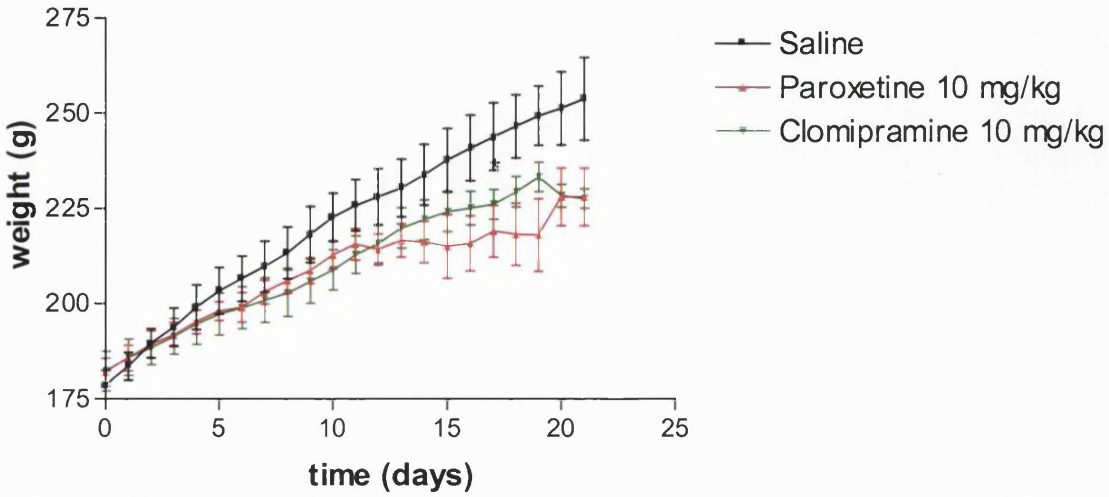

Figure I The effect of chronic paroxetine and clomipramine dosing on body weight over 21 days. Data are the mean ± SEM of six animals in each group.

I.II Effect of chronic (21 days) dosing of paroxetine with 48 hours 'drug holiday' on body weight

Over a period of 21 days, saline-treated rats were observed to steadily gain weight even after the cessation of drug/vehicle treatment. At the start of the study, before any drug treatment, rats weighed 146g and at the end of the study, the control rats weighed 230g. Similarly rats chronically treated with paroxetine were observed to gain weight but at a slower rate to the control rats. The paroxetine-treated rats gained weight at a rate of 2g/day compared to saline-treated rats which gained weight at a rate of 4g/day. However, after day 11 of the dosing regime, the paroxetine treated rats were gaining weight at a much slower rate (1g/day) (Figure II). Thus at the end of drug treatment these rats weighed 192g. After 48 hours of cessation of drug treatment, the rats did not gain any extra weight beyond 192g.

I.III Effect of chronic (21 days) dosing of paroxetine with 14 days 'drug holiday' on body weight

Over a period of 21 days, the results for saline and paroxine-treated rats was similar to above. At the start of the drug holiday, saline-treated rats weighed 223g and after cessation of drug treatment, these rats carried on gaining weight. After 14 days at the end of the study, the control treated rats weighed 273g (Figure 37). Thus rats were gaining weight on average 3g/day. The paroxetine-treated rats at the start of the drug holiday weighed 205g but these rats gained weight at a greater rate than during treatment and weighed identical to the control rats after 14 days of 'drug holiday'.

