EFFECTS OF DRUGS WHICH ALTER NMDA RECEPTOR FUNCTION ON IN VIVO NEUROCHEMICAL CHANGES FOLLOWING ACUTE AND REPEATED ANTIDEPRESSANT DRUG TREATMENT IN THE FREELY MOVING RAT

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Thesis submitted in part fulfilment of the requirements of the University of London, Faculty of Medicine for the degree of Doctor of Philosophy

January 2001
Abstract

It has been suggested that the psychiatric disorder of depression is functionally associated with insufficient synaptic serotonin, noradrenaline and to a lesser extent dopamine. However, this alone does not seem to explain the mechanisms involved in depression, especially as all current antidepressants must be taken chronically for the clinical effects to be observed. The levels of the monoamines are seen to change earlier than this suggesting that some form of adaptation must be occurring before the antidepressant effect is observed. More recently it has been suggested that a dysfunction of N-methyl-D-aspartate (NMDA)-glutamatergic receptors may play an important role.

Using in vivo microdialysis in freely moving male rats, the effects of drugs that alter NMDA receptor function were studied following acute and repeated dosing of antidepressant drugs. Clomipramine, a relatively selective serotonin reuptake inhibitor, was used as an example of a classical antidepressant in clinical use today. Reboxetine, a drug that is believed to exert its antidepressant activity through its selective noradrenaline reuptake inhibitory action, was used as an example of a novel antidepressant. Levels of serotonin (5-HT), dopamine (DA), noradrenaline (NA), and glutamate (GLU) were measured in the raphe nuclei and frontal cortex – these two regions are believed to be involved in depression and low levels of these monoamines in the frontal cortex are usually indicative of the disorder.

Interestingly only one class of antagonist given in combination with either antidepressant was observed to increase frontal cortex monoamines after both acute and sub-chronic dosing. Amantadine is a weakly selective ion channel blocker that is used in the treatment of Parkinson's disease and has already been shown to exhibit antidepressant properties in these patients. The combination of reboxetine and amantadine results in frontal cortex levels of 5-HT and DA after 4 days of treatment equivalent to those observed after 14 days of reboxetine alone. It is therefore suggested that the combination of an antidepressant drug with this particular class of NMDA receptor antagonist may prove to be more effective than an antidepressant alone due to its ability to reduce the latent period seen with current antidepressant treatment.
Acknowledgements

I would like to thank my supervisor, Dr. Peter Whitton, for his help, guidance, continual interest and much appreciated scientific advice during the course of this research.

My gratitude to Prof. Trevor Smart for allowing the use of the facilities of the Department of Pharmacology, which have contributed to the formation of this thesis.

Special thanks to Dr. Les Fowler, Dr. Chris Biggs, Dr. Jeff Dalley and Dr. Rob Harvey for their scientific and technical advice throughout my three years in the Department of Pharmacology. I would also like to thank Steve Coppard, Donna Howell and Dave Zeraschi for all their help with the ordering and maintenance of the animals and Fiona Richmond and Dan Bishop for their help with proof reading.

Thank you to all the friends who I have made in the department and supported me during my time here, including James Philips, Emma Dunne, Jo Watts, Mike Postlethwaite, Andy Fisher, Helena Da Silva, Joanna Segieth and Tracy Assari.

Finally I would like to thank my Mum and Mike for all their help and encouragement throughout, I could not have got this far without their support and understanding.
For my Grandad
"Every person who has experienced a severe depression has his own sad, awful tale to tell, his own mess to live through. Sadly Kurt Cobain will never get that far. Every day, I thank God that I did."

Elizabeth Wurtzel

(1995)
Publications arising from this thesis

The following publications have resulted from original work carried out during the course of this study:

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AADC</td>
<td>Aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>ACPC</td>
<td>1-aminocyclopropyacetic acid</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>(D)-AP5</td>
<td>2-amino-5-phosphopentanoic acid</td>
</tr>
<tr>
<td>AP7</td>
<td>D-2-amino-7-phosphonoheptanoic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBT</td>
<td>Cognitive behaviour therapy</td>
</tr>
<tr>
<td>CGI</td>
<td>Clinical global impressions scale</td>
</tr>
<tr>
<td>CGP40116</td>
<td>(R)-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid</td>
</tr>
<tr>
<td>CGS-19755</td>
<td>Cis-4-phosphonoethyl-2-piperidine carboxylic acid</td>
</tr>
<tr>
<td>CIM</td>
<td>Clomipramine</td>
</tr>
<tr>
<td>CMS</td>
<td>Chronic mild stress</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CPP</td>
<td>3-carboxy-piperazin-propyl phosphonic acid</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic adenosine 3',5' monophosphate response element binding protein</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>5,7 DCKA</td>
<td>[3H]-5,7 dichlorokynurenic acid</td>
</tr>
<tr>
<td>DCIM</td>
<td>Desmethylclomipramine</td>
</tr>
<tr>
<td>DDC</td>
<td>L-DOPA decarboxylase</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,5-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DRL72</td>
<td>Differential reinforcement of low rate 72 operant responding test</td>
</tr>
<tr>
<td>DSP-4</td>
<td>N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ECT</td>
<td>Electroconvulsive therapy</td>
</tr>
<tr>
<td>EPDS</td>
<td>Edinburgh postnatal depression scale</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory post-synaptic potential</td>
</tr>
<tr>
<td>FC</td>
<td>Frontal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDS</td>
<td>Geriatric depression scale</td>
</tr>
<tr>
<td>GHQ-12</td>
<td>General health questionnaire-12</td>
</tr>
<tr>
<td>(L)-GLU</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GLY</td>
<td>Glycine</td>
</tr>
<tr>
<td>GP</td>
<td>General practitioner</td>
</tr>
<tr>
<td>HAM-D</td>
<td>Hamilton depression rating scale</td>
</tr>
<tr>
<td>5HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenocortical axis</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HPLC-ED</td>
<td>HPLC with electrochemical detection</td>
</tr>
<tr>
<td>HPLC-FD</td>
<td>HPLC with fluorometric detection</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine; serotonin</td>
</tr>
<tr>
<td>5-HP // 5-HTP</td>
<td>5-hydroxytryptophan</td>
</tr>
<tr>
<td>5-HTPDC</td>
<td>5-HTP decarboxylase</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration to give 50% of the maximum possible inhibition of a response for a given drug</td>
</tr>
<tr>
<td>I.D.</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Dose to give 50% of the maximum possible inhibition of a response for a given drug</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KA</td>
<td>Kainate</td>
</tr>
<tr>
<td>KD</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coeruleus</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>LSD</td>
<td>Lysergic acid diethylamide</td>
</tr>
<tr>
<td>LTP</td>
<td>Long term potentiation</td>
</tr>
<tr>
<td>MADRS</td>
<td>Montgomery-Asberg depression rating scale</td>
</tr>
<tr>
<td>MAOI</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
</tbody>
</table>
Abbreviations

MHPG | 3-methoxy-4-hydroxy-phenylglycol
MK801 | Dizocilpine
mRNA | Messenger ribonucleic acid
NA | Noradrenaline; Norepinephrine
NMDA | N-methyl-D-aspartate
NMN | Normetanephrine
NO | Nitric oxide
NOS | Nitric oxide synthase
NRI | Noradrenaline reuptake inhibitor
OB | Olfactory bulbectomized
O.D. | Outer diameter
ODS | Octadecylsilicate
8-OH DPAT | 8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide
OPA | σ-pthalidialdehyde
PC | Personal computer
PCP | Phenycyclidine
PDE | Phosphodiesterase
PKA | Protein kinase A
PKC | Protein kinase C
RIMA | Reversible inhibitors of monoamine oxidase type A
RN | Raphé nuclei
SAD | Seasonal affective disorder
s.c. | Subcutaneous
S.e.mean | Standard error of the mean
SNRI | Selective noradrenaline reuptake inhibitor
SSRI | Selective serotonin reuptake inhibitor
T\(_{1/2}\) | Terminal half-life of elimination
T\(_{\text{max}}\) | Maximum rate of absorbance
TB | Tuberculosis
TCA | Tricyclic antidepressant
TMS | Transcranial magnetic stimulation
VTA | Ventral tegmental area
WAY100635 | N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridil)cyclohexanecarboxamide trihydrochloride
1. Depression: a common psychiatric disorder

The term ‘affective disorder’ is used to describe a major class of psychoses, distinct from schizophrenia, that are characterised by extreme changes in mood (depression or mania). Depression has long been recognised as both a common and serious group of illnesses that is typically initiated by external events. The two main types are unipolar and bipolar depression. Unipolar depression is the more common of the two. Symptoms appear later in life and are associated with adverse conditions, such as bereavement. This type of depression is often associated with symptoms of anxiety and agitation. In bipolar depression both mood and behaviour oscillate, often alarmingly, between depression and mania. During the depressive phase patients are more likely to be inert, and apathetic, the opposite to unipolar patients. Other differences compared to unipolar depression include an earlier onset, a strong hereditary link, and some bipolar patients display features associated with schizophrenia.

1.1 The impact of depression

Marano (1999) summed up the effects of depression by saying “Depression appears to hold the very soul hostage, with total lack of energy, disturbed sleep, loss of interest in food and sex, inability to experience pleasure, difficulty concentrating and thinking clearly, impaired short-term memory, self-blame, and inability to see alternatives”. As an illness, depression is frequently described as existing across a spectrum (Figure 1.1) and in reality covers a range of distinct states such that the boundary between depression and other psychiatric symptoms may become blurred. Depression is known to affect the patient’s ability to function in everyday life: this includes social and economic function as well as family and marital relationships. Studies have shown that social functioning in depression is worse than observed in chronic medical conditions, such as hypertension, arthritis and diabetes (Wells et al. 1989). What is more worrying is that depression has also been associated with high degrees of morbidity and mortality (Lane 1995). Increased morbidity from almost all types of physical illness (e.g. cardiovascular disease, respiratory disorders) is seen in association with depression (Sims & Prior 1978, Sims 1988). Cardiovascular disease becomes particularly deadly in association with depression (Marano 1999) and a higher than expected rate of mortality is seen in these patients (Roose & Dalack 1992). The risk of suicide is also well known.
to be increased in depression – an estimated 30-70% of all suicides are depressed (Monk 1987). In addition to suicide, mortality from causes other than depression is higher in those suffering from depression than in the general population (Lane 1995). To sum this up, if you’re 45, in perfect health, and depressed, you’re somewhere between 50% and 100% more likely to have a heart attack than if you weren’t depressed (Marano 1999).

**Figure 1.1 Dimensions of depression.**
(Reproduced from Taylor 2000)

### 1.2 Epidemiology of depression

It is estimated that over 80 million working days per year are lost in the U.K. through mental illness – 30 times the number lost due to industrial disputes (Creed 1993). Depression therefore imposes a significant burden on the National Health Service (NHS). Direct costs (related to diagnosis and treatment in hospital and primary care) were estimated at £420 million a year for England and Wales in 1993 (Kind & Sorensen 1993) and by 2000 this figure had risen to £750 million (Taylor 2000). In total, depression costs the U.K. ten times this figure, which is equivalent to 1% of the gross national product (Taylor 2000).
The epidemiology of depression has been shown to vary from country to country (Lane 1995). In general, however, the lifetime risk for depression for males is 4-12% and for females 12-26% (Weissman & Myers 1978, Helgason 1979, Tsuang et al. 1984, Hagnell 1989). It should be noted that the prevalence of depression is not stable and several authors have suggested that the frequency is rising (Klerman 1978, 1988, Schwab et al. 1979).

1.2.1 Social epidemiology of depression

Some factors have been shown to be major determinants of the frequency of depression. These include social class, sex, domestic position (Bebbington 1998) and inheritance (Rang & Dale, 1991). A list of statistics showing the effect of depression is shown in table 1.1.

Nowadays social class is more often referred to as occupational class. Over the years some studies have found associations between class and the prevalence of depression (e.g. Kessler et al. 1994), while others have found none (e.g. Weissman & Myers 1978, Bebbington et al. 1981). The overall consensus seems to be that frequency of depression is indeed increased in the lower classes, due to the direct effect of the disadvantages faced (as opposed to any downward drift by those people suffering with depression) (Bebbington 1998).

Females may be up to twice as likely to suffer from depression than males and data shows sex to be a major determinant of the frequency of depression. The female bias is extremely robust and is highlighted by the vast majority of studies (Bebbington 1998). However, although women are more likely to report depressive symptoms, the suicide rate in men is 2-3 times higher than in women (Taylor 2000) and recently some studies have suggested that sex ratios for reported mental health are converging (Jenkins et al. 1998).

Changes in domestic situation are perhaps one of the most obvious causes of depression. Stress-related events, such as bereavement, may trigger up to 50% of all depression and early life stress may prime people for depression later in life (Marano
Table 1.1 The natural history of depression
(Reproduced from Marano 1999)

<table>
<thead>
<tr>
<th>Likelyhood that a person will develop major depression/dysthymia in their lifetime</th>
<th>6.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Likelyhood that a person will suffer some depressive symptoms in their lifetime</td>
<td>23.1%</td>
</tr>
<tr>
<td>Average age of first onset of major depression</td>
<td>25-29 years</td>
</tr>
<tr>
<td>Average duration of all depressive episodes</td>
<td>20 weeks</td>
</tr>
<tr>
<td>Percentage of patients who recover within a year after onset of symptoms</td>
<td>74%</td>
</tr>
<tr>
<td>Likelyhood of a second or more episodes of major depression</td>
<td>80%</td>
</tr>
<tr>
<td>Likelyhood of a second or more episodes of minor depression</td>
<td>100%</td>
</tr>
<tr>
<td>Median number of major depressive episodes during a patient's lifetime</td>
<td>4</td>
</tr>
<tr>
<td>Percentage of patients whose depression takes a chronic, unremitting course</td>
<td>12%</td>
</tr>
<tr>
<td>Incidence of depression in women vs. men</td>
<td>3.62 vs. 1.98 per 1000 per year</td>
</tr>
<tr>
<td>Female: male ratio of depression in cultures with low rates of alcoholism</td>
<td>1:1</td>
</tr>
<tr>
<td>Rank of unipolar major depression in the world league of disabling diseases in 1990</td>
<td>4</td>
</tr>
<tr>
<td>Rank of unipolar major depression among disabling diseases in westernised countries</td>
<td>2</td>
</tr>
<tr>
<td>Rank of depression among disabling diseases the world over, projected 2002</td>
<td>2</td>
</tr>
</tbody>
</table>

1999). There is little doubt that becoming unemployed increases the risk of depression, although most resulting cases are minor (Warr 1984). The most straightforward influence in this area is marital termination, for whatever reason. Marital disruption, for example, has been shown to increase scores on a standard depression screening scale, but, surprisingly, women were shown to cope better than men in many studies (Aneshensel et al. 1981).

The hereditary nature of depression has been clearly shown for both of the major types, though little cross-inheritance has been observed. The siblings of patients suffering
from bipolar depression show a morbid risk of bipolar depression of 20.7% and of unipolar illness of 0.4%. Siblings of unipolar depressives show morbid risks of 0.5% and 12.6% respectively (Rang & Dale 1991).

1.2.2 The causes of depression

Data from a variety of studies have shown that depression has psychological, environmental and biological roots. It is a difficult illness to define precisely, mainly due to problems in discriminating between unpleasant but normal reactions to stressful events, and states of abnormal functioning which can be reasonably defined as illnesses (Taylor 2000). Studies have shown relationships between depression and neuroendocrinology and chronic stress (Checkley 1996), genes such as the tryptophan hydroxylase gene (Bellivier et al. 1998), changes in the activity of discrete brain regions (Drevets et al. 1997) and even stress and activation of the immune system (Leonard 2000). A number of drugs, including beta-blockers, steroids, the oral contraceptive pill, opiates and L-DOPA, have also be shown to cause depression (Nutt et al. 1997). However it is the monoamine theory of depression, which has formed the cornerstone of research into the illness since the 1960’s (see 1.5.1). The areas of the brain related to mood and emotion contain a high density of monoaminergic neurones, and the hypothesis suggests that a deficiency of noradrenaline, serotonin (Baldessarini 1975) and, to a lesser extent, dopamine (Willner 1983, Maj et al. 1984) may be to blame. Figure 1.2 summarises the determinants of depression.

1.3 Recognising depression

Depression is common in general practice, however half of people with depression are ‘missed’ by their GP on the first consultation (Jackson et al. 1997). Very few people present with a clear-cut diagnosis of depression, instead they present with a mixture of less obvious symptoms. As well as looking at recent life events, physical illness and family history there a number of symptoms which are indicative of major depression.
Chapter One: General Introduction

Vulnerability Factors
Genetic/Biological constitution

Precipitating factors
High or low risk phenotype
Fear of future losses.
Lost role or relationship.
Infections.
Medicine side-effects.

Recovery Factors
POSITIVE
Social support,
Positive role,
effective treatment

NEGATIVE
Poverty,
Lack of support,
Ongoing problems,
Poor treatment

Early Life Experiences
Psychological variables eg. learnt helplessness or cognitive resilience

Figure 1.2 Determinants of depression
(Reproduced from Taylor 2000)

Five or more of the following symptoms must have been present most of every day during the same 2 week period for a diagnosis to be made (Jackson et al. 1997, Nutt et al. 1997).

- Depressed mood – may be diurnal with mood lifting towards evening
- Loss of interest and enjoyment
- Changes in appetite/weight disturbance
- Changes in sleep pattern
- Agitation or retardation
- Fatigue/loss of energy
- Feelings of worthlessness or excessive/inappropriate guilt
- Reduced concentration
- Recurrent thoughts of self-harm/suicide or death
- Other identifying features may include decreased eye contact, tearfulness, decreased libido and reduced self-confidence (Jackson et al. 1997, Nutt et al. 1997).
1.3.1 Screening tests

A number of screening tests are available to detect depression, some of which are used in clinical trials (Jackson et al. 1997). There are three obvious times when screening by a GP is both appropriate and easy to apply:

1. New patients – depression should be considered when assessing the patient’s well being. GHQ-12 (General Health Questionnaire-12) is an easily administered self-report health questionnaire. It uses a rating scale commonly used in primary care settings to assess mental health.

2. Postnatal patients – the Edinburgh Postnatal Depression Scale (EPDS) is a 5min test designed to establish the feelings of the patient over the last seven days. It is useful in the early diagnosis of post-natal depression.

3. Elderly patients – depression is common in the over 75 year age group and can be assessed using the Geriatric Depression scale (GDS).

There are a number of screening tests, which, due to the time constraints involved, are usually restricted to clinical trials and research. They can be used to classify depressive illness and also to monitor the effectiveness of antidepressant treatments. Examples of these tests include Hamilton Depression Rating Scale (HAM-D), Clinical Global Impressions Scale (CGI) and Montgomery-Asberg Depression Rating Scale (MADRS) (Jackson et al. 1997).

1.3.2 Types of depression

As outlined in 1. depression can be split into unipolar and bipolar, however within these broad bands exist further divisions:

1. Dysthmia: A low-grade, chronic depression that does not meet the requirement for major depression although individual symptoms are similar.