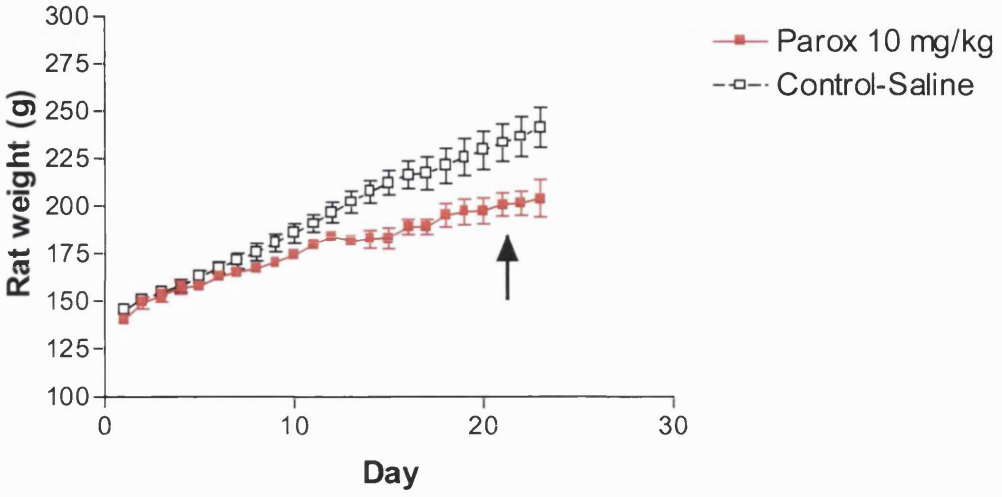

Figure II Effect of chronic (14 days) dosing of paroxetine with 48 hours 'drug holiday' on body weight. The arrow indicates the point at which daily dosing was stopped. Data are the mean ± SEM of six animals in each group.

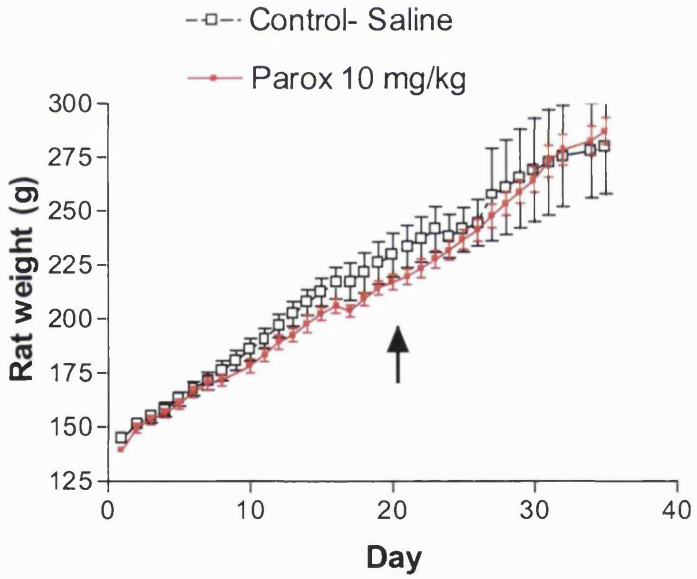

Figure III Effect of chronic (14 days) dosing of paroxetine with 14 days 'drug holiday' on body weight. The arrow indicates the point at which daily dosing was stopped. Data are the mean ± SEM of six animals in each group.

I.IV Effect of chronic (14 days) dosing of clomipramine with 48 hours 'drug holiday' on body weight

Over a period of 14 days, saline-treated rats were observed to steadily gain weight even after the cessation of drug treatment. At the start of the study, before any drug treatment, rats weighed 140g and at the end of the study, the control rats weighed 210g (Figure IV). Similarly rats chronically treated with clomipramine were observed to gain weight but at a slower rate to the control rats. The clomipramine-treated rats gained weight at a rate of 2g/day compared to saline-treated rats which gained weight at a rate of approximately 4g/day. Thus at the end of drug treatment these rats these rats weighed 195g. After 48 hours of cessation of drug treatment, the rats were observed to carry on gaining weight.

I. V Effect of chronic (14 days) dosing of clomipramine with 14 days 'drug holiday' on body weight

Similar to rats given a shorter 'drug holiday', rats which were receiving the saline treatment initially gained more weight in comparison to clomipramine treated-rats. Clomipramine-treated rats carried on gaining weight even after drug cessation and in fact at the end of the study, clomipramine treated rats weighed 255g which was higher than saline-treated rats which only weighed 230g at the end of the study (Figure V). An interesting point is that after 72 hours of 'drug holiday' the saline-treated rats started gaining weight at a much reduced rate of 2g/day compared to 4g/day as earlier observed in this study.