2. Seasonal Affective Disorder (SAD): Recently identified, this condition is characterised by recurrent episodes of depression at the same time of the year with euthymia/hypomania in others (Nutt et al. 1997). The most common pattern is onset
in autumn/winter with recovery in spring/summer. Studies have shown the benefits of artificial light during the hours of darkness in some patients.

3. Atypical Depression: Features include low mood, increased appetite (especially carbohydrate) with consequent weight gain, increased sleep, low energy with severe fatigue and leaden paralysis, mood reactivity (anxiety may be severe) and interpersonal rejection sensitivity (Nutt et al. 1997). It is important to recognise atypical depression as there is a preferential response to one class of antidepressant – the Monoamine Oxidase Inhibitors (MAOIs).

4. Postnatal Depression: 10% of new mothers develop postnatal depression (Jackson et al. 1997). It is often hard to recognise due to the reluctance of the mother to admit that they are unable to cope. Typical symptoms include extreme anxiety over the baby, feelings of inadequacy as a mother, as well as those symptoms seen in major depression. Support, help and counselling may be sufficient but early recognition is important.

5. Treatment-resistant depression: Some depressions may not initially respond to treatment. If a treatment is not effective within two months it is necessary to consider one of the following: higher doses, changing the drug class, drug combinations or referral to a specialist. Lithium has been shown to be particularly effective in such cases.

6. Recurrent Depression: Defined as two episodes within 5 years. It is more common in patients with previous history of depression, severe illness, inadequate treatment at initial presentation, no social support and residual symptoms at the end of the initial course. Long-term treatment, sometimes for the lifetime of the patient, may be required (Jackson et al. 1997). Recent evidence has suggested that this form of depression may be a neurodegenerative disorder where nerve cell connections and certain brain cells are destroyed (Marano 1996).

It may be easier to categorise depression according to its severity and a table showing characteristics of the different diagnoses of depression expressed in this way can be seen in table 1.2.
Table 1.2 Severity of depression and treatment
(Reproduced from Freeman et al. 1997)

<table>
<thead>
<tr>
<th>Severity</th>
<th>Diagnosis</th>
<th>Characteristics</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>Adjustment Disorder</td>
<td>Mild depressive symptoms for at least 2 weeks, following a stressful event</td>
<td>Cognitive behaviour therapy</td>
</tr>
<tr>
<td></td>
<td>Dysthymic Disorder</td>
<td>Depression lasting at least 2 years, in which pessimistic, negative outlook becomes an ingrained part of the person</td>
<td>Counselling (possibly)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Possibly drugs</td>
</tr>
<tr>
<td>Moderate</td>
<td>Major depression</td>
<td>An overwhelming depressed mood with significant sleep and appetite disturbances, including weight loss</td>
<td>Drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cognitive behaviour therapy</td>
</tr>
<tr>
<td>Severe</td>
<td>Major depression with melancholia</td>
<td>Pervasive, stultifying depression with total loss of interest or pleasure, marked by psychomotor retardation and early morning awakening</td>
<td>Drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cognitive behaviour therapy</td>
</tr>
</tbody>
</table>

1.4 Management of depression

Once depression has been diagnosed it is important to develop a treatment plan. Antidepressants are not the only form of treatment and it is often more effective to include therapy in addition to any drug prescribed. However, the choice between medication and a psychological approach is dependent on individual presentation, as well as the willingness of the patient to comply. Reassuring the patient that depression
is a treatable illness with good prognosis and dispelling the stigma associated with mental illness is essential.

There are three phases of treatment – acute, continuation/consolidation and prophylaxis (Lane 1995). Acute treatment will resolve symptoms, whilst continuation treatment will ensure the maintenance of the response. Early return of symptoms in either of these stages is thought to represent the return of the same episode of depression (Montgomery et al. 1989). The risk is at its highest just after antidepressant treatment is ended, and reduces with time. The final stage of treatment, prophylaxis, should keep the patient well and prevent relapse. The distinction between relapse (return of symptoms of acute episode after apparent response to treatment) and recurrence (appearance of a new episode of depression) is extremely important (Lane 1995).

1.4.1 The pharmacological approach

There are a number of classes of drugs which have been shown to act as antidepressants, some are used more commonly than others. The three main classes of drugs currently available include:

- Tricyclic antidepressants (TCAs) e.g. amitriptyline, imipramine, dothiepin,
- Selective serotonin reuptake inhibitors (SSRIs) e.g. fluoxetine, paroxetine
- Serotonin noradrenaline reuptake inhibitors (SNRIs) e.g. venlafaxine

Other classes include the MAOIs (e.g. phenelzine) and the reversible inhibitors of monoamine oxidase type A (RIMAs, e.g. moclobemide), which have a specialised role in treating atypical depression and are often used in the psychiatric setting. Noradrenaline reuptake inhibitors (NRIs) represent a new generation of antidepressants and are just starting to become more generally available (e.g. reboxetine). Outside these classes are a number of ‘atypical’ antidepressants which can also be useful in the treatment of depression (e.g. trazodone and mianserin). Finally, lithium may be considered for use in some patients, primarily those suffering from bipolar depression.
1.4.1.1 Monoamine Oxidase Inhibitors

Drugs of this type were the first to be introduced as antidepressants. MAO is an enzyme which catalyses the oxidative deamination of monoamine neurotransmitters to their appropriate aldehydes within nerve terminals. Inhibition of MAO increases monoamine concentration within the brain. MAOIs have been shown to be effective in the treatment of atypical depression and should also be considered for treatment-resistant patients (Nutt et al. 1997). Most of the clinically available MAOIs are unable to discriminate between the A and B isoform, which may explain some of the side effects seen with this class of antidepressant. The 'cheese reaction' is a direct consequence of MAO inhibition; interaction with foods containing tyramine (e.g. mature cheese, marmite, and pickled herrings) can lead to catastrophic hypertension. The use of MAOIs has largely been superseded by the newer types of antidepressants, whose clinical efficacies are considered to be better and whose side effects are generally less severe. However, newer MAOIs are selective for the MAO-A isoform (RIMAs), which is the subtype concerned with the metabolism of 5-HT and NA. Moclobemide is the only RIMA currently available and, as it does not affect the B-subtype, this remains free to metabolise tyramine. The result is that the risk of the 'cheese reaction' is reduced and a low tyramine diet is not required.

1.4.1.2 Tricyclic antidepressants

TCAs are believed to act by blocking the reuptake of monoamines into the presynaptic terminal, probably by competition for the carrier that forms part of this membrane transport system. Most inhibit uptake of 5-HT and NA into brain synaptosomes but have a reduced effect on dopamine. It is not known which type of activity is most important in relation to antidepressant effects. Many of the side effects associated with TCAs are due to the fact that they act on other transmitter systems including the cholinergic receptors and this may affect their use. Side effects common to TCAs are listed in table 1.3. Increases in brain monoamine levels are seen within 24 hours but 10-20 days of treatment are required before the therapeutic benefit is seen. It has been suggested that changes in the sensitivity of pre-and post-synaptic receptors may be a requirement (Artigas et al. 1996). TCAs have been shown to be as effective as more
### Table 1.3 Side effects and interactions seen with antidepressants
(Reproduced from Jackson et al. 1997)

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>Side Effects</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAs</td>
<td>Dry mouth, Constipation, Impaired visual accommodation, Urinary retention, Worsening of glaucoma, Confusion, Psychosis, Tachycardia, Hypotension, Cardiac conduction effects - especially in overdose, Seizures, Drowsiness, Sexual dysfunction</td>
<td>Alcohol, MAOIs, Antiepileptics, Antihistamines, Sympathomimetic drugs</td>
</tr>
<tr>
<td>SSRIs</td>
<td>Headache, Nervousness, Drowsiness, Vision disturbances, Nausea, Dry mouth, Sweating, Fatigue, Anorexia, Confusion, Dizziness</td>
<td>Anticoagulants, MAOIs, Dopaminergics, Lithium, Sumatriptan, Theophylline</td>
</tr>
<tr>
<td>SNRIs</td>
<td>Nausea, Headache, Dry mouth, Somnolence, Dizziness, Constipation, Weakness, Sweating, Nervousness</td>
<td>MAOIs, CNS active drugs - except lithium and diazepam</td>
</tr>
</tbody>
</table>
modern antidepressants and may be more effective in the treatment of severe depression (Stahl 1999). Clomipramine, which is the most potent 5-HT and NA reuptake blocker in the TCA class, is also effective in the treatment of obsessive compulsive disorder but it should be noted that high doses can lead to increased risk of seizures (Stahl 1999). A more detailed review of clomipramine can be found in chapter 3 (3.1).

1.4.1.3 Selective Serotonin reuptake inhibitors

The group of antidepressants referred to as SSRIs have revolutionised the treatment of depression. This is principally due to their improved safety and tolerability in comparison to TCAs and MAOIs (Stahl 1999). SSRIs act by selectively blocking the reuptake of 5-HT into the presynaptic terminal. The time course of events is similar to that observed with TCAs and can be explained in a similar manner (Artigas 1996). Although there is considerable variability in pharmacokinetics and drug interactions within this class of antidepressants, their side effects are similar and usually well tolerated (see table 1.3 for side effects and drug interactions). Most resolve over about 3 weeks with the exception of sexual dysfunction, which may continue (Nutt 1997). The SSRIs are particularly useful in depressions where other symptoms, known to have a serotonergic component, co-exist (eg. obsessive compulsive disorder, panic syndrome). Their relative efficacy in severe depression is, however, controversial (Hirshfeld & Schatzberg 1994).

1.4.1.4 Selective serotonin and noradrenaline reuptake inhibitors

Venlafaxine is one of the only SNRIs currently available. It blocks the uptake of both 5-HT, NA and possibly DA (Stahl 1999), but is devoid of the anticholinergic and α₁-adrenergic side effects that plague the older TCAs. Reuptake block is dependent on the dose given: low dose blocks 5-HT reuptake, medium dose blocks both 5-HT and NA reuptake and very high doses block the reuptake of all three monoamines (Stahl 1999). It is of particular use in retarded, hypersomnic, weight gaining, atypical depressives and should not be considered for use in those patients who are anxious, insomniac, suffering from weight loss or sexual dysfunction or who have borderline or labile hypertension (Stahl 1999).
More recently a new class of drugs has been developed, the first of which to become available is reboxetine. Reboxetine is a selective noradrenaline reuptake inhibitor (NRI) (Dostert et al. 1997) which increases levels of NA in the brain. This is achieved without the typical side effects associated with TCAs as it lacks affinity for 5-HT and DA reuptake sites as well as being devoid of affinity for muscarinic, α1-adrenergic, H1-histaminergic receptors. (Burrows et al. 1998, Riva et al. 1989). Side effects associated with reboxetine have been classed as mild to moderate in severity in both acute- and chronic-treatment (Burrows et al. 1998). On the basis of available data, reboxetine is believed to have a similar onset of action to other antidepressants of 2 to 3 weeks (Holm & Spencer 1999). A more detailed review of reboxetine can be seen in chapter 4 (4.1).

1.4.1.5 Lithium

Lithium is not regularly prescribed as an antidepressant. It should only be used under the care of a psychiatrist, in recurrent depression or in patients with bipolar disorder (Jackson et al. 1997). This is due to its narrow therapeutic window, meaning that overdose can be fatal. A number of toxic effects are associated with lithium treatment including tremor, pallor, irritability, dehydration, ataxia, dysarthria, nystagmus, renal impairment and convulsions. It is therefore important that levels of lithium in the blood are monitored regularly to ensure an effective and safe dose and that kidney and thyroid function is monitored every twelve months. Lithium is known to control the manic phase of bipolar disorder and giving lithium prophylactically can prevent the characteristic changes in mood that are seen in this disorder. However, when lithium is given acutely it will reduce mania but no effect will be observed during the depressive phase (Rang & Dale 1991). Little is known about its mechanism of action, but reports suggest that it affects calcium ion dynamics and the platelet intracellular ion concentration (Dubovsky 1993).

1.4.2 Alternative forms of treatment

Although prescription drugs are often the first to be considered in the treatment of depression, there are a number of alternatives that may be used instead of or to augment antidepressant therapy.
1.4.2.1 Counselling

In cases of mild depression counselling may be used without the need for drug treatment. Counselling and social intervention is an important step in dealing with depression, but it will take time. In cases of moderate to severe depression counselling is still an important part of treatment, but parallel drug treatment should always be considered. Some form of counselling should accompany all forms of antidepressant therapy, be it from a GP or, in more severe cases, an expert counsellor. Support groups may also be of benefit, as they provide an environment of understanding. However the requirement of illness for membership may well prolong symptoms.

1.4.2.2 Cognitive behaviour therapy

Cognitive behaviour therapy (CBT) aims to identify abnormal, negative cognitions, challenge them and devise alternative explanations. It is believed that these negative cognitions, when dwelled upon, lead to depression or maintain an episode once it has started (Jackson et al. 1997). Activities are planned so that opportunities for experiencing pleasure are maximised and an effective balance is achieved over the course of a day.

CBT is helpful in the treatment of mild to moderate depression (Nutt et al. 1997) and also for those at risk of relapse (Jackson et al. 1997). There are a number of pros and cons associated with this type of treatment. Pros include patients not needing to take drugs and feeling in more control of their treatment, whilst examples of cons include expense, time, lack of effectiveness in severe depression and the need for an effective dose of an antidepressant in order to fully benefit from CBT.

1.4.2.3 Psychotherapy

Only a small number of patients need to be referred to psychiatric services. Reasons for referral include uncertainty about diagnosis, depression associated with other symptoms (eg. anorexia, alcoholism), concern over self-harm or suicide, history of psychiatric illness or for consultation about management in non-responders (Jackson et al. 1997). Psychotherapy is not generally available as an emergency form of treatment but in the
longer term it can be useful to help patients explore issues in their past and present which have made them vulnerable to depression. Therapy of this nature focuses on interpersonal relationships and social functioning, but as with CBT, adequate treatment with an antidepressant may be required to make best use of the psychotherapy.

1.4.2.4 Electroconvulsive Therapy

Electroconvulsive therapy (ECT) involves "the induction of a modified epileptiform seizure by the passage of an electrical current. The production of seizure is necessary for therapeutic benefit. Changes in neurotransmitters and their receptors occur, many of which are similar to those seen during antidepressant treatment" (Nutt et al. 1997). Treatment of this nature is usually restricted to patients hospitalised for severe depression but it may also be used in the elderly, as it may be safer than antidepressants. It is a safe, effective and in some cases life-saving treatment when used appropriately but it remains surrounded by stigma.

ECT can be administered in two ways: unilateral ECT involves both electrodes placed on the scalp covering the non-dominant hemisphere, whilst bilateral ECT has electrodes applied to both sides of the brain. Of the two, bilateral ECT is preferable because of its overall effectiveness but it does produce a higher level of cognitive impairment (Nutt et al. 1997). Side effects include headaches, states of confusion and slight or transient amnesia.

A new procedure more acceptable to both patients and public opinion is rapid transcranial magnetic stimulation (rTMS) (Zyss 1994). Clinical effects are favourable (George et al. 1995, 1997, Pascual-Leone et al. 1996) and rTMS produces similar neurochemical effects to those induced by ECT (Fleischman et al. 1995, Zyss et al. 1997).

1.5 Neurotransmitters in depression

The idea that a deficiency in one or more of the monoamine neurotransmitters is a biological component in the aetiology of depression will be outlined in 1.6.1. In order to understand the complex actions of antidepressants it is first necessary to understand the
normal behavioural functions of the monoamine systems and how their dysfunction may result in depression.

Brain monoamine systems are highly divergent with few specialised post-synaptic structures. A small number of neuronal cell bodies situated in discrete brain stem nuclei give rise to widespread projections, with monoamine release occurring along the length of the long pre-terminal regions of the axon (Deakin & Crow 1986). This results in monoamines being released over substantial areas of the brain in response to neuronal cell discharge and suggests that monoaminergic systems are involved in exerting general effects, as opposed to detailed information transfer (Deakin & Crow 1986).

1.5.1 5-HT systems

About 1-2% of 5-HT in the body is present in the brain (Cooper et al. 1996) and as it is unable to cross the blood-brain barrier (BBB) synthesis must occur here. Tryptophan is hydroxylated in a reaction catalysed by tryptophan hydroxylase to form 5-hydroxytryptophan (5-HTP), which then undergoes decarboxylation by aromatic amino acid decarboxylase (AADC) to yield 5-HT (5-hydroxytryptamine). Destruction of 5-HT to an inactive metabolite is catalysed by the enzyme MAO.

5-HT-containing neurones are found in clusters of cells lying in or near the midline or raphe regions of the pons and upper brain stem. Fig 1.3 shows the ascending and descending serotonergic pathways in the brain. Nine 5-HT nuclei (B₁-B₉) have been described and more recently immunocytochemical studies have suggested that 5-HT reactive cells are also present in the area postrema, caudal locus coeruleus and around the interpeduncular nucleus. The more caudal groups (B₁-B₃) project largely to the spinal cord, whilst the intermediate groups (B₄-B₆) may project to either ascending or descending groups and an extensive innervation of the cortex has been observed (Cooper et al. 1996). The remaining nuclei, which are situated towards the anterior, project extensively to the telencephalon and diencephalon (forebrain). The B₇-B₉ nuclei are also termed the dorsal raphe, median raphe and centralis superior. The dorsal raphe distributes 5-HT terminals to areas innervated by dopamine (e.g. the amygdala, basal ganglia, cortical areas), whilst the median raphe innervates the hippocampus and cortex.
in a similar but more limited distribution to NA (Azmitia & Segal 1978, Bobillier et al. 1976).

The existence of multiple subtypes has been shown for 5-HT. There are four major categories of receptors, which can be further subtyped depending on pharmacological or molecular properties. Pre-synaptically there is a serotonin transporter and the 5-HT$_{1A}$ and 1D receptors, whilst post-synaptically several receptors have been identified (5-HT$_{1A}$, 1D, 2A, 2C, 3, 4, 6, 7). Presynaptic 5-HT$_{1A}$ receptors are termed somatodendritic autoreceptors. They are activated by the presence of 5-HT and shut down neuronal impulse flow, therefore down-regulation will result in increased terminal 5-HT. It is this down regulation that is believed to be behind the delayed onset of action of antidepressants such as the SSRIs (Blier & de Montigny 1994, Artigas et al. 1996). Presynaptic 5-HT$_{1D}$ receptors are called terminal autoreceptors and regulate 5-HT in a similar manner to the 5-HT$_{1A}$ receptors but only inhibit release (they have no effect on cell firing). Finally a 5-HT neurone can be inhibited by a presynaptic $\alpha_2$-adrenergic heteroreceptor on the axon terminal. Thus NA binding to this receptor can inhibit the release of 5-HT. See Appendix I for full 5-HT receptor nomenclature.
1.5.2 DA systems

DA is synthesised from the amino acid precursor tyrosine, which must be transported across the BBB into the DA neurone. The rate-limiting step is the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase. L-DOPA then undergoes decarboxylation by AADC to produce DA. The breakdown of DA is by the same enzymes that destroy NA: MAO and catechol-O-methyltransferase (COMT).

The central dopaminergic systems are considerably more complex than the noradrenergic systems. As well as a larger number of DA-containing cells, there are several major DA-containing nuclei and specialised dopamine neurones that make connections within the retina and olfactory bulb (Cooper et al. 1996). Fig 1.4 shows the ascending dopaminergic pathways in the brain. DA-containing nerve terminals are localised in subcortical structures (e.g. basal ganglia, amygdala, nucleus accumbens). However it has become apparent that DA cells within A8, A9 and A10 form an anatomically heterogeneous population in terms of their projection areas – the frontal, cingulate and entorhinal areas of the limbic cortex (Bjorklund & Lindvall 1984). These cells arise from a continuous sheet of dopamine cells in the brain stem (Cooper et al. 1996). The A10 group refers to the ventral tegmental tract area and projects to the mesolimbic terminals, whilst the A9 group of the substantia nigra innervates the basal ganglia.

Fig 1.4 Ascending dopamine neurones in the rat brain
(Reproduced from Cooper et al. 1996)
Receptors for DA modulate dopaminergic transmission within the CNS. As with 5-HT, a number of pharmacological subtypes exist along with several molecular isoforms. The D2 group of receptors has been extensively studied as agonists of the receptor are used in the treatment of Parkinson’s disease, whilst antagonists acts as neuroleptics for the treatment of schizophrenia (Stahl 1999). Postsynaptic receptors include D1 and D2 and are found in the projection areas of midbrain dopamine neurones. They can regulate the activity of neuronal feedback pathways e.g. postsynaptic receptors in the striatum regulate the pathways which control communication between striatal neurones and the DA cell bodies in the substantia nigra (Cooper et al. 1996). Chronic exposure of DA receptors to either antagonists or agonists may result in adaptive changes occurring. These may be relevant in understanding the involvement of DA receptors in diseases believed to involve changes in DA. The importance of D1 receptors being linked to adenylyl cyclase (Deakin & Crow 1986) in terms of intracellular signalling pathways will be discussed in 1.6.4. D2 autoreceptors exist on most portions of dopamine cells and this determines their effects. Activation of somatodendritic autoreceptors reduces the rate of firing of the neurone, whilst terminal autoreceptors inhibit the synthesis and release of DA (Cooper et al. 1996). Similarly to presynaptic receptors, autoreceptors are believed to undergo adaptive changes in response to chronic exposure to dopaminergic drugs. See Appendix II for full dopaminergic receptor nomenclature.

Although often overshadowed by 5-HT and NA it has been proposed that DA plays an important part in the aetiology of depression (Willner 1995b). A number of studies have shown that antidepressant drug treatment increases receptor expression (Ainsworth et al. 1998), extracellular DA (Tanda et al. 1994) and behavioural responses to the DA agonist apomorphine (Maj et al. 1984). There is good evidence that the nigro-striatal DA system is underactive in some depressed patients, and that treatment with DA agonists was effective in some cases. What does remain unclear is whether the changes observed in DA are primary or secondary (Willner 1983).

1.5.3 NA systems

The initial steps in the synthesis of NA are the same as for DA. The final hydroxylation to convert DA to NA is catalysed by the non-specific enzyme dopamine-β-hydroxylase. This enzyme may be non-specific but its distribution is restricted to catecholamine-
producing cells. MAO and COMT are the two principal enzymes involved in the breakdown of NA.

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. Fibres from the LC form five major noradrenergic tracts and it is the efferent pathways that have been closely studied, especially those projecting to the cortex and hippocampus (Cooper et al. 1996, Deakin & Crow 1986). The major effect of activating this pathway seems to be inhibition of spontaneous discharge and seems to be related to the intracellular messenger scheme that will be discussed in 1.6.4. The second group of cells (A1, A2, A3) lie outside the LC and contribute mainly descending fibres within the mesencephalon and spinal cord, although some from the more anterior tegmental levels innervate the forebrain and diencephalon (Cooper et al. 1996). Due to the complex nature of these neurones it is difficult to analyse their function. A diagram representing noradrenergic pathways in a sagittal section of rat brain is shown in fig 1.5.

Noradrenergic transmission can be regulated by a number of pre- and post-synaptic receptors. The three main receptors are $\alpha_1$, $\alpha_2$ and $\beta_1$ adrenergic receptors. Both groups

---

**Fig 1.5 Ascending noradrenergic pathways in the rat brain**

(Reproduced from Cooper et al. 1996)
are present postsynaptically in the cortex. These receptors respond to NA setting up a molecular cascade in the postsynaptic neurone, allowing neurotransmission to pass from neurone to neurone. β receptors linked to adenylate cyclase mediate the ability of noradrenaline to increase the formation of the second messenger cAMP. The α2 receptor that is located presynaptically acts as an autoreceptor, so in response to NA it reduces further release of NA. This is believed to occur physiologically to prevent the NA neurone over-firing, thus ‘turning off’ the receptor (Stahl 1999). NA also regulates the release of other monoamines, especially 5-HT via a series of interactions. These will be covered in 1.5.4.1. See Appendix III for full adrenoceptor nomenclature.

1.5.4 Interactions between monoaminergic neurotransmitter systems

Interactions between different transmitter systems can occur within the CNS. Described here are interactions between NA-5-HT and DA-5-HT that may be important in the understanding of the mechanisms of action of antidepressant drugs, especially as no one transmitter system can fully explain the pathophysiology of depression.

1.5.4.1 NA-5-HT interactions

There are a number of types of interaction between NA and 5-HT. A postsynaptic interaction exists where NA enhances the release of 5-HT by activating α1 receptors located on the 5-HT neurones. There is also a presynaptic interaction, which inhibits the release of 5-HT. NA binds to an inhibitory α2 heteroreceptor located on the 5-HT neurone, inhibiting its release. It has been suggested that NA from the LC tonically regulates the serotonergic output from the raphe nuclei (RN) via this interaction (Haddjeri et al. 1996).

However it has also been suggested that LC neurones are regulated by 5-HT. The spontaneous activity of the LC appears to be under the influence of several factors, including the serotonergic system (Gorea & Adrien 1988). A number of studies have suggested the existence of an inhibitory control of the LC noradrenergic system by the RN 5-HT system (Pickel et al. 1977, Leger & Descarries 1978, Crespi et al. 1980, McRae-Degueuerce et al. 1985, Segal 1979) using anatomical, biochemical and electrophysiological methods. Gorea & Adrien (1988) showed 5-HT2-mediated
influence on LC neuronal activity to be indirect but suggest that their data provides strong evidence for a predominantly inhibitory serotonergic influence, acting via post-synaptic 5-HT$_2$ receptors on the spontaneous discharge of LC noradrenergic neurones.

1.5.4.2 DA-5-HT interactions

Evidence has been found for dopaminergic regulation of the serotonergic raphe-striatal neurones that project mainly to the dorsal striatum, showing that stimulation of D$_2$ receptors increases the local concentration of 5-HT (Ferré & Artigas, 1993). This in turn activates somatodendritic RN 5-HT$_{1A}$ autoreceptors, reducing the release of 5-HT in the striatum (Ferré et al. 1994).

Morphological data also suggests that RN serotonergic neurones can modulate the function of brain dopaminergic systems. Several 5-HT receptor subtypes (e.g. 5-HT$_{1B/1D}$, 5-HT$_2$, 5-HT$_3$) can be found at moderate to high densities within DA-containing structures (Pazos & Palacios 1985, Pazos et al. 1985, Waeber et al. 1989,1990, Hoyer 1990) and functional evidence of a 5-HT-mediated regulation of mesencephalic dopaminergic systems at both somatodendritic and terminal levels has been observed (Nedergaard et al. 1988, Guan & McBride 1989, Blandina et al. 1989, Benloucif & Galloway 1991, Chen et al. 1991, Parsons & Justice 1993).

1.6 Neurochemical theories of depression

A number of distinct brain regions which communicate with each other via axons extending from the cell bodies of one region to another are believed to be involved in depression (Fig 1.6). The seminal region is probably the frontal cortex (FC), which lies just behind the forehead. It is important in the processing of emotions and it is thought that depressed patients have a malfunctioning left FC, which is known to regulate positive feelings in normal subjects (Marano 1999). The FC is linked to the amygdala, which acts as a centre for negative emotions. Ordinarily the activation of the left frontal cortex modulates the negative outflow from the amygdala, but in depressed patients this control is removed and negative emotions are left unchecked (Marano 1999). Two other
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The prefrontal cortex, when activated on the left side, normally generates positive feelings and dampens flow of negativity from the amygdala.

The amygdala, center of negative emotions, informs the brain of threat. It runs unchecked in depression.

The hypothalamus, center of the stress response, is overactive in depression.

The hippocampus, center of memory, loses nerve-to-nerve links in depression.

**Fig 1.6 Major brain regions important in depression**

(Reproduced from Marano 1999)

regions of importance are the hypothalamus and hippocampus. The hypothalamus instigates the response of the body to stress and is connected to a discrete region of the FC known as the subgenual cortex, which is also important in managing the hormonal responses to stressful stimuli. The hippocampus is the brain centre that deals with memory; its links to other limbic regions of the brain are lost in depression (Duman et al. 1997a, Marano 1999). It has been suggested that stress-associated cases of depression may result from the atrophy of vulnerable pyramidal neurones in the CA3 region of the hippocampus (Duman et al. 1997a). Elevations in the levels of glucocorticoids are known to play a major role in the stress-induced damage of CA3 neurones (Magarinos et al. 1996, Sapolsky et al. 1985, 1990, Stein-Behrens et al. 1994, Uno et al. 1989, Watanabe et al. 1982, Woolley et al. 1990). This may result in decreased levels of brain-derived neurotrophic factor (BDNF), as long-term antidepressant pre-treatment blocks BDNF down-regulation in response to stress (Nibuya et al. 1995). A number of neurochemical theories of depression are discussed below.
1.6.1 The monoamine theory of depression

The monoamine theory of depression was first formulated over 30 years ago (Bunney & Davis 1965, Schildkraut 1965). It proposes that there is a biological basis for depression, namely that alterations in the metabolism of biogenic amines in the central nervous system (CNS) may be the cause or at least be involved in the pathophysiology of the illness (Baldessarini 1975). The monoamines usually referred to by the hypothesis are the aromatic catecholamines, DA and NA, and the indoleamine 5-HT (Baldessarini 1975). Today depression is primarily thought of as being caused by a deficiency of synaptic 5-HT and NA, with DA involved to a lesser extent. Although the hypothesis has a huge amount of support, it is inadequate in its original form and has evolved over the years.

Key observations made during the 1950s were formulated to produce the monoamine theory. The major research area at this time was the investigation of the action of the hallucinogen and mood-altering drug lysergic acid diethylamide (LSD). LSD was shown to block peripheral 5-HT receptors (Woolley & Shaw 1954) and this, along with its central effects, led to the suggestion that LSD may have similar effects in the brain and therefore that 5-HT was involved in mood regulation. Meanwhile studies of the antihypertensive agent reserpine showed that it precipitated depression in a number of hypertensive patients (Muller et al. 1955). Shore et al. (1955) noted that it depleted brain 5-HT stores and increased the concentration of its metabolite, 5-hydroxyindoleacetic acid (5HIAA) in urine. It is currently believed that reserpine interferes with the vesicular storage of 5-HT and NA, thereby depleting the presynaptic levels available for release at the synapse (Hirschfeld 2000). The first major class of antidepressants appeared in 1951, with the development of the MAOIs isoniazid and its isopropyl derivative iproniazid. However they were initially developed as antimycobacterial agents for use in the treatment of tuberculosis (TB) and it was only by chance that they were seen to improve the mood of depressed TB patients (Crane 1956, Kline 1961). Development of the MAOIs specifically for their antidepressant properties produced agents that increased levels of 5-HT and NA within the brain, correlating with behavioural excitation and reinforcing the hypothesis that the antidepressant effects of MAOIs are due to increased monoamine levels (Hirschfeld 2000). Similarly, the TCAs were initially developed as potential neuroleptics (Rang &
Dale 1991). Imipramine was ineffective in calming agitated psychotics but had an effect on those patients who also exhibited symptoms of depression (Kuhn 1958). TCAs do not inhibit MAO and this initially cast doubt on the monoamine hypothesis in its original form. Instead imipramine appeared to act by inhibiting the reuptake of NA and 5-HT. Further developments have led to a range of effective TCAs which exhibit varying degrees of inhibition but none are completely specific for 5-HT or NA (Richelson 1996). However, as TCAs are non-specific, they exhibit side effects due to their actions at other transmitter sites. This led to the development of a new class of drugs – the SSRIs, which were specifically developed for use as antidepressants and to be selective to a single transmitter system. The first NRI has recently been launched onto the market: reboxetine is as efficacious as TCAs (including imipramine) in both hospitalised and outpatients, with a tendency towards higher response rates with reboxetine (Berzewski et al. 1997, Montgomery 1997). When compared to fluoxetine, in two outpatient studies, only patients classified as severely depressed showed reboxetine to be significantly more effective (2.6 points advantage on the HAM-D rating scale total score; p<0.05) than fluoxetine (Montgomery 1997).

As stated, the monoamine hypothesis has been refined over the years as more has become known about the physiology of both the brain regions and neurotransmitters involved in depression. A major revision was made to include the understanding of the roles of autoreceptors in regulating the release of monoamines and studies currently being undertaken into the intracellular events which occur following receptor binding will further expand the hypothesis (Hirschfeld 2000). For further information on BDNF and other intracellular messengers see below. However, the major issue that the hypothesis does not address is the delay in onset of antidepressant action that is common to all current antidepressant drug treatments. It is known that antidepressants affect the monoaminergic system within hours of treatment, yet it can take several weeks before the antidepressant effect becomes apparent. Early work suggested that the persistent activation of these receptors would lead to adaptations in the receptors themselves, which, in turn, contribute to the delayed action of the antidepressant (Sulser et al. 1978). This may be explained by the knowledge that some antidepressants act as agonists at neurotransmitter receptors on the postsynaptic membrane (eg. 5-HT_{1A} receptor). Thus the consequence of binding to inhibitory somatodendritic autoreceptors is initially a reduction in neuronal firing, which is overcome by sustained treatment
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(Blier & de Montigny 1994). This theory is reinforced by studies of the effects of 5-HT$_{1A}$ antagonists (primarily N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridil)cyclohexanecarboxamide trihydrochloride (WAY100635) and pindolol), which accelerate the effect of selected antidepressants (Artigas et al. 1996). Pindolol, however, is a 5-HT$_{1A}$/β-adrenoceptor antagonist and it is not known whether its ability to enhance the extracellular concentration of 5-HT is due to its serotonergic or adrenergic activity. However, WAY100635, a selective 5-HT$_{1A}$ receptor antagonist, has been shown to be effective in increasing extracellular FC 5-HT in conjunction with acute and chronic fluoxetine treatment (Dawson & Nguyen 1998, Invernizzi et al. 1996). Cremers et al. (2000) recently suggested that augmentation of 5-HT release with WAY100635 is dependent on the concentration of the antidepressant (in this case citalopram) used. It is currently believed that long-term antidepressant treatment down regulates the density of both NA and 5-HT receptors in the hippocampus and cerebral cortex (Duman et al. 1997), but there are a number of problems with this hypothesis:

1) Not all antidepressants effectively down regulate NA and 5-HT receptors (Heninger & Charney 1987).
2) The time-course for down-regulation of NA and 5-HT receptors is more rapid than the therapeutic onset of antidepressant treatment (Heninger & Charney 1987).
3) One of the most effective treatments for depression, ECT, increases levels of 5-HT receptors (Heninger & Charney 1987).
4) Reduction of NA function by selective NA receptor antagonists, e.g. salbutamol, is not effective in treating depression and may even trigger depression in susceptible individuals (Duman et al. 1997). This suggests that chronic antidepressant treatments actually result in increased activation of the intracellular signal transduction pathways of the monoamine receptors.

1.6.2 Neuroendocrine involvement in depression

A variety of alterations can be seen in the regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis in depressed patients (Hatzinger 2000). These mainly consist of increased pituitary-adrenocortical hormone secretion at baseline and a number of abnormal neuroendocrine function tests (e.g. the dexamethasone suppression/corticotrophin-releasing hormone (CRH) challenge test). The abnormalities involve the
impairment of central corticosteroid receptor function, leading to enhanced synthesis and release of vasopressin and CRH. It is these neuropeptides which mediate not only neuroendocrine but also behavioural effects. Studies have shown that this phenomenon is causally related to the action of antidepressants (Holsboer & Barden 1996). CRH, via CRH1 receptors, can trigger depression-like symptoms in animals (Owens & Nemeroff 1992, Dunn & Berridge 1990, Kalin et al. 1990), suggesting that CRH1 receptor antagonists may be a promising approach to the treatment of depression (Hatzinger 2000, Holsboer 2000).

It is also worth noting that brain monoamine pathways regulate the release of certain anterior pituitary hormones (Cowen & Anderson 1986) and the interaction between the two has been extensively investigated in animals (Muller et al. 1977). It is not so easy in man, as available 5-HT antagonists are not selective enough and changes in brain 5-HT function can frequently cause nausea and psychological symptoms, which themselves can stimulate anterior pituitary hormone release (Cowen & Anderson 1986). This may, however, allow the unification of two of the current theories of depression (ie. monoamine theory and endocrine), resulting in a greater degree of understanding of the illness.

1.6.3 A calcium hypothesis of antidepressant action

Abnormal parathyroid function has long been associated with mood disturbances and over the years a relationship between hypoparathyroidism and depression has been identified (Clark et al. 1962). Other changes in calcium metabolism have also been associated with depression, including vitamin D intoxication (Carmen & Wyatt 1979). Simply stated, the calcium hypothesis of antidepressant action states “serum ionized calcium may be lower among depressives when compared with controls; serum ionized calcium may increase in direct proportion to amelioration of depressive symptoms irrespective of treatment regimens, ie. ECT, TCA, MAOIs and/or thyroid hormone” (Ortolano et al. 1983). Three effects of calcium on monoamine metabolism may help to explain the hypothesis (Ortolano et al. 1983):

1) Calcium is essential for normal neuronal function and is required for the release of neurotransmitters (Phillis 1974, Rubin 1970, Blaustein et al. 1972).

3) Calcium has been shown to be capable of changing the activity states of tyrosine hydroxylase (Kuczenski 1975).

It can therefore be hypothesised that calcium antagonists may influence the psychopathology of affective disorders (Höschl 1991). Lithium, for example, affects calcium ion dynamics and the platelet intracellular ion concentration (Dubovsky 1993). The administration of calcium antagonists is safe and they have already been used, primarily in the treatment of cardiac tachyarrhythmias, angina and hypertension (Höschl 1991). Verapamil, however, is the most widely studied drug in terms of calcium and depression, and it is the second drug of choice in the treatment of mania, with effects comparable to those of lithium (Höschl 1991).

1.6.4 A molecular and cellular theory of depression

It is now possible to characterise the actions of antidepressants beyond the neurotransmitter and receptor level. The pre- and post-synaptic actions of monoamines are mediated by the coupling of the receptors to their respective intracellular signalling transduction pathways. It is therefore believed that the therapeutic action of many antidepressants involves the regulation of these pathways.

There are two broad categories of intracellular pathways. The first involves pathways that are controlled by receptor-coupled second messengers, e.g. cyclic adenosine monophosphate (cAMP), and regulated by classical transmitters (e.g. monoamines, neuropeptides, amino acids). The second category includes pathways controlled by receptors that contain or interact with protein tyrosine kinases and are regulated by neurotrophic factors and cytokines (see Fig 1.7).