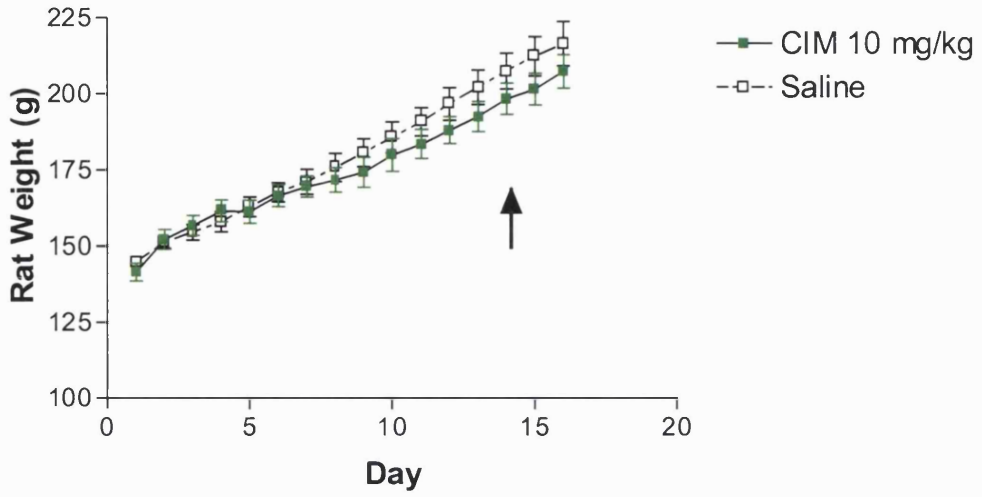

Figure IV. Effect of chronic (14 days) dosing of clomipramine with 48 hours 'drug holiday' on body weight. The arrow indicates the point at which daily dosing was stopped. Data are the mean ± SEM of six animals in each group.

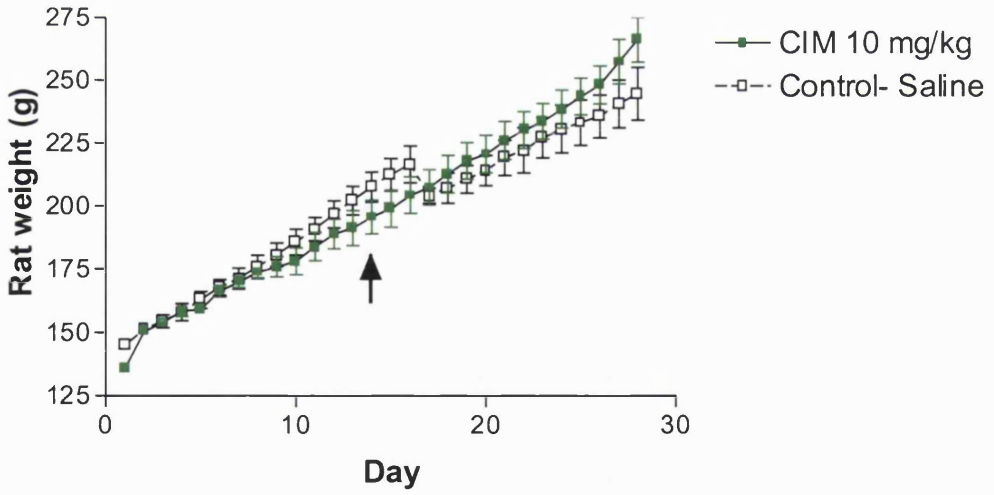

Figure V. Effect of chronic (14 days) dosing of clomipramine with 14 days 'drug holiday' on body weight. The arrow indicates the point at which daily dosing was stopped. Data are the mean ± SEM of six animals in each group.