These pathways control all aspects of neuronal function and ultimately underlie the ability of the brain to adapt and respond to pharmacological and environmental stimuli. Examples of these include the morphological changes, such as the atrophy or sprouting
Fig 1.7 A general model for regulation of transcription factors by receptor-coupled signal transduction pathways.
(Reproduced from Duman et al. 1997b).

of neurones, seen in response to damaging or growth-promoting stimuli respectively (Small et al. 1998).

A complex interaction of stimulatory and inhibitory factors regulates the cAMP system, the most common and best characterised of which are the neurotransmitter and hormone receptors that regulate the production of cAMP via adenylyl cyclase (Duman & Nestler 1995). Adenylyl cyclase catalyses the formation of cAMP from adenosine triphosphate via the appropriate G protein (Duman & Nestler 1995). Several monoamine subtypes can regulate adenylyl cyclase, either by stimulation or inhibition. Subtypes which stimulate the production of cAMP include 5-HT4, 5A, 6, 7 and β-adrenergic receptors, whilst 5-HT1A, 1B, 1D, 1E and α2-adrenergic receptors inhibit cAMP (Duman & Nestler 1995).

The different monoamine receptors selectively couple to and regulate several intracellular pathways, including the cAMP, phosphatidylinositol and calcium pathways. The first evidence that the cAMP system may be involved in the action of
antidepressants was suggested by the observation that β-adrenergic receptor activation of cAMP production was decreased by antidepressant treatment (Vetulani & Sulser 1975), a finding which has proved consistent with many forms of antidepressant. This finding could be explained by the down-regulation of β-adrenergic receptors in response to the sustained level of NA resulting from antidepressants that affect the metabolism of this compound. Fig 1.8 shows antidepressant regulation of the β1-adrenergic receptor and cAMP levels in the brain. It has been suggested that the cAMP pathway is modulated by chronic antidepressant treatment, despite decreased levels of β1-adrenergic receptors. Acute treatment results in increased levels of NA, activating β1-adrenergic receptors and increasing the formation of cAMP. However, chronic treatment leads to down-regulation of β1-adrenergic receptors, reducing the formation of cAMP. The result of continued antidepressant treatment, however, is elevated synaptic NA, so the level of cAMP formation is still increased relative to the no-treatment condition (Duman et al. 1997b). Recent work by Duman et al. (1997a) and Takahashi et al. (1999) has demonstrated that long-term antidepressant treatments result in sustained activation of the cAMP system in specific brain regions. The post-receptor elements that are increased include adenylyl cyclase activity, protein kinase A (PKA) activity and cyclic adenosine 3',5' monophosphate response element binding protein (CREB) function and expression (Nibuya et al. 1996). It can therefore be suggested that the action of antidepressant treatment goes beyond the level of neurotransmitters, to a change in function of the intracellular signal transduction cascade (Takahashi et al. 1999). Indeed phosphorylation, caused by messenger-regulated protein kinases, is the main route of activation or deactivation of cellular proteins. Popoli et al. (2000) have suggested that antidepressants interfere with intracellular phosphorylation processes and this may contribute to their therapeutic effects (Vetulani & Nalepa 2000). Studies supporting this theory include Mori et al. 1998, Perez et al. 1991, 1995 and Popoli et al. 1995. These findings may be relevant for desensitisation of presynaptic 5-HT autoreceptors and adaptive changes in the molecular machinery regulating transmitter release at serotonergic terminals (Vetulani & Nalepa 2000). The discrepancy between the effects of 5-HT1A agonists on cAMP levels in vivo and ex vivo (Hoyer & Boddeke 1993) may be explained by the form of adenylyl cyclase expressed to the largest extent in the hippocampus. The type II enzyme can be activated by the βγ- and α-s units of G
Fig 1.8 Antidepressant regulation of β₁-adrenergic receptors and cAMP levels in the brain.
(Reproduced from Duman et al. 1997b).

proteins (Federman et al. 1992, Tang & Gilman 1991). Dissociation of the G protein G-o yields large quantities of βγ, which acts synergistically with α-s to activate adenylyl cyclase. In the ex vivo situation, 5-HT₁A receptor activation results in the formation of Gα-i and therefore only small quantities of βγ. In vivo, larger amounts of βγ are available due to the stimulation of G-o dissociation by other endogenous transmitters and the stimulatory effects of these subunits on adenylyl cyclase to overcome inhibition due to the α-i subunits (Newman et al. 2000).

1.7 Animal models of depression

Most animal models used for the selection of putative antidepressants have been based on the simple amine deficiency theory. The criteria necessary for the establishment of an animal depression model were summarised as: comparable symptomatology, comparable aetiology, comparable neurophysiological basis and the concordant effect of antidepressants (McKinney 1977) and more recently under the headings of predictive validity, face validity and construct validity (Willner 1995a, 1997).
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Animal depression models can be split into three groups:

1) Those based on the effects of stress. The stressors used can vary from mild to severe as exemplified by foot shock, restraint stress and immersion in water. Willner (1987, 1990) developed the chronic mild unavoidable stress model, which has the advantage over other models, as the behavioural deficits can only be corrected by chronic, not acute, antidepressant treatment.

2) Those based on the effects of social isolation. Studies are largely based on non-human primates, but studies in rats have been shown to be useful. Little change in the models has taken place since their introduction 20 years ago (Leonard 1998).

3) Those models involving the reversal of changes that follow discrete lesions of the limbic system. Effects of the lesions of the amygdala, septum and olfactory bulbs can be reversed by chronic antidepressant treatment (Leonard 1998).

Models of depression such as these have been used in the development of new antidepressants as well as expanding our knowledge of depression by suggesting possible mechanisms of action. They have a practical advantage of allowing hypotheses from clinical observations to be to be tested in animals (Leonard 1998), so despite their widely-recognised limitations they will continue to have a major impact on our understanding of depression.

1.8 The N-methyl-D-aspartate receptor

The excitatory amino acid glutamate (GLU) is used as a messenger by most of the excitatory synapses in the CNS (Pin & Duvoisin 1995). It acts at a number of receptors that can be categorised as metabotropic or ionotropic receptors. Metabotropic receptors are coupled to G-proteins and allow GLU to regulate the production of second messengers (Pin & Duvoisin 1995), whilst the second group consists of ligand-gated cationic channels: N-methyl-D-aspartate (NMDA), α-amin-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (KA) receptors (Hollmann & Heinemann 1994, Monaghan et al 1989). For a summary of functional characteristics see table 1.4.

The agonist NMDA is a synthetic analogue of aspartate (see Fig 1.9) and does not occur naturally within the CNS (Watkins 1962). However, it selectively agonises one of the
Table 1.4: Summary of functional characteristics of excitatory amino acid receptors in the mammalian CNS.
(Reproduced from Watkins 1994).

<table>
<thead>
<tr>
<th>Ionotropic</th>
<th>Metabotropic</th>
</tr>
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<tbody>
<tr>
<td><strong>NMDA</strong></td>
<td><strong>A family of receptors linked positively to inositol triphosphate or negatively to cyclic AMP formation. Subtypes differentially activated by L-GLU, quisqualate, ibotenate, (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylate (ACPD), (1S,3S)-ACPD, (2S,3S,4S)-α-(carboxyxyxlopropyl) glycine, and L-AP4, but not by AMPA, NMDA, or kainate. Not antagonised by NMDA or non-NMDA antagonists but sensitive to pertussis toxin. May be involved in developmental plasticity.</strong></td>
</tr>
<tr>
<td>Widely distributed in the CNS – especially enriched in the hippocampus and cerebral cortex. Demonstrated most easily by pharmacological antagonism under Mg$^{2+}$-free or depolarised conditions or in binding experiments (NMDA-sensitive $[^3]$H$\text{-GLU}$, $[^3]$H$\text{-AP5}$, $[^3]$H$\text{-CPP}$, and other tritiated antagonists). Usually recognised as a slow component in repetitive activity generated primarily by non-NMDA receptors. Important in synaptic plasticity.</td>
<td></td>
</tr>
<tr>
<td><strong>AMPA</strong></td>
<td>Widespread in CNS; parallel distribution to NMDA receptors. Involved in the generation of fast component of EPSPs in many central excitatory pathways.</td>
</tr>
<tr>
<td><strong>Kainate</strong></td>
<td>Concentrated in a few specific areas of CNS, complementary to NMDA/AMPA receptor distribution (e.g. region of hippocampus). Difficult to distinguish from AMPA receptors pharmacologically due to lack of sufficiently selective antagonists. However, present specifically (in absence of AMPA receptors) on some dorsal root C fibres and dorsal root ganglion cells.</td>
</tr>
</tbody>
</table>
ionotropic GLU receptors mentioned above. The NMDA receptor exhibits an unusual property for a ligand-gated ion channel: a combination of high Ca\(^{2+}\) permeability and strong voltage dependence (McBain & Mayer 1994). This confers integrative properties which are utilised in a range of physiological functions e.g. generation of rhythmic motor activity (Travén et al. 1993), regulation of neuronal development (Cline et al. 1990, Constantine-Paton 1990, Komuro & Rakic 1993) and initiation of the complex biochemical cascade (long term potentiation – LTP) underlying some forms of memory and learning (Blanton et al. 1990) as well as being of importance in the plasticity of synapses. Activation of NMDA receptors can also trigger the mechanisms responsible for cell death (Meldrum & Garthwaite 1990) and electrographic seizures (Dingledine et al. 1990), as well as being suspected of involvement in a number of neurodegenerative disorders (Meldrum & Garthwaite 1990). These diverse mechanisms have resulted in the NMDA receptor generating considerable interest in terms of its properties and functions.

The ability of glutamatergic pathways to modulate neuronal plasticity may explain the mechanism behind the therapeutic effects of antidepressants. Although antidepressants display a range of acute actions, they have a common factor: for all known antidepressants clinical improvement only begins following chronic drug administration. The lag between onset of treatment and response has been suggested as proof that the immediate neurochemical actions of antidepressants cannot be responsible for their therapeutic effects (Hyman & Nestler 1996). This delayed onset may be mediated by a mechanism based on neuronal plasticity, with the ability to restore
neurotransmitter balance in areas of the limbic system linked to emotion (Trullas 1997). The NMDA receptor has been shown to mediate long-term synaptic responses induced by GLU (Collingridge & Bliss 1987) and it has been suggested that the therapeutic action of diverse antidepressants is mediated by a mechanism related to the neuronal plasticity processes of learning and memory (Trullas 1997). 1.8.6 summarises the evidence for NMDA receptor involvement in the aetiology of depression and antidepressant action.

1.8.1 GLU systems

The status of GLU as a neurotransmitter was uncertain for many years, as it is also involved in intermediary metabolism in neural tissue (e.g. detoxification of ammonia). Its occurrence in the CNS is ubiquitous and at high levels, but the unequal regional distribution in the spinal cord provides evidence for GLU functioning as a excitatory transmitter released from primary afferent nerve endings (Cooper et al. 1996). The regulation of brain GLU is not determined by the transport of circulating GLU; the influx from the blood (via the BBB) is much lower than the efflux of glutamate from the brain (Cooper et al. 1996). Brain GLU is synthesised in the nerve terminals via two pathways (Salway 1994):

1) From glucose via the Krebs cycle: by reductive amination of the Krebs cycle intermediate α-ketoglutarate by glutamate dehydrogenase.

2) From glutamine: synthesised in glial cells from GLU and NH₄⁺ by glutamine synthetase, which is then transported to the nerve terminals where glutaminase converts glutamine to GLU.

Termination of synaptic GLU action is mediated by a high-affinity Na⁺-coupled glutamate transporter that is present on both the presynaptic nerve terminal and/or glial cells (Cooper et. al 1996). GLU that is transported into the glial cells is converted back to glutamine by glutamine synthetase and therefore is ready to be transported back to the nerve terminal by a low-affinity process for conversion to GLU.

Mapping of the GLU system is proving to be a difficult task due to the involvement of the transmitter in intermediary metabolism (see above). However the development of
antibodies against excitatory amino acids has facilitated the process. As summarised in table 1.4, GLU can act at a number of different receptors, which can be split into two families: ionotropic and metabotropic. The NMDA receptor is a member of the ionotropic receptor family and is discussed in detail below.

1.8.2 The NMDA receptor-ionophore complex

Unlike many fast-acting receptors, the NMDA receptor is not a simple ligand-gated ion channel (Foster & Fagg 1987). It is subject to additional regulation at several sites by, for example, the amino acid glycine (GLY) and by voltage-dependent binding of magnesium (Mg²⁺) within the ion channel.

A schematic illustration of the NMDA receptor is shown in Fig 1.10 and shows the known sites of action of different pharmacological compounds. These include:

1) Transmitter binding site for GLU and related agonists, promoting the opening of a high conductance channel allowing influx of Na⁺ and Ca²⁺.

2) Regulatory binding site for the co-agonist, GLY. GLU is ineffective without GLY occupying this strychnine-insensitive modulatory site.

3) A site found within the channel, which binds phencyclidine (PCP) and related non-competitive antagonists (e.g. ketamine, MK801).

4) A voltage-dependent Mg²⁺ binding site. Depolarising conditions remove this blockade and the ion channel responds to GLU with the conductance of Na⁺ and Ca²⁺.

5) An inhibitory divalent cation site near the extracellular end of the channel. Zinc (Zn²⁺) binds here to produce a voltage-independent block.

6) A polyamine regulatory site that facilitates NMDA receptor-mediated transmission when activated.
Fig 1.10 The NMDA receptor

The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^{+} and is gated by Mg^{2+} in a voltage-dependent fashion; K^{+} is the counter-ion. The NMDA receptor channel is blocked by phencyclidine (PCP) and MK801, and the complex is regulated at two modulatory sites by glycine and polyamines; AP5 and CPP are competitive antagonists at the NMDA site.

(Reproduced from Cooper et al. 1996).

Two distinct binding sites are also believed to be associated with the transmitter binding site, one that preferentially binds agonists and one that prefers antagonists (Cooper et al. 1996).

The glycine site may be important in the action of antiepileptic drugs, as well as those for the prevention of ischaemic brain damage. Submicromolar amounts are required to increase the frequency of channel opening, therefore conditions which alter the
extracellular concentration of GLY may have a dramatic effect on NMDA-receptor mediated events.

1.8.3 Molecular and pharmacological diversity of NMDA receptor subtypes

NMDA receptors are heteromultimers, and, like all ligand gated ion channels, they are composed of multiple protein subunits. Three subunits are now recognised — NR1, NR2 and NR3. The NR3 subunit was most recently identified (Adams et al. 1995, Das et al. 1998) and is expressed primarily during development. Its role in the regulation of receptor activity in adult brain appears to be minimal (Chenard & Menniti 1999). The NR1 (ξ in mice) subunit is encoded by one gene and has eight splice variants (Moriyoshi et al. 1991) and the NR2 subunits, NR2A-NR2D (ε1-4 in mice), are derived from four different genes (Kutsuwada et al. 1992, Monyer et al. 1992). Functional receptors can be formed in expression systems from NR1 - but not NR2 - subunits alone, but a physiological NMDA receptor of an NR1 subunit plus one or more modulatory NR2 subunits is the norm.

Studies have shown that the glutamate binding site is found on the NR2 subunit (Laube et al. 1997), whilst the glycine binding site is located on the NR1 subunit (Kuryatov et al. 1994). Many potential configurations for the arrangement of subunits have been suggested, however for optimum channel activation the binding of two glutamates and two glycines is required (Clements & Westbrook 1991), and this is consistent with a tetrameric configuration of two NR1 and two NR2 subunits. The pairing of an NR1 subunit with NR2 subunits results in the formation of a functional ion channel characterised by high Ca\(^{2+}\) conductance (Boyer et al. 1998). Over-activation of this channel, resulting in a sustained rise in the cytosolic Ca\(^{2+}\) concentration, is believed to trigger neurotoxicity. The prevention of elevated Ca\(^{2+}\) levels following NMDA receptor activation may therefore limit the neuropathological changes associated with GLU.

The NMDA receptor subunits are differentially expressed throughout the CNS (Monyer et al. 1994) and are localised, primarily, if not exclusively, to postsynaptic dendritic spines of glutaminoceptive neurones (Mayer & Westbrook 1987, McBain & Mayer 1994). The mRNA for the NR1 and NR2A subunits are ubiquitously expressed throughout the adult brain. In contrast NR2 subunit mRNA expression exhibits a more
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restricted, region-specific distribution (Boyer et al. 1998, Laurie & Seeburg 1994). NR2B subunit expression is restricted to forebrain regions (e.g. cortex, hippocampus, striatum), the NR2C subunit is expressed in the cerebellum and the NR2D subunit is restricted to the midbrain regions (Chenard & Menniti 1999).

Using Xenopus oocytes it has been shown that homomeric NR1 receptors respond to GLU, but NR2 do not. When heteromeric receptors are formed, the receptor properties are dependent on the subunit composition (Laurie & Seeburg 1994). It seems that it may be the NR2 subunit that is important in determining the affinities of various NMDA receptor ligands at the GLU, GLY and ion channel sites (Laurie & Seeburg 1994), and each heteromeric recombinant receptor type displays an individual profile of affinities for ligands at these sites. It is therefore possible that differences in subunit composition of the NMDA receptor may underlie the different functional and pharmacological properties. The affinities of different recombinant receptors have been studied and compared to homomeric receptors. NR1 receptors exhibit a lower affinity for GLU than heteromeric receptors (NR1-NR2B>NR1-NR2A=NR1-NR2D>NR1-NR2C>NR1) (Laurie & Seeburg 1994), whilst NR1-NR2A receptors have a much lower affinity for the co-agonist GLY as compared to NR1-NR2C (Laurie & Seeburg 1994). The complete reproduction of native receptors may require a third element, NR3 perhaps (Adams et al. 1995, Das et al. 1998), or even post-translational modification not available with recombinant receptors. Although these studies were carried out using recombinant, as opposed to native, receptors they demonstrate the possibility that NMDA receptor function may be regulated by subunit composition and therefore changes in the expression of subunit mRNA may induce pharmacological changes.

1.8.4 Modulation of the NMDA receptor

The activity of NMDA receptors is subject to regulation by allosteric mechanisms as well as a number of second messenger systems.

The ion channel associated with the receptor is permeable to both Ca\(^{2+}\) and Na\(^{+}\) and it is the accumulation of extracellular Ca\(^{2+}\) that appears to be significant in mediating the consequences of NMDA receptor activation (Mattson 1990). The polarisation state of the postsynaptic membrane is also a limiting factor and this affects the degree of Mg\(^{2+}\)
binding to its binding site in the channel pore. Upon depolarisation, Mg$^{2+}$ binding is reduced and ion flux may occur. However, once the membrane repolarises, this allows the binding of Mg$^{2+}$ to its site within the pore, occluding the channel and inhibiting ion flux. Therefore in order for NMDA receptor activation, presynaptic GLU release and postsynaptic depolarisation are required.

Activation of the NMDA receptor can only occur in the simultaneous presence of the agonist (e.g. GLU) and the co-agonist GLY (Johnson & Asher 1987, Kleckner & Dingledine 1988). Kinetic analysis of NMDA receptor activation suggests that two molecules of both GLY and GLU must bind to the receptor in order to activate ion channel gating (Benveniste et al. 1990, Benveniste & Mayer 1991, Clements & Westbrook 1991, Patneau & Mayer 1990). However, there is now substantial evidence for interactions between GLY and GLU binding sites based on allosteric mechanisms (McBain & Mayer 1994), and it has been suggested that GLU binding decreases NMDA receptor affinity for GLY sevenfold (Benveniste et al. 1990). This is of physiological importance, as GLY is present continuously in the extracellular space, whilst GLU is only released transiently from nerve terminals. GLY transporter mRNA is widely expressed within the CNS and its distribution overlaps that of the NR1 subunit mRNA, therefore suggesting that the extracellular concentration of GLY may be closely regulated (Smith et al. 1992).

The NMDA receptor is also allosterically modulated by polyamines including spermine. Although they potentiate NMDA currents in the presence of saturating levels of both GLU and GLY, their presence is not a requirement for the activation of the NMDA receptor. During pathological conditions, the levels of polyamines increase and it is possible that they mediate the excitotoxic mechanisms responsible for neuronal damage. Ifenprodil and eliprodil are potent antagonists of the polyamine site (Carter et al. 1989) and have been shown to exhibit neuroprotective properties (Gotti et al. 1988, Graham et al. 1992, Tamura et al. 1993). However, recent evidence suggests that these two antagonists may be selective to the NR2B subunit of the NMDA receptor (Williams 1993) and together these findings strongly suggest that NMDA receptors containing the NR2B subunit contribute to excitotoxic damage (Mott et al. 1998).
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In addition to the modulation of the receptor by GLY and polyamines, the NMDA receptor is negatively modulated by \( \text{Zn}^{2+} \) and excess protons in the range of physiological values of pH (6.6-8.0) (McBain & Mayer 1994, Mott et al. 1998, Tang et al. 1990). Zinc acts as a potent antagonist at NMDA receptors and the mechanism of action involves components with strong and weak voltage sensitivity (Christine & Choi 1990, Legendre & Westbrook 1990, Westbrook & Mayer 1987). Alternative splicing of NR1 subunits is believed to regulate the sensitivity of the NMDA receptors to protons, as opposed to the assembly of different NR2 subunits (Traynelis & Heinemann 1993). The difference in proton sensitivity of the NR1-1a and NR1-1b splice variants is of potential physiological significance as at pH 7.3, 50% inhibition of the a-variant occurs whilst the b-variant is fully active (McBain & Mayer 1994). However, it is not known whether native NMDA receptors exist as homomeric or heteromeric receptors in terms of the NR1 splice variants and it has been suggested that NMDA receptors are not active under normal conditions (Traynelis & Cull-Candy 1990).

A more complex system of modulation is through one or more redox sites (Aizenman et al. 1989, McBain & Mayer 1994) and a number of endogenous substances, including glutathione (Gilbert et al. 1991), pyrroloquinoline quinone (Aizenman et al. 1989), oxygen free radicals (Aizenman et al. 1990) and nitric oxide (Lei et al. 1992, Lipton et al. 1993, Maricq et al. 1991, Smith & Whitton 2000) have been shown to modulate NMDA receptor activity in this way. The activity of the NMDA receptor is inhibited by oxidising agents (e.g. 5,5'-dithiobis(2-nitrobenzoic acid)-DTNB) and potentiated by reducing agents (e.g. dithiothreitol-DTT). The complex effects seem to be dependent on subunit composition (McBain & Mayer 1994) and it has been suggested that there are in fact two redox sites, one in NR2A, NR2B and NR2C subunits and a second site only found in the NR2A subunit (Köhr et al. 1993).

In 1992, it was demonstrated that Xenopus oocytes injected with whole rat brain mRNA expressed NMDA receptors whose function could be potentiated by the protein kinase C (PKC) activator 12-O-tetradecanoylphorbol-13-acetate (Urushihara et al. 1992). Subsequent cloning and expression of functional NMDA receptor subunits from both the mouse and rat demonstrated that phosphorylation was peculiar to specific subunits and heteromeric combinations. Little information exists on the possible phosphorylation sites contained within the NMDA receptor subunits that underlie the action of PKC
activators. PKC-mediated phosphorylation has been shown to occur on several sites of the NR1-1A subunit (Tingley et al. 1993) and it has been suggested that NR2 subunit sites within the carboxy-terminal may regulate sensitivity to phosphorylation (McBain & Mayer 1994)

1.8.5 NMDA receptor antagonists

Antagonists acting at the NMDA receptor can be classified as either competitive or non-competitive. Competitive antagonists act at the GLU recognition site and include CGP40116, CPP and 2-amino-5-phosphopentanoic acid (D-AP5). Non-competitive antagonists can be further divided into channel blockers (e.g. amantadine, memantine), polyamine site antagonists (e.g. ifenprodil, eliprodil) and the GLY site antagonists (e.g. D-cycloserine, HA966). Each class has a different pharmacological profile, especially with respect to their side effects, which results in limited clinical use.

The first target syndrome identified for the use of competitive NMDA receptor antagonists was epilepsy (Croucher et al. 1982), but other proposed targets include ischaemic brain damage, stroke and anxiety (Meldrum 1985). The possibility of use in long-term neurodegenerative disorders seems remote due to the problems of side effects. Early competitive antagonists were highly polar substances and were poorly brain penetrant when given peripherally, thus the objective in developing newer competitive drugs was to increase NMDA receptor affinity (Jane et al. 1994).

The excessive release of glutamate and subsequent over-activation of post-synaptic receptors results in neuronal damage (Choi 1991, McCulloch 1992). Work concentrated on non-competitive antagonists of the NMDA receptor in an attempt to develop drugs for the treatment of neurodegenerative disorders. Potent channel blockers, such as PCP and dizocilpine (MK801), penetrate easily into the CNS but possess undesirable side effects e.g. behavioural stimulation (Tricklebank et al. 1989) and cardiovascular effects (Lewis et al. 1989). Despite their therapeutic usefulness being limited by these side effects, the compounds have been successfully used as neuroprotectants in animal models of neurodegeneration. More recently work has concentrated on drugs which act at other sites on the NMDA receptor complex, including the glycine co-agonist site.
1.8.5.1 Side effects of NMDA antagonists

In comparison with other transmitter systems (e.g. 5-HT, NA, DA), excitatory amino acid drugs have very few peripheral side effects, due to the receptors being primarily confined to the CNS. It should be noted that there is evidence for the presence of excitatory amino acid receptors in the periphery (Bertrand et al. 1992, Erdoe 1991, Moroni et al. 1986, Wiley et al. 1991), but not to the same extent as for 5-HT, for example.

The use of NMDA antagonists as therapeutically effective drugs is dependent on the degree of side effects. Side effects of NMDA antagonists include:

- **Muscle relaxation and sedation**: NMDA antagonists block synaptic responses involved in spinal reflexes (Davies 1988) and could be used in cases of spasticity, however this effect may be a limiting factor in the treatment of epilepsy.

- **Psychotomimetic effects**: Ketamine is used as an anaesthetic in man and has displayed psychotomimetic properties that are believed to be associated with its NMDA channel blocking property (Herrling 1994). These effects may restrict the use of such agents to acute indications, e.g. head injury, where clinical benefit outweighs side effects. It may be possible to separate out the psychotomimetic effect from the beneficial as they may be dose-dependent (Herrling 1994).

- **Effects on learning performance and neuronal plasticity**: NMDA antagonists have been shown to inhibit learning performance in rats (Morris et al. 1986, Morris & Davis 1994, Parada-Turska & Turski 1990), as well as influencing neuronal plasticity during development (Artola & Singer 1994, Kleinschmidt et al. 1987).

- **Effects on sensory systems**: Excitatory amino acid receptors (both NMDA and non-NMDA) are involved in all levels of sensory transmission. Tang & Ho (1988) have shown that NMDA antagonists affect brightness discrimination in rats. It is not known whether these effects are of such a magnitude to limit their clinical use (Herrling 1994).
1.8.6 NMDA receptors and affective disorders

Stressful experiences have been shown to contribute, provoke or exacerbate clinical depression (Anisman & Zacharko 1982, Anisman & Zacharko 1992). Behavioural depression, however, only results when there is no control over the stressful stimuli or when such stimuli are considered uncontrollable (Trullas 1997). It can therefore be perceived that depression is not a direct outcome of stress, but of the inability to activate the mechanisms required to generate an adaptive response to stressful stimuli (Anisman & Zacharko 1992, Stone 1983). A number of recent studies have supported this theory by showing that NMDA receptors are involved in both acute and long-term adaptive neurochemical and behavioural effects of stress (Kim et al, 1996, Nowak et al. 1995, Shors et al. 1989, Shors & Servatius 1995). The CA1 cell body layer of the hippocampus contains a high density of NMDA receptors (Cotman et al. 1987) and it has been shown that exposure to inescapable stress impairs the induction of LTP in this region (Shors et al. 1989).

1.8.6.1 NMDA antagonists as antidepressants: Pre-clinical findings

Inescapable stress also induces behavioural depression that can be antagonised by clinically available antidepressants (Desan et al. 1988, Leshner et al. 1979, Petty & Sherman 1980, Shanks & Anisman 1989), therefore it was hypothesised that NMDA receptors may be modulating the behavioural deficits induced by inescapable stress (Trullas & Skolnick 1990). The hypothesis could be tested by using two models that are commonly used to detect drugs with antidepressant properties: the forced swim test (Porsolt et al. 1977) and the tail suspension test (Steru et al. 1985). Both were designed as screening models for potential antidepressant drugs and are based on the ability of clinically effective antidepressants to reduce the immobility displayed after animals are exposed to inescapable stress. The competitive NMDA antagonist 2-amino-7-phosphonoheptanoic acid (AP7) (Evans et al. 1982), the non-competitive NMDA antagonist Dizocilpine (MK801) (Wong et al. 1986) and the partial agonist at the GLY site on the NMDA receptor 1-aminocyclopropanecarboxylic acid (ACPC) (Marvizon et al. 1989) have all been shown to mimic the effects of clinically effective antidepressants, such as imipramine, when tested in these models (Trullas & Skolnick 1990), therefore supporting the hypothesis that pathways subserved by NMDA
receptors are involved in the pathophysiology of depression. Additional studies carried out at a number of laboratories using a variety of pre-clinical models have confirmed that compounds which reduce the activity at NMDA receptors mimic the effects of clinically active antidepressants (Heresco-Levy & Javitt 1998) and these are summarised in table 1.5 below.

The chronic treatment of both rats and mice with a diverse range of antidepressant treatments (including ECT) produced a 2-4 fold reduction in the potency of GLY to inhibit \[^3H\]-5,7 dichlorokynurenic acid (5,7-DCKA) binding to the NMDA receptor-associated GLY sites in neocortical membrane (Nowak et al. 1993, Paul et al. 1993, Paul et al. 1994). This slowly developing reduction was observed in 16 of the 17 antidepressants tested (Paul et al. 1994) and is associated with 40-100% reduction in GLY-displaceable \[^3H\] CGP-39653 binding to the GLU recognition site of the NMDA receptor. It is therefore suggested to be a more accurate predictor of antidepressant activity than either β-adrenoreceptor down-regulation or efficacy in the forced swim test (Heresco-Levy & Javitt 1998).

NMDA receptor antagonists have also been shown to be active in the chronic mild stress (CMS) model of anhedonia, but the model has yet to be validated with as many drugs as the forced swim and tail suspension tests. However, chronic antidepressant treatment reverses the reduction in both sucrose consumption and intracranial self-stimulation produced by chronic application of inescapable, uncontrollable stressors (Willner 1997). Traditional antidepressants, including TCAs and SSRIs, require treatment of more than three weeks to reverse stress-induced deficits. A study by Papp & Moryl (1993, 1994) tested NMDA antagonists in the CMS model and demonstrated that MK801 and the competitive antagonists CGP 37849 and CGP 40116 were as effective as imipramine in restoring stress-induced deficits in sucrose consumption. A similar study using ACPC demonstrated that at the higher dose of 200mg/kg/day, ACPC was effective within 2 weeks, compared to 5 weeks for imipramine (Papp & Moryl 1996).
Table 1.5 NMDA antagonists are active in pre-clinical models that predict clinical efficacy.

(Reproduced from Skolnick 1999).

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-7, MK 801, and ACPC</td>
<td>Trullas &amp; Skolnick 1990,</td>
</tr>
<tr>
<td></td>
<td>Trullas at al. 1991,</td>
</tr>
<tr>
<td>MK 801</td>
<td>Maj et al. 1992a, c.</td>
</tr>
<tr>
<td>CGP 37849 and CGP 39551</td>
<td>Maj et al. 1992b.</td>
</tr>
<tr>
<td>Eliprodil</td>
<td>Layer et al. 1995.</td>
</tr>
<tr>
<td>ACPC is active in the chronic mild stress model. The onset of effect is dose-dependent, and significantly more rapid than imipramine (rats).</td>
<td>Papp &amp; Moryl 1996.</td>
</tr>
</tbody>
</table>

A study by Boyer et al. (1998) has suggested that chronic administration of imipramine and citalopram alters the expression of NMDA receptor subunit mRNAs in mouse brain. They hypothesised that the effect of chronic antidepressant treatment was due to an alteration in NMDA subunit composition and examined their theory using in situ hybridisation. Both antidepressants altered the levels of mRNA encoding the ζ-subunit in a similar fashion, either reducing transcript levels (e.g. cortex, cerebellum, thalamus, striatum) or having no significant effect (e.g. hippocampus). The effects on the ε-subunit family were often distinct and region-specific. These findings therefore suggest that chronic antidepressant treatment results in changed expression of NMDA receptor subunit transcripts and therefore altered NMDA receptor composition. This is important...
as the pharmacological and physiological properties have been shown to be dependent on individual subunits (Laurie & Seeburg 1994). It should however be noted, that an earlier study which looked at the effects of a range of antipsychotic and antidepressant drugs on glutamate receptor subunit (NMDA and metabotropic) mRNA levels in rat brain were unable to show a significant effect when compared to controls (Oretti et al. 1994), although any effect may have been diluted by the use of whole brain.

There is strong evidence for interactions between the glutamatergic and serotonergic systems in the brain and that these may play a role in the development and treatment of depression. Antidepressant treatment may stabilise the effect of NMDA receptor stimulation on 5-HT transmission and this could be important in the mode of action of these drugs. It is widely accepted that in vivo 5-HT release is, in part, regulated by glutamatergic receptors in several brain regions (Becquet et al. 1990, Whitton et al. 1992a, Whitton et al. 1994a,b). Tao & Auerbach (1996) used microdialysis to demonstrate that NMDA infusion into the RN leads to a concentration-dependent increase in extracellular 5-HT, associated with increased extracellular 5-HT in the nucleus accumbens. Pallotta et al. (1998) observed NMDA to have an inverse-concentration effect over 5-HT release and resultant transmission to the FC. The role of NMDA receptors in regulating serotonergic transmission in ascending pathways is evidently complex, since Lejeune et al. (1994) observed that NMDA receptor antagonists increased serotonergic transmission to the striatum but did not observe any effect of NMDA alone on firing of RN neurones. Pallotta et al. (2001) have studied the effects of acute and chronic clomipramine (CIM) on NMDA-mediated 5-HT transmission between the RN and FC. They found that acute treatment had no effect but chronic (15day) treatment with CIM (10 or 20mg/kg) caused a dose-dependent increase in basal extracellular 5-HT in both the RN and FC which could be greatly attenuated or abolished by the infusion of NMDA into the RN (Pallotta et al. 2001). These findings suggest a clear association between chronic antidepressant treatment with CIM and serotonergic transmission by NMDA receptors located in the RN (Pallotta et al. 2001), a consideration lacking in much of the work in which the role of antidepressants acting at the NMDA receptor has been studied. The observation that acute CIM did not increase FC 5-HT, along with evidence from other groups (Adell & Artigas 1991), may be explained by the action of 5-HT at 5-HT$_{1A}$ somatodendritic autoreceptors.
Behavioural evidence is also available to suggest that antidepressant treatment affects the function of NMDA receptors. A recent study by Popik et al. (2000) looked at the effects of the GLY/NMDA receptor antagonist L-701,324, which has been shown to have anxiolytic actions (Kotlinska & Liljequist 1998). Chronic treatment with citalopram, imipramine and ECT produced a reduction in the anxiolytic-like actions of L-701,324 that could not be reversed by GLY. No change was seen with acute treatment. The apparent reduction in the anxiolytic-like actions of a specific GLY/NMDA receptor antagonist following chronic treatment with a variety of antidepressants is consistent with previous neurochemical and molecular studies indicating that chronic antidepressant treatment can affect NMDA receptor function (Popik et al. 2000).

A study of alterations in the NMDA receptor complex in the frontal cortex of suicide victims provided the first evidence supporting the theory that dysfunction of the glutamatergic system is involved in the psychopathology underlying suicide and depression in humans (Nowak et al. 1995). A diagnosis of major depressive disorder can be rendered in 50% of suicide victims and therefore can be used as a good approximation to identify sufferers of depression by post-mortem. Nowak et al. (1995) reported a reduction in the proportion of high affinity, glycine displaceable $[^3]$H CGP-39653 binding to GLU receptors, from ~45% in controls to ~27% in age- and post-mortem interval matched suicide victims. However the following factors were unaffected in the frontal cortex of suicide victims when compared to controls: the potency of GLY to inhibit $[^3]$H 5,7 DCKA binding to the GLY site (strychnine-insensitive GLY receptor), the specific binding of $[^3]$H 5,7-DCKA and basal GLY- or GLU- enhanced non-equilibrium binding of $[^3]$H MK801 (Nowak et al. 1995).

1.8.6.2 NMDA antagonists as antidepressants: Clinical findings

Surprisingly, the compelling findings outlined above have resulted in little clinical evaluation of the use of NMDA receptor antagonists as antidepressant drugs. Support for the hypothesis has been shown in preliminary studies with D-cycloserine, a partial agonist at the GLY site, (Crane 1959, 1961) and the weak NMDA channel blocker amantadine (Vale et al. 1971), but until recently little more has been known. Ketamine hydrochloride is a potent NMDA antagonist and was initially tested as an antidepressant.
Chapter One: General Introduction

in a proof-of-concept study (Cappello et al. 1997, Cappello et al. 1998). It was hypothesised that a dysfunction of NMDA receptor function contributes to depression and depressed patients show abnormal NMDA receptor function that results in increased sensitivity to drugs such as ketamine. Secondly, that the mechanism of antidepressant drugs is regulated by NMDA receptors as an apparent reduction in NMDA receptor function is observed. In this study, 7 patients with major depression were administered an intravenous infusion of low-dose (0.5mg/kg) ketamine or saline over a 40min period. Those patients who were previously unresponsive to antidepressant treatment responded to the ketamine infusion and a substantial and significant improvement in mood was observed for up to 72hours afterwards. No response to the saline infusion was observed (Cappello et al. 1998). A second study confirmed that low-dose ketamine infusion, as compared to placebo, is associated with robust decreases in depressive symptoms over 3 days (Berman et al. 2000). These results are consistent with limited reports of the use of NMDA receptor antagonists in animal models of depression. However, the method of delivery may be the major determinant of the rapidity of the response as some (Malhotra & Santosh 1996, Sallee et al. 1997) but not all (Pollock et al. 1989) studies show rapid antidepressant response to intravenous infusion of TCAs (Berman et al. 2000).

Both the pre-clinical and clinical findings above strongly suggest that a behavioural stressor selectively and differentially affects recognition sites on the NMDA receptor complex. There are a number of possibilities for these changes in affinity e.g. alterations in subunit expression, post-translational modifications of the NMDA receptor complex, alteration in gene expression of enzymes linked to the NMDA receptor, such as PKC. This coupled with the evidence that functional NMDA receptor antagonists are active in the forced swim test (Trullas & Skolnick 1990, Trullas et al. 1991, Maj et al 1992b, Skolnick et al. 1992), learned helplessness and chronic mild stress models of depression (Papp & Moryl 1993, Meloni et al. 1993) and reduce β-adrenoceptor density (Paul et al. 1992, Klimek & Papp 1994) suggest that the NMDA receptor plays an important role in the action of antidepressants and the pathophysiology of depression.
1.8.7 The effect of alterations in NMDA receptor subunit composition

The induction of conformational changes in NMDA receptors by antidepressants may be a means of dampening elevations in intracellular Ca\(^{2+}\) concentration in specific neuronal populations evoked by the release of GLU. As outlined in 1.6.3, Ca\(^{2+}\) homeostasis may be important in the pathophysiology of depression. Alterations in cerebrospinal fluid (CSF) and serum Ca\(^{2+}\) levels have been reported in depressed patients and these changes could not be directly associated with a dysfunction of the endocrine system (Laurie & Seeburg 1994). Conversely, reduced plasma, serum and CSF Ca\(^{2+}\) levels have been reported after both successful ECT and antidepressant drug therapy (Huang et al. 1997).

Studies showing antidepressant induced changes in radioligand binding to NMDA receptors were the first to suggest a link between a selective adaptation of a neurotransmitter receptor in response to chronic administration of all classes of antidepressants. Such changes may also reflect changes in post-translational modification, as NMDA receptor subunits serve as substrates for PKC, PKA and many other kinases (see Fig 1.11). Emerging evidence suggests that NMDA receptor function can be modulated by phosphorylation (see 1.8.4), therefore it can be suggested that changes in one or more of the kinase enzymes associated with the NMDA receptor complex could be directly responsible for the change in radioligand binding.
Fig 1.11 Schematic of the NMDA receptor complex.

Components of the NMDA receptor complex include cytoskeletal and scaffold proteins, as well as signalling enzymes and receptors. Known protein-protein interactions are represented by contacts between proteins.
(Reproduced from Sheng & Lee 2000).

1.8.8 Molecular links between conventional antidepressants and NMDA receptors

Most antidepressant drugs increase synaptic concentrations of NA and/or 5-HT by stimulating adenylyl cyclase via G proteins (Gs) coupled receptors (Duman et al. 1997a,b, Rossby & Sulser 1997). This results in an increase in cAMP levels and the activation of cAMP-dependent PKA (see 1.5.4), triggering the events that initiate gene transcription. It has been demonstrated that chronic antidepressant treatment elevates levels of CREB mRNA and protein in rat hippocampus (Nibuya et al. 1995, 1996). Fig 1.12 summarises a cellular model for the action of antidepressant treatments and outlines the intracellular pathways involved. CREB could serve as a common
post-receptor target for monoamine receptors that stimulate the cAMP-PKA cascade (e.g. 5-HT$_4$,6,7 and β-adrenergic receptors) or receptors that lead to activation of Ca$^{2+}$-dependent kinases (e.g. 5-HT$_2$ and α$_1$-adrenergic receptors). The up-regulation of CREB may lead to the regulation of specific genes such as BDNF and its receptor, TrkB, which are also increased in response to antidepressant treatment (Nibuya et al. 1995). The function and survival of hippocampal neurones or neurones innervating the region could be influenced by BDNF and TrkB (Duman et al. 1997b).

1.8.8.1 BDNF – evidence for involvement in depression

BDNF is a member of a structurally related family of trophic factors that includes neurotrophin-3 and neurotrophin-4. Expression of its mRNA is activity dependent and shows marked, transient changes in response to a number of neuronal insults (e.g. ischaemia, hypoglycaemia, stress, trauma). A complex interaction between different neurotransmitter systems regulates the levels of BDNF mRNA under basal conditions, as well as in response to insult (Thoenen 1995). Glutamate is involved in the up-
regulation of BDNF mRNA, whilst GABAergic pathways down-regulate BDNF expression in the hippocampus (Zafra et al. 1991). Studies have also shown that both cholinergic and noradrenergic pathways modulate BDNF expression in vivo (Zafra et al. 1992), further demonstrating the large overlap between the roles of monoamines and neurotrophic factors in regulating brain function.

The actions of BDNF are mediated by its receptor (TrkB), which exists as a receptor dimer in its active form. Activation results in autophosphorylation of intracellular sites as well as other cellular proteins involved in the regulation of the MAP cascade. The trophic and neuroprotective properties of BDNF (Mamounas et al. 1995, Tong & Perez-Polo 1998) have led to the hypothesis that BDNF induction is a crucial step in blunting the ability of chronic stressors to damage vulnerable neurones (Altar 1999, Duman et al. 1997a,b).

Several lines of evidence point to BDNF being involved in depression:

1) ECT increases the expression of BDNF and its receptor TrkB in the brain (Nibuya et al. 1995).
2) Chronic infusion of BDNF has an antidepressant effect in animal behavioural models of depression (Siuciak et al. 1996)
3) Stress has been shown to decrease hippocampal BDNF expression. Chronic stress is known to cause atrophy of vulnerable hippocampal neurones (Condorelli et al. 1994), a region which has long been implicated in the pathophysiology of depression (Heninger & Charney 1987).

The importance of these changes is highlighted by recent brain imaging studies of patients with depression that demonstrate that they have a small, but significant, reduction in the volume of certain brain structures, including the hippocampus (Sheline et al. 1996). Therefore, if down-regulation of BDNF contributes to the atrophy of hippocampal neurones in response to stress, and depression results, it may be classified as a neurodegenerative disorder.
1.8.9 BDNF and the NMDA receptor

NMDA antagonists have been shown to be protective against a number of neuronal insults (Choi 1988, Kornhuber & Weller 1997), an effect shared with the neurotrophic factor BDNF (see 1.8.8.1). It can therefore be hypothesised that conventional antidepressants (via BDNF) and NMDA antagonists reach the same cellular endpoint – the protection of vulnerable neurones. Brandoli et al. (1998) have suggested that the two treatment strategies reach an identical functional endpoint – dampening of NMDA receptor function (see Fig 1.13). They demonstrated that long-term exposure (>6 hours) to BDNF reduced the levels of NR2A and NR2C mRNA and protein in cerebellar granule cell neurones, which express high levels of NMDA receptors. The reduction in NR2A mRNA is in the range produced by chronic imipramine treatment (Boyer et al. 1998) and is accompanied by a reduction in NMDA-evoked increases of Ca\textsuperscript{2+} (Brandoli et al. 1998), an effect mimicked by direct application of NMDA antagonists (Dildy & Leslie 1989).

1.9 The future of antidepressants

Fig 1.13 outlines potential targets for new antidepressants by illustrating how the elevation of synaptic monoamine levels affects G\textsubscript{S}-coupled pathways and how this in turn affects NMDA receptor function (Skolnick 1999). Indeed if increased BDNF levels are the key to the antidepressant action of current therapies, it may be possible to develop new treatments which bypass the monoaminergic synapse altogether. The cAMP system too may play a role in the mechanism of current antidepressants, a selective inhibitor of phosphodiesterase (PDE) IV (Rolipram; Hughes et al. 1997) has been shown to be a clinically effective antidepressant (Horowski et al. 1985). Problems exist with its clinical use due to the degree of side effects suffered, but the potential for future development is there. Combining rolipram with imipramine has been shown to produce a more rapid increase in hippocampal BDNF and CREB mRNA levels than either compound alone (Duman et al. 1997a).

It is worth noting that there are other signal transduction pathways which are known to be affected by conventional antidepressant treatments, e.g. 5-HT\textsubscript{2}-activation of an inositol-phosphate cascade (Rossby & Sulser 1997). These events may affect
Fig 1.13 Linking conventional antidepressant treatment to a reduction in NMDA receptor function.
A solid bar preceding an arrow denotes inhibition. (Reproduced from Skolnick 1999).
transcription of an additional set of target molecules, which in turn may interact with the sequence of events outlined in Fig 1.13. Therefore, it is possible that the activation of multiple signal transduction pathways may underlie the basis of therapeutic lag (Skolnick 1999). Other potential targets include the glucocorticoid receptor (see 1.6.2 for detail) and genes whose expression is altered by conventional antidepressant treatment.

1.10 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. Although modern antidepressant treatment is successful in most cases, 20% of patients are treatment resistant and a further 20-30% are only partial responders (Fawcett & Kravitz 1985). Therefore, the need for more effective treatments has led to the suggestion that combination treatments may reduce the lag time before therapeutic effect and increase the efficiency of the antidepressant. Artigas et al. (1994) were the first to suggest that the combination of an SSRI with the 5-HT1A/β-adrenoceptor antagonist pindolol induced a rapid improvement in the mood of depressed patients. However, the results of subsequent double-blind, placebo-controlled studies have proved to be inconsistent (Berman et al. 1999, Coryell 2000, Dawson et al. 2000, Perez et al. 1999, Rasanen 1999, Shiah et al. 2000).

Given the new information concerning the effectiveness of NMDA receptor antagonists in animal models of depression and the evidence outlined above, this study was undertaken in order to clarify the following points:

1) Do antidepressants of different classes, e.g. TCA and NRI, have a similar effect on NMDA evoked-monoamine and amino acid release in the RN and FC?
2) Do NMDA antagonists have a similar effect on monoamine and amino acid release in the RN and FC?
3) Do NMDA antagonists and currently used antidepressants show synergism that may lead to a more rapid onset of increased monoamine release in the FC and thereby antidepressant effect? Is this effect restricted to a particular class of antagonist?
Chapter Two

Materials and Methods
Chapter Two: Materials and Methods

2.1 In vivo microdialysis

2.1.1 In vivo methods for determining brain extracellular fluid constituents

In order to study the complex biochemical reactions in both the normal and neuropathological states of the brain a number of techniques have been developed. The criteria for the development of these techniques were the need for instantaneous and reliable measurement of neurotransmitter, neuromodulator and metabolite levels in discrete brain regions. However, it was also important to ensure that normal tissue structure and metabolism were unaffected following application of the technique. Of the techniques developed to date, none fulfil these requirements (Benveniste 1989).

The techniques can be further split into in situ and ex situ techniques. In situ techniques allow detection and measurement directly in the extracellular space and include ion-selective microelectrodes. The main problem with microelectrodes is their lack of selectivity due to several compounds having similar oxidation potentials. Ex situ techniques collect substances from the extracellular space to be analysed elsewhere, for example, by high-pressure liquid chromatography (HPLC). The cortical cup was introduced in 1953 (Macintosh & Oborin 1953), the push-pull cannula in 1961 (Gaddum 1961) and microdialysis in 1966 (Bito 1966), although it has been continuously refined (Ungerstedt & Pycock 1974, Ungerstedt et al. 1982).

2.1.2 Introduction to in vivo microdialysis

Microdialysis is now the method of choice for in vivo measurements of the extracellular space. In principle it allows changes in the chemistry of the extracellular space to be monitored in the intact brain during normal and neuropathological states by the dialysis or diffusion of soluble molecules across a semi-permeable membrane (Benveniste & Huttemeier 1990). In practice a microdialysis probe is stereotaxically implanted into the brain region of interest and is perfused with artificial cerebrospinal fluid (aCSF), which initially lacks the substance(s) of interest but closely mimics the endogenous extracellular fluid in both ionic composition and pH. Diffusion occurs across the resultant concentration gradient until steady state equilibrium is regained. The continuous flowing of the aCSF through the probe allows the samples to be collected at
set time points and assayed using highly sensitive assay procedures such as HPLC. The technique allows the manipulation of the extracellular environment by drugs that can enter the CNS, either infused by the microdialysis probe or administered systemically. Microdialysis therefore has a wide variety of uses including measuring neurotransmitter release, as a drug delivery system and also as a pharmacokinetic tool. There are a number of advantages of the technique including regional specificity, the blood-brain barrier remains intact, modest tissue trauma, a wide range of substances can be analysed and the technique can be carried out in awake animals therefore overcoming anaesthetic-drug interactions. It should be noted that all microdialysis experiments presented here used conscious animals and therefore overcame any problems with anaesthesia-induced neurochemical changes, for example the compromising of neuronal function and possible interactions of drugs which may affect neurotransmitter release (Marsden 1985). However there are a number of disadvantages of in vivo microdialysis. Firstly a specific and sensitive assay method for the desired substances is required, secondly anatomical resolution is limited by the external diameter of the membrane (~250-350µm) and finally microdialysis suffers from poor temporal resolution, resulting in longer sampling times which makes transient changes harder to resolve. It should be noted that the development of more sensitive assay techniques (microbore HPLC, capillary electrophoresis, for example) has gone some way to addressing these problems. A summary of the advantages and disadvantages of microdialysis compared to the other in vivo methods described above is given in Table 2.1.

2.2 Animal Husbandry

Male albino Wistar-derived rats (250-350g: Bantin & Kingman Ltd., Hull, U.K.) were used in all in vivo microdialysis experiments in this thesis. Animals arrived in the unit a minimum of 7 days prior to start of experiments to allow habituation to new surroundings. Prior to experiments the animals were group housed (6 animals per cage) under conditions of constant humidity (50%) and temperature (18-22°C) and conditioned to a light-dark cycle of 12 hours (light from 0700-1900 every day) in accordance with Home Office regulations. Food (standard rodent diet) and water were available ad libitum. All experimental procedures were carried out during the light cycle and in strict adherence to the terms of the 1986 Animals (Scientific Procedures) Act.
Table 2.1 Advantages and disadvantages of *in vivo* techniques (modified from Benveniste 1989).

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td><strong>In situ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion-selective microelectrode</td>
<td>i. Examination of all brain regions.</td>
<td>i. Only detects ions.</td>
</tr>
<tr>
<td></td>
<td>ii. Time resolution &lt;1s.</td>
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</tr>
<tr>
<td></td>
<td>iii. Tip of electrode &lt;4μM</td>
<td></td>
</tr>
<tr>
<td>Carbon fibre microelectrode</td>
<td>i. Examination of all brain regions</td>
<td>i. Only detects oxidisable compounds</td>
</tr>
<tr>
<td></td>
<td>ii. Used in conscious animals</td>
<td>ii. Selectivity poor without prior HPLC analysis</td>
</tr>
<tr>
<td></td>
<td>iii. Time resolution &lt;1min</td>
<td>iii. Drainage</td>
</tr>
<tr>
<td></td>
<td>iv. Tip of electrode &lt;300μM</td>
<td>iv. Some electrodes have short working life in <em>vivo</em></td>
</tr>
<tr>
<td><strong>Ex situ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical cup</td>
<td>i. No tissue penetration</td>
<td>i. Time resolution &gt;10min</td>
</tr>
<tr>
<td></td>
<td>ii. Used in conscious animals</td>
<td>ii. Only cortex can be analysed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iii. Drainage</td>
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<tr>
<td></td>
<td></td>
<td>iv. Deproteinization required before HPLC analysis</td>
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<tr>
<td></td>
<td></td>
<td>v. Enzymatic degradation of collected compounds</td>
</tr>
<tr>
<td>Push-Pull cannula</td>
<td>i. Examination of all brain regions</td>
<td>i. Time resolution &gt;10min</td>
</tr>
<tr>
<td></td>
<td>ii. Used in conscious animals</td>
<td>ii. Drainage</td>
</tr>
<tr>
<td></td>
<td>iii. BBB intact following implantation</td>
<td>iii. Deproteinization required before HPLC analysis</td>
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<tr>
<td></td>
<td></td>
<td>iv. Enzymatic degradation of collected compounds</td>
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<td></td>
<td>v. Tissue trauma following implantation</td>
</tr>
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<td></td>
<td>vi. cannula O.D. &gt;1mm</td>
</tr>
<tr>
<td>Microdialysis probe</td>
<td>i. Examination of all brain regions</td>
<td>i. Time resolution &gt;5 min</td>
</tr>
<tr>
<td></td>
<td>ii. BBB intact following implantation</td>
<td>ii. Drainage</td>
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<tr>
<td></td>
<td>iii. probe O.D. &lt;600μM</td>
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<tr>
<td></td>
<td>iv. Minute tissue trauma within first 2 days</td>
<td></td>
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<td></td>
<td>v. No need for deproteinization</td>
<td></td>
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<tr>
<td></td>
<td>vi. No enzymatic degradation</td>
<td></td>
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<tr>
<td></td>
<td>vii. Used in conscious animals</td>
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</tbody>
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2.3 Principles of in vivo microdialysis

2.3.1 Concentric microdialysis probe preparation

The concentric microdialysis probes were constructed following the method outlined below and consisted of a semi-permeable dialysis membrane, steel cannulae and fused silica tubing. The body of the probe was formed from a steel cannula (24g stainless steel, 15mm length, Tomlinson Tube and Instrument Ltd., Warwick) through which two lengths of fine fused silica tubing (Scientific Glass Engineering, Milton Keynes) were inserted. The piece of tubing for the probe inlet emerged from the opposite (bottom) end of the cannula, whilst the tubing for the outlet was fed approximately half way into the cannula. This left 5mm for the inlet and 10mm for the outlet at the top end of the cannula. A small drop of adhesive (Araldite rapid epoxy resin, Ciba-Geigy Plastics Ltd.) was used to secure the two lengths of silica to the top end of the cannula and allowed to dry for 24 hours.

In order to strengthen the silica, steel cannulae (27g stainless steel, 8mm length, Tomlinson Tube and Instrument Ltd., Warwick) were fitted over the inlet and outlet silica at the top end of the 24g cannula and secured with adhesive. The inlet tube was marked with permanent marker to distinguish it from the outlet. Once the adhesive had dried, two pieces of non-sterile, fine-bore polythene tubing (pp10 tubing, 0.28mm I.D., 0.61mm O.D.; Portex Ltd., U.K.) were fitted to form the inlet (50mm) and outlet (75mm) of the probe. The junction between the cannulae and the polythene tubing was coated with adhesive and allowed to dry for 24 hours.

To complete the probe, the silica tube inlet at the bottom end of the 24g cannula was trimmed to 3mm and a hollow fibre dialysis membrane (10KD molecular weight cut-off point; Cuprophan capillary membrane, type F1 8 200, 0.2mm O.D., GFE9; Gambro, Hechingen, Germany) was fed over the trimmed silica. The dialysis membrane was trimmed so that it extended 1mm beyond the end of the silica, resulting in an active membrane length of 4mm. A drop of adhesive was used to secure the dialysis membrane to the inner circumference of the 24g cannula and the cut end of the membrane was carefully sealed in the same way. A schematic diagram of the finished probe is shown in Figure 2.1.
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Fig. 2.1 Schematic representation of a concentric microdialysis probe.
Arrows depict direction of flow.
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After allowing the adhesive to dry for 24 hours, the probes were tested for patency by gently perfusing deionised water through at a flow rate of 0.8μL/min using a model 22 infusion pump (Harvard Apparatus, U.S.A.) and 500μl gas tight syringes (Hamilton, Australia). Probes that showed high resistance or 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 and used within 7 days of construction in an attempt to prevent the dialysis membrane from drying out prior to implantation. For all experiments, very low internal volume tubing (1.2μl/100mm, FEP tubing; Carnegie Medicine, Sweden) was connected to the outlet tube in order to ensure rapid transfer of the perfusate to the collecting vial. This allowed a more accurate temporal correlation between the collection of substances at the membrane/tissue interface and their appearance in the collecting vial, which is especially important in the study of monoamines, as they are prone to oxidation.

2.3.2 In vitro recovery of constructed microdialysis probes

The concentrations of substances in the perfusate from the dialysis probe are only a reflection of the true extracellular brain concentrations. It is therefore important to ‘calibrate’ the microdialysis probe before its use in vivo. A number of factors can affect the recovery including the length of the dialysis membrane, the flow of the perfusion liquid, the speed of diffusion of the substance through extracellular fluid and properties of the dialysis membrane (Ungerstedt 1991). There are two types of recovery: Relative and Absolute. Relative recovery is defined as the concentration of a substance in the perfusate when it leaves the probe expressed as a per cent value of the concentration in the surrounding medium. Absolute recovery is the total amount of a substance recovered during a defined period of time, expressed in moles/litre. In vivo the relative recovery remains constant as long as the perfusion conditions remain the same, however absolute recovery will vary depending on the production or release of the substance of interest (Ungerstedt 1991).

In vitro recovery experiments were carried out in order to obtain an estimation of the extracellular concentration of monoamines and amino acids from the microdialysis experiments. The probe was connected to the syringe by a length of pp10 tubing and continuously perfused at a constant flow rate of 0.8μl/min. The perfusion fluid was
identical to the bathing medium with the exception of the substances of interest. For the
monoamines a 1μM solution of dopamine (DA), 5-hydroxytryptamine (5-HT) and
noradrenaline (NA) was made up in aCSF. L-cysteine (20mg/100ml) was added to limit
oxidation (Chai & Meltzer 1992). For the amino acid glutamate the bathing medium
consisted of a 1μM solution of glutamate (L-GLU) in aCSF. All monoamines and the
amino acid were obtained from Sigma, U.K. The probe was inserted into the bathing
medium and perfused for a minimum of 60min before samples were collected at fixed
intervals of 30min. The monoamine or amino acid concentration in the outflow was
then immediately determined using HPLC with electrochemical detection (HPLC-ED)
for the monoamines and HPLC with fluorometric detection (HPLC-FD) for L-GLU.

The relative recoveries for the monoamines and L-GLU were calculated using the
following relationships (reproduced from Benveniste 1989) and are shown in Table 2.2:

\[
\text{In vitro recovery} = \frac{C_{\text{out}}}{C_{\text{in}}}
\]

where \(C_{\text{out}}\) is the substance concentration in the outflow and \(C_{\text{in}}\) is the substance
concentration in the bathing medium.

The same procedure is then performed \textit{in vivo} and the extracellular concentration of the
substance can be calculated as:

\[
C_1 = \frac{C_{\text{outflow}}}{\text{In vitro recovery}}
\]

where \(C_1\) is the true extracellular concentration and \(C_{\text{outflow}}\) is the concentration of the
substance in the \textit{in vivo} outflow solution.

The validity of these relationships assumes that conditions \textit{in vitro} are equal to those \textit{in
vivo}. As this is obviously not the case we must assume the conditions to be
approximately equal to allow us to make estimates of actual brain extracellular levels
from subsequent microdialysis experiments.
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Table 2.2 In vitro recovery of monoamines and L-GLU from standard solutions.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% In vitro recovery (mean ± s.e.m., n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>25.7 ± 7.1</td>
</tr>
<tr>
<td>DA</td>
<td>40.8 ± 4.0</td>
</tr>
<tr>
<td>NA</td>
<td>37.14 ± 2.07</td>
</tr>
<tr>
<td>L-GLU</td>
<td>37.2 ± 2.0</td>
</tr>
</tbody>
</table>

2.3.3 Stereotaxic surgery and probe implantation

For implantation of the microdialysis probe rats were anaesthetised with Isoflurane (5% v/v in O₂ for induction and 2% v/v in O₂ for maintenance; Abbot Laboratories Ltd., Kent) and placed in a stereotaxic frame (David Kopf, U.S.A.). Anaesthesia was maintained during implantation via a mouthpiece fitted over the incisor bar. The incisor bar was then adjusted according to the weight of the animal (-3.3mm to 2.5mm) and the head of the animal was placed parallel to the frame base plate. Using a scalpel the skull surface was exposed by laterally reflecting two skin flaps and excess membranous material and blood were removed. Bregma was located visually and using stereotaxic co-ordinates from the atlas of Paxinos and Watson (1982), the raphé nuclei (RN) and frontal cortex (FC) were located. The co-ordinates for the RN were posterior 7.8mm, lateral 0.5mm from bregma and 8mm below dura and for the FC anterior 3.2mm, lateral 3mm from bregma and 4.5mm below dura. Once located, a dental drill fitted with a tungsten carbide burr tip (2mm) was used to drill an insertion point through the skull, exposing the dura. Two additional holes were drilled in the skull, one anterior and one posterior of bregma, and fitted with 2mm stainless steel grub screws (size 10B, Clerkenwell Screw Company, Holborn, London). These screws were used to anchor the probes in position with the dental acrylic applied later.

A microdialysis probe was prepared by perfusing deionised water at a constant flow rate of 0.8μl/min and then mounting it in a probe clip that was attached to the stereotaxic
frame by means of a bolt. 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 an attempt 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 initiation of dialysis. The probe tip was inserted into the hole over the FC and adjusted until contact with the dura was made. The probe was then gently lowered to the required depth using a graduated thumbwheel on the stereotaxic frame and secured using dental acrylic (Duralay inlay resin, Henry Schein Procare, U.K.). Once the acrylic had set the probe holder was removed and, if required, a second probe was implanted into the RN following the procedure outlined above. Dental acrylic was then used to secure the whole area and ensure durability. The inlet and outlet of the probes were temporarily sealed with a drop of dental acrylic to prevent the formation of air blocks within the probe.

When the acrylic had completely set, the animal was removed from the stereotaxic frame, given an injection of saline (0.9% s.c.) to prevent dehydration during recovery and wrapped in paper towels to prevent hypothermia. Animals were then placed in individual, purpose-built Perspex cages and allowed food and water ad libitum.

A minimum postoperative recovery period of 19 hours was allowed prior to the start of microdialysis experiments. This has been proved to be essential as several studies have raised concerns about the time between probe implantation and the start of experimental sampling. Acute implantation (~2 hours) is associated with destabilisation of 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 (Major et al. 1990, Allen et al. 1992, Morgan et al. 1996). Therefore the implanted probes often recover excessive quantities of analytes that do not reflect true extracellular basal levels. Westerink and De Vries (1988) have shown that following acute implantation the source of dialysate neurotransmitters was damaged nerve terminals as opposed to steady-state release from neuronal stores. However chronic implantation (~18 hours+) sees these parameters return towards normal, basal levels. At this time the release of 5-HT and DA is almost totally abolished (>90%) by the Na$^+$ channel blocker tetrodotoxin (Whitton et al. 1990).
1994b), an effect which is less pronounced in amino acids (Biggs et al. 1995), and therefore suggests that normal tissue neurochemistry has been restored. It should also be noted that longer periods of implantation also have their limitations and therefore carrying out the experiments the day after implantation seems to be the optimum for accurate measurement of extracellular transmitter release.

2.3.4 General protocol for *in vivo* microdialysis experiments

The protocol outlined here was common to all microdialysis experiments carried out in the course of this study. Microdialysis experiments were carried out using freely moving rats the day after probe implantation. A gas-tight syringe was filled with aCSF (2.5mM KCl, 125mM NaCl, 1.18mM MgCl₂·6H₂O, 1.26mM CaCl₂; Sigma, U.K.) and the needle connected to a piece of pp10 tubing and flushed through. Using a short piece of wider bore tubing as a connector the syringe was connected to the probe inlet and the syringe placed on the syringe infusion pump set at a constant flow rate of 0.8μl/min. Once the probe was found to be working a piece of FEP tubing (~20cm) was connected to the probe outlet and the system was flushed with aCSF. Throughout the experiment the flow rate was maintained at 0.8μl/min. An equilibration period of 60min was allowed in order to eliminate abnormally high levels of neurotransmitters which accumulate in the vicinity of the dialysis membrane following implantation: this pre-dialysate was discarded. Further dialysate samples were collected in 30min fractions and the first four consecutive samples were used to establish basal extracellular levels of monoamines and L-GLU. At this point various drugs were either infused by one of the dialysis probes or injected (i.p.) and dialysates collected for up to a total of 360min. At the end of the experiment rats were sacrificed using chloral hydrate (500mg/kg i.p.; Sigma, U.K.) followed by cervical dislocation.

2.3.5 Anatomical verification of probe placement

Following the completion of each microdialysis experiment, the location of the probe in the brain was verified. Once the rat had been sacrificed the brain was quickly removed, frozen over dry ice and stored at −80°C prior to sectioning. Coronal brain sections (10μm) were cut around the areas of interest using a refrigerated cryostat (-20°C, CM3050, Leica, Germany) and mounted on microscope slides (Superfrost plus
406/0179/00; BDH, U.K.). Prior to staining the dry sections were fixed in ice-cold paraformaldehyde (4%; Sigma, U.K.) for 5 min and then transferred into phosphate buffered saline (Sigma, U.K.). Staining of the sections was carried out using the protocol below, adapted from Wisden & Morris 1994.

1. Rehydrate Tap water Wash
2. Staining Ehrlich's Haematoxylin (BDH, U.K.) 10 min
3. Differentiate Acid-Alcohol (1% HCl in 70% Ethanol) 15-30 sec
4. Hydrate Tap water Wash
5. Staining Eosin Aqueous (BDH, U.K.) 5 min
6. Hydrate Tap water Wash

The stained sections remained in the final hydrate wash until the mounting medium (glycerol jelly; BDH, U.K.) was applied and coverslips (24x60 mm, no.1; BDH, U.K.) put on. Inspection of the probe placement was made by visualisation of the probe tract using a microscope (Eclipse E600, Nikon, Japan). Typical probe placement for the FC can be seen in Fig 2.2 and for the RN in Fig 2.3 and was verified using a stereotaxic atlas of the rat brain (Paxinos & Watson 1982).

2.4 High performance liquid chromatography

Dialysates obtained from in vitro recovery and in vivo microdialysis experiments were analysed for monoamine and amino acid content. No pre-analysis treatments, such as acidification or deproteinisation, were required for analysis of monoamines. However in order to visualise L-GLU, a fluorescent moiety was conjugated to the amino acid. As a general procedure dialysates were loaded onto the appropriate HPLC system on the day of collection or stored at -80°C for a period not exceeding one week. All chemicals used in the preparation of HPLC reagents were obtained from Fluka, Switzerland, unless otherwise stated.

2.4.1 HPLC-ED

Two HPLC systems were used for the detection and quantification of monoamines. The first, used to detect 5-HT and DA, consisted of the following components: Bischoff
Fig 2.2 Photomicrograph illustrating typical probe placement in the FC.
The arrow indicates the entry tract of the probe into the FC, with the dialysis membrane of the probe extending 4mm below this (magnification X20).

Fig 2.3 Photomicrograph illustrating typical probe placement alongside the RN.
The arrow indicates the entry tract of the probe alongside the RN, with the dialysis membrane of the probe extending 4mm below this (magnification X20).
compact HPLC pump (model 2250; Bischoff Analysentechnik und -geräte GmbH), Triathlon refrigerated autosampler (4°C; Spark-Holland, Netherlands), C18 reverse phase column maintained at 40°C (ODS 3μM, 4.6mm I.D. x 100mm; Rainin Dynamax Instrument Co. INC., U.S.A.) protected by a microsorb guard column (C18 5μM, 4.6mm I.D. x 15mm; Rainin Dynamax Instrument Co. INC., U.S.A.) and a Antec-Intro electrochemical detector (Antec Leyden BV, Holland) fitted with a VTO3 flow cell ($V_{cell}$ +625mV filtered to 5abu with range set on 0.5nA/volt for a full scale deflection).

Data capture was achieved and analysed by a Dell Corporation PC system 310 (Dell Corporation, U.S.A.) equipped with Chromperfect for Windows software (Justice Innovations chromatography data systems, CA, U.S.A.).

The separation technique used was based on that of Hutson et al. (1989) with modifications to allow the concurrent detection of DA and its metabolites. All separations were isocratic and the mobile phase had the following composition: Sodium acetate (90mM), citric acid (35mM), EDTA (0.34mM), 1-octane-sulfonic acid (ion pairing reagent; 0.06mM), 5.5% methanol and pH was adjusted to 4.2 using citric acid. The mobile phase was de-gassed using an in-line degassing unit (Jour Research) and pumped at a flow rate of 0.650ml/min.

The second system used to detect NA, consisted of the following components: Severn analytical isocratic solvent delivery HPLC pump (model SA 6410B; Severn Analytical, Bedfordshire, U.K.), CMA 200 refrigerated autosampler (4°C; CMA, Sweden), C18 reverse phase column (MD-150, ODS 3μM, 1mm I.D. x 150mm; ESA, INC., U.S.A.) and a ESA electrochemical detector (model 5100A) with an amperometric porous carbon electrode analytical microdialysis cell (model 5014B; electrode one: -175mV, electrode two: +160mV with range set on 20nA/volt for a full scale deflection).

Data capture was achieved and analysed by a Dell Corporation PC system 310 (Dell Corporation, U.S.A.) equipped with Drew Laboratory Computing DS4000 software (Drew Scientific, U.K.).

All separations were isocratic and the mobile phase was purchased from ESA, INC. (MD-TM mobile phase) with the following composition: Sodium dihydrogen phosphate
monohydrate (75mM), Triethylamine (100μL/L), EDTA (25μM), 1-octane-sulfonic acid (1.7mM), pH was adjusted to 3.0 using phosphoric acid and 10% acetonitrile. The mobile phase was de-gassed using helium and pumped at a flow rate of 0.3ml/min.

2.4.1.1 Maintenance and calibration of HPLC-ED

At the end of a run the flow rate was reduced to 0.1ml/min and the electrochemical cells switched off. The mobile phase used in detecting NA was recirculated during the sample runs and was replaced every 2-3months as required. However for 5-HT and DA the mobile phase was not recirculated during sample runs and was therefore topped up when necessary. In both cases a minimum of 6hours was allowed for equilibration of the electrochemical cell after it was switched on and also when new mobile phase was introduced.

Both HPLC-ED's were regularly calibrated using standard solutions of monoamines. Stock solutions (5mM) containing 5-HT, DA and NA (Sigma, U.K.) were prepared in double-distilled, deionised water containing a few drops of 1mM HCL and stored in 1ml aliquots at -80°C. When required an aliquot was removed from the freezer, diluted in double-distilled, deionised water and stored in the fridge for use. Fresh standards were made up daily. Dilutions of this standard where injected into the HPLC-EC systems via an injection valve (Rheodyne, U.S.A.). Partial loop fill was employed as this ensures low sample loss and allowed good reproducibility. Sample HPLC traces can be seen in Fig 2.4 (5-HT and DA) and Fig 2.5 (NA) showing standard and dialysate traces. Typical calibration plots for each of the monoamine standards can be seen in Fig 2.6.

Monoamine peaks were quantified by measuring the peak area generated by the analysis software. The peaks were identified by the comparison of retention times with authentic monoamine standards.
Fig 2.4 Typical Chromatograms obtained following separation of monoamines with HPLC-ED.

A) shows a representative standard mixture and B) is a typical representation of basal monoamine output from the RN. The horizontal axis represents retention time in min. Peaks noted are 1) DA, 2) DOPAC, 3) 5-HT, 4) 5HIAA.
Fig 2.5 Typical Chromatograms obtained following separation of NA with HPLC-ED.
A) shows a representative standard mixture and B) is a typical representation of basal monoamine output from the FC. The horizontal axis represents retention time in min. Peak noted is 1) NA.
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5-HYDROXYTRYPTAMINE

DOPAMINE

GluTAMATE

Fig 2.6 Typical calibration plots obtained using HPLC.
5-HT, DA and NA are detected by electrochemical and L-GLU by fluorimetric detection. For details of chromatographic protocol refer to text.
2.4.2 HPLC-FD

The HPLC-FD consisted of the following components: BAS solvent delivery system with gradient manager (PM-80; Bioanalytical Systems INC., Indiana), CMA 200 refrigerated autosampler (9°C; CMA, Sweden), column (MF 6213 phase-II ODS 3µM, 3.2mm I.D. x 100mm; Bioanalytical Systems INC., Indiana) with a temperature controller (30.5°C, LC-22C, Bioanalytical Systems INC., Indiana) and a CMA 280 fluorescence, fixed wavelength detector (excitation: 350nm, emission: 495nm, amplification x20, filtering 0.3s; CMA, Sweden).

Data capture was achieved and analysed by a Dell Corporation PC system 310 (Dell Corporation, U.S.A.) equipped with Chromperfect for Windows software (Justice Innovations chromatography data systems, CA, U.S.A.).

The separation of amino-acid containing dialysates was carried out by a gradient, reverse phase HPLC system using the method of Lindroth & Mopper (1979). The gradient conditions meant that the mobile phase became progressively non-polar with retention time allowing fuller separation of amino acids from the dialysate. Prior to delivery to the HPLC-FD two mobile phase components were mixed: 100% methanol and mobile phase. The mobile phase consisted of Sodium dihydrogen phosphate dihydrate (50mM) adjusted to pH 5.5 using NaOH (1mM) and 20% methanol and was degassed using helium. The gradient manager controlled the proportions of mobile phase and methanol in the final mixture. Flow rate was constantly maintained at 0.5ml/min throughout the gradient.

2.4.2.1 Preparation of α-phthalaldehyde/thiol derivitisation reagent

To allow subsequent detection of dialysate L-GLU by HPLC-FD, the amino acid was conjugated to a fluorescent moiety by a non-enzymatic reaction. Pre-column derivitisation with α-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol was carried out under alkaline conditions (sodium tetraborate buffer, pH 9.5) resulting in an isoindole derivative sufficiently stable enough to undergo chromatographic separation (Fig 2.7). The derivitisation solution was prepared 24 hours prior to use. OPA (27mg) was dissolved in absolute ethanol (500µl) and sodium tetraborate buffer (5ml, 0.1M),
Fig 2.7 Reaction of L-GLU with OPA and 2-mercaptoethanol to form a fluorescent derivative for detection by HPLC-FD.

and mixed thoroughly. Mercaptoethanol (50µl) was added to the mixture to enable thiol reduction and the derivitisation solution was stored in the dark at 4°C for a maximum of two weeks.

2.4.2.2 Maintenance and calibration of HPLC-FD

At the end of a run the fluorimetric detector was switched off and the system was flushed with 100% methanol. This prevented crystallisation of mobile phase within the pumps once they were switched off and also removed any contamination from the column. On the day of a run a minimum of 6 hours was allowed for the progressive change from 100% methanol to 100% mobile phase in order to equilibrate the column and the fluorimetric detector.

The HPLC-FD was regularly calibrated using a standard solution of L-GLU. A stock solution (5mM) containing L-GLU (Sigma, U.K.) was prepared in double-distilled,
deionised water and stored in 1ml aliquots at -80°C. When required an aliquot was removed from the freezer, diluted in double-distilled, deionised water and stored in the fridge for use. Fresh standards were made up daily. Dilutions of this standard were injected into the HPLC-FD system via an injection valve (Rheodyne, U.S.A.) fitted with a 20μl loop. Partial loop filling was employed as this method has shown good reproducibility with low sample loss. Sample HPLC traces can be seen in Fig 2.8 showing standard and dialysate traces. A typical calibration plot for the L-GLU standard can be seen in Fig 2.6.

Amino acid peaks were quantified by measuring the peak area generated by the analysis software. The peaks were identified by the comparison of retention times with authentic amino acid standards.

2.5 Experimental drugs

Drugs used throughout this study are listed below along with the supplier:

CGP40116 Gift from Ciba-Geigy, U.K.
Reboxetine methanesulphonate Gift from Pharmacia, MI, U.S.A.
Ifenprodil Tartrate Research Biochemicals International (RBI), MA, U.S.A.
Amantadine Hydrochloride, Clomipramine Hydrochloride, D-Cycloserine, NMDA, Yohimbine Hydrochloride Sigma, U.K.

2.6 Statistical analysis

Data included in this study was expressed in one of two ways: acute data was expressed as the percentage of basal analyte levels while subchronic and chronic data were expressed as absolute values using the relationship shown in 2.3.2. Statistical comparisons were made between drug and aCSF-perfused control for each of the collection periods and also between two drug treatments. These temporal data series were analysed by means of analysis of variance (ANOVA) for repeated measures followed by Dunnett’s test to compare pre- and post-treatment periods as outlined by Adell & Artigas (1998). P<0.05 was considered to be statistically significant.
Fig 2.8 Typical Chromatograms obtained following separation of L-GLU with HPLC-FD.

A) shows a representative standard mixture and B) is a typical representation of basal amino acid output from the RN. The horizontal axis represents retention time in min. Peak noted is 1) L-GLU.
Chapter Three

Effects of NMDA antagonists on clomipramine-induced monoamine and amino acid release in the raphe nuclei and frontal cortex as measured by in vivo microdialysis in the freely moving rat
3.1 Introduction

For over 30 years the tricyclic antidepressant (TCA) clomipramine (CIM) has been recognised as an effective first-line treatment of depression (Kornhaber & Horwitz 1984, Lemoine et al. 1981, Moron et al. 1988). It is a unique TCA in that it allows more aggressive dosing, faster onset of action (Perel et al. 1995, Pollock et al. 1989), and exhibits a broader range of therapeutic properties than other drugs of the same class (Browne et al. 1993, McTavish & Benfield 1990, Sunblad et al. 1993). Studies have shown CIM to be particularly effective in the management of treatment-resistant depression (Drago et al. 1983, Dudley et al. 1980, Kielholz 1986) and obsessive-compulsive disorder (McDougle et al. 1993).

The TCAs share the pharmacological characteristic of inhibition of 5-HT and NA reuptake, resulting in increased levels of the neurotransmitter at postsynaptic receptors. The relative potencies with respect to inhibition of neurotransmitter reuptake vary, but the tertiary amine CIM (Potter et al. 1995) is the most potent inhibitor of 5-HT reuptake within this drug class (Balant-Gorgia et al. 1991, McTavish & Benfield 1990, Tulloch 1993). This property may explain its success in the treatment of obsessive-compulsive disorder (McDougle et al. 1993). CIM has also been shown to modulate levels of DA in both the RN and FC (Pallotta et al. 1999a,b).

The effects of CIM have been studied both in vitro and in vivo. In receptor binding studies CIM has been shown to be a potent inhibitor of 5-HT reuptake, with a similar level of inhibition of NA observed as with other antidepressants (McTavish & Benfield 1990). However, CIM is biotransformed to the secondary metabolite desmethylclomipramine (DCIM) (Fujita et al. 1991) and this has been shown to be a more potent inhibitor of NA reuptake than its parent compound (Benfield 1980, Thomas & Jones 1977). Increased inhibition of NA from the metabolic formation and possible accumulation of DCIM is believed to significantly contribute to the pharmacological profile of CIM (McTavish & Benfield 1990, Maj et al. 1982). CIM has also been shown to have a relatively high affinity for central dopamine-D2, histamine-H1, and α1-adrenergic receptors. Both CIM and its metabolite exert a potent anticholinergic effect in vivo and in vitro, which may explain the side effect profile associated with TCAs (see 1.4.1.2 and table 1.3). In vivo administration of CIM markedly reduces levels of 5-HT in
Chapter Three: Clomipramine, NMDA antagonists and neurotransmitter release

platelets and of its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the cerebrospinal fluid of depressed patients (McTavish & Benfield 1990).

All antidepressants are highly lipophillic compounds that undergo biotransformation in the liver to produce progressively more polar metabolites that are readily excreted by the kidneys (Potter 1995, Rudorfer & Potter 1987). CIM is extensively metabolised with only 1-3% of the dose being excreted unchanged. The major metabolic pathway forms DCIM by N-demethylation, but 8-hydroxylation (to produce 8-hydroxy-clomipramine) and N-oxidation are also important (see Fig 3.1).

![Figure 3.1 The major metabolic pathways of clomipramine](image)

Figure 3.1 The major metabolic pathways of clomipramine
Acute and chronic CIM dosing has been shown to result in changes to the extracellular levels of 5-HT and DA in the RN and FC of freely moving rats. Acute CIM (10-20mg/kg, i.p.) results in decreased extracellular levels of DA in both regions (Pallotta et al. 1999a,b) and increased extracellular 5-HT in the RN with a slight, but not significant, decrease in the FC (Pallotta et al. 2001). Chronic (15day, 10-20mg/kg/day, i.p.) dosing was shown to dose-dependently increase the basal levels of both 5-HT and DA in both regions (Pallotta et al. 1999a,b, Pallotta et al. 2001). It is also worth noting that chronic (28day, 10mg/kg/day) CIM has been shown to alter presynaptic 5-HT1B receptor sensitivity in rat hypothalamus and hippocampus respectively (Newman et al, 2000). Taken together with experiments involving the cerebral cortex (Gur et al. 1999b), these in vivo studies suggest that chronic CIM exerts effects both pre- and post-synaptically, but also that the effects are highly regional-specific (Newman et al, 2000).

NMDA receptors are believed to modulate the release of a number of neurotransmitters. Activation of NMDA receptors results in the release of GLU and monoamines in many brain regions including the nucleus accumbens (Imperato et al. 1990), prefrontal cortex (Karremann & Moghaddam 1990), striatum (Carter et al. 1988, Young & Bradford 1991), RN (Pallotta et al. 1998, Pallotta et al. 1999a,b) and hippocampus (Whitton et al. 1994a,b). The 5-HT response to NMDA has been shown to be biphasic, with lower doses (25μM) resulting in decreased extracellular 5-HT in the RN and higher doses (100μM) increasing 5-HT in this region (Pallotta et al. 1998, Tao & Auerbach 1996). The converse effects are observed in the FC (Pallotta et al. 1998, Tao & Auerbach 1996), suggesting that the degree of NMDA receptor activation results in dramatically different outcomes in terms of serotonergic transmission to the FC. The effects of NMDA (25-100μM) on DA release are more straightforward, a concentration-dependent decrease is observed in both the RN and FC (Pallotta et al. 1999a,b, Smith & Whitton 2001). Infusion of NMDA into the striatum, for example, has been shown to promote both DA and GLU release (Carter et al. 1988, Whitton et al. 1994a), whilst systemic administration of the non-competitive NMDA receptor antagonist MK801 leads to a reduction in the extracellular levels of both neurotransmitters. However similar studies in the hippocampus have shown an elevation of GLU levels accompanied by an inhibition of DA release in response to NMDA infusion (Whitton et al. 1994a). This suggests that GLU may have the ability to modulate DA release depending on the brain area under investigation.
Chapter Three: Clomipramine, NMDA antagonists and neurotransmitter release

Acute CIM (10-20mg/kg, i.p.) had no effect on NMDA-evoked transmitter release in either the FC or RN. However, following chronic (15day, 10-20mg/kg/day, i.p.) CIM administration, NMDA-evoked decreases in DA release in the RN (Pallotta et al. 1999a) and FC (Pallotta et al. 1999b) were reversed. Similar changes were seen with 5-HT (Pallotta et al. 2001). This suggests that adaptive changes must occur in NMDA receptor function during treatment with CIM.

3.1.1 NMDA antagonists

As outlined in 1.8.5, a number of compounds can act as antagonists at the NMDA receptor. For this study one representative from each classification was used, with the exception of ifenprodil all have been previously shown to display some antidepressant properties (Huber et al. 1999, Moryl et al. 1993, Papp & Moryl 1994, 1996).

1) Competitive antagonists:

Act at the GLU recognition site e.g. (R)-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP40116). They have been observed to have a number of behavioural effects (Schmidt 1994) and when given systemically or by local injection into the striatum or nucleus accumbens, competitive NMDA antagonists show psychomotor stimulating effects (Schmidt 1994). CGP40116 contains the structure of the competitive antagonist D-AP5, a highly polar substance that is poorly absorbed by brain tissue. Therefore the objective in developing new competitive NMDA receptor antagonists has been to make structural alterations that increase NMDA receptor affinity and improve penetration into brain tissue. CGP40116 was the result of the unsaturation of the interacidic side chain (see fig 3.2).

A review of the current literature showed that the dose of CGP40116 administered i.p ranged from 0.5mg/kg (Bienkowski et al. 1997) to 5mg/kg (Fisher et al. 1998, Fisher & Starr 2000, Wedzony et al. 1996, Wlaz et al. 1999). Behavioural studies performed in our department have shown that higher doses of CGP40116 markedly reduce the responsiveness of rats when scoring for locomotor activity and exploratory behaviour (A.Fisher, Personal communication). Therefore, based on this information and the data of Biggs et al. (1996), a dose of 1mg/kg i.p. was deemed to
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Figure 3.2 Structures of NMDA antagonists
(Reproduced from \textsuperscript{1}Jane \textit{et al}. 1994, \textsuperscript{2}Kornhuber \textit{et al}. 1991, \textsuperscript{3}Scatton \textit{et al}. 1987)
be appropriate for this study. A higher dose would be neither pharmacologically specific in its action nor clinically relevant and therefore it would be impossible to relate any observations purely to the NMDA antagonistic action of CGP40116.

2) Non-competitive antagonists:
These compounds act at sites other than the GLU recognition site and can be further divided into channel blockers, polyamine site antagonists and GLY site antagonists. They are known to exhibit strong psychomotor stimulating effects and to elicit rewarding effects, unlike competitive antagonists (Schmidt 1994). Maze tests sensitive for hippocampal learning also showed that non-competitive antagonists produced learning difficulties over the whole dose range tested (Schmidt 1994).

a) Ion channel blocker e.g. amantadine
Several distinct classes of compounds have been shown to interact with the MK801 binding site of the NMDA receptor-gated ion channel (Collingridge & Lester 1989) and the 1-amino-adamantane derivative amantadine (see fig 3.2) has been shown to possess this type of activity (Stoof et al. 1992). Amantadine has been shown to possess a number of properties including functioning as a drug that releases catecholamines (Grelak et al. 1970), inhibits dopamine reuptake (Heikkila & Cohen 1972), stimulates dopamine reuptake (Allen 1981, Gianutsos et al. 1985), alters the conformation of the dopamine receptor (Gianutsos et al. 1985) or has anticholinergic properties (Nastuck et al. 1976). Originally developed as a treatment for influenza, its use in the treatment of Parkinson's disease has been well documented (Danysz et al. 1997, Dalvi & Ford 1998).

Again after a review of the literature (Danysz et al. 1997, Fisher et al. 1998, Moryl et al. 1993) and based on studies carried out in the department (A. Fisher, Personal Communication) it was decided that a dose of 40mg/kg i.p. was appropriate for this study.

b) GLY site antagonists e.g. D-cycloserine
The strychinidine-insensitive modulatory GLY site has been shown to be essential for the activation of the NMDA receptor ion channel (Johnson & Ascher 1987),
where it is believed to modulate NMDA function through an allosteric mechanism. D-cycloserine is a D-serine analogue (see figure 3.2) that has been found to have antagonist properties at this site (Emmett et al. 1991, Henderson et al. 1990, Hood et al. 1989). D-serine is known to act as an endogenous agonist for glycine\textsubscript{B} receptors (Chouinard et al. 1993, Hashimoto et al. 1993, Kumashiro et al. 1995, Schnell et al. 1997) and D-cycloserine is a systemically active partial agonist at this site (Parsons et al. 1998). This property may be important in preserving a certain level of NMDA receptor function, even at high concentrations (Parsons et al. 1998). D-cycloserine has been demonstrated to show different levels of intrinsic activity at different NMDA receptor subtypes expressed in *Xenopus* oocytes (O'Connor et al. 1996). Receptor subtype selectivity may cause glycine\textsubscript{B} full antagonists to block NMDA receptor function in a similar manner to partial agonists on cells expressing heterologous populations of NMDA receptors (Parsons et al. 1998). Studies have shown that D-cycloserine displays cognitive enhancing properties in animals (Herberg & Rose 1989, Monahan et al. 1989, Thompson et al. 1992).

From the results of the study by Papp & Moryl (1996) it was decided that a dose of 10mg/kg i.p. was appropriate for this study.

c) Polyamine site antagonists e.g. ifenprodil

The profile of these non-competitive NMDA receptor antagonists does not allow them to be classified as NMDA channel blockers or as antagonists at the GLY modulatory site. Ifenprodil (see figure 3.2) was developed as a commercial antihypertensive agent and possesses potent activity at several brain receptors, including NMDA and \(\alpha_1\) adrenergic receptors (Chenard et al. 1991, Karbon et al. 1990). Although the compound was reported to be a structurally unique NMDA receptor antagonist (Carter et al. 1988, Scatton et al. 1987, Shalaby et al. 1992), other models known to be sensitive to NMDA receptor inhibition showed ifenprodil to be only weakly effective (Chenard et al. 1999). However the molecular cloning and expression of NMDA receptor subunits (Monyer et al. 1992, Nakanishi 1992) allowed the demonstration that ifenprodil was >100-fold selective for receptors containing the NR2B subunit compared to those containing the NR2A subunit (Williams 1993).
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A review of the literature showed that 0.9mg.kg is the ID$_{50}$ value which antagonises the stimulatory effect of intrastriatally dialysed NMDA on rat striatal DA release (Carter et al. 1988), therefore this dose was used in the following study.

The following questions were addressed:

1) Do NMDA receptor antagonists modulate the release of 5-HT, DA and GLU in the RN and FC of freely moving rats?

2) Does acute or sub-chronic treatment with an NMDA receptor antagonist effect CIM-induced release of 5-HT, DA or GLU in the RN and FC?

3) Are the effects restricted to one subtype of NMDA receptor antagonist?
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3.2 Results

A summary of the results is presented in table 3.2 prior to the discussion.

3.2.1 Basal levels of 5-HT, DA and GLU measured in frontal cortex and raphe nuclei dialysates

Basal levels of extracellular 5-HT, DA and GLU were derived from the first four samples collected in each dialysis experiment. Basal levels are expressed as mean ± s.e.mean, n=70 and are shown in table 3.1 below. A review of the literature showed these values to be within the acceptable range (Whitton et al. 1992a, b) and similar to those previously obtained in our laboratory. In the results, data are expressed as percentage of basal for acute studies and as absolute values for chronic studies.

Table 3.1 Basal extracellular levels in frontal cortex and raphe nuclei dialysates

<table>
<thead>
<tr>
<th></th>
<th>Frontal Cortex</th>
<th>Raphe Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>19 ± 3 fmol/10μl</td>
<td>30 ± 5 fmol/10μl</td>
</tr>
<tr>
<td>DA</td>
<td>88 ± 6.3 fmol/10μl</td>
<td>56 ± 6.3 fmol/10μl</td>
</tr>
<tr>
<td>GLU</td>
<td>15 ± 3 pmol/10μl</td>
<td>8 ± 2 pmol/10μl</td>
</tr>
</tbody>
</table>

3.2.2 Effect of clomipramine on the release of 5-HT, DA and GLU in the frontal cortex

The injection of CIM elicited a dose-dependent decrease in the extracellular levels of both 5-HT and DA (see fig 3.3 A, B). A decrease in GLU efflux was also observed in response to 20mg/kg i.p. (see fig 3.3 C).

The effect of 20mg/kg CIM on 5-HT was fairly rapid in onset – extracellular levels were decreased 30mins post-injection and reached a minimum of 57 ± 5% of basal 120mins after the injection. The effect persisted for 240mins before returning to basal
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Figure 3.3 The effect of clomipramine on extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex

The arrow indicates the time of the i.p. injection of clomipramine. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.0001).

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levels at the end of the experiment. 10mg/kg CIM was observed to have a similar but lesser effect, with a minimum of 87 ± 10% recorded before levels returned to basal.

CIM was observed to have a similar effect on DA levels, in terms of both magnitude and time. A minimum of 45 ± 15% of basal levels was observed with 20mg/kg CIM.

GLU efflux was observed to decrease in response to CIM, although the onset was delayed until 150mins post-injection, possibly due to the metabolism of CIM. Levels of GLU were observed to fall to 29 ± 10% and remained low for the duration of the experiment.

3.2.3 Effect of clomipramine on the release of 5-HT, DA and GLU in the raphe nuclei

A dose-dependent effect of CIM was observed in the raphe nuclei for both 5-HT and DA. However the effects were not the same, 5-HT levels were seen to increase whilst those of DA decreased (see figure 3.4 A, B). Figure 3.4 C shows that a dose of 20mg/kg of CIM did not significantly alter the levels of GLU in the extracellular space.

CIM caused a dose-dependent increase in extracellular levels of 5-HT which was both rapid in onset and persistent (figure 3.4 A). At both doses the increase peaked 120mins post-injection (290 ± 25% at 20mg/kg, 220 ± 15% at 10mg/kg). Levels of 5-HT were observed to remain significantly high throughout the experiment, although the trend suggested that levels would return to basal with time.

The effect of CIM on DA was a rapid and persistent decrease in efflux. At 10mg/kg, levels of DA fell to 63 ± 10% of basal, but were observed to return to basal levels by the end of the experiment. However the higher dose of CIM resulted in a decrease of greater magnitude (40 ± 15%) which persisted until the end of the experiment.
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A)

![Graph A](image)

B)

![Graph B](image)

C)

![Graph C](image)

Figure 3.4 The effect of clomipramine on extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei

The arrow indicates the time of the i.p. injection of clomipramine. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control (p<0.05).
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3.2.4 Effect of NMDA infusion into the raphe nuclei on 5-HT, DA and GLU release in the frontal cortex

The 30min infusion of two different concentrations of NMDA into the RN was observed to have a biphasic effect on both 5-HT and DA levels in the FC (see figure 3.5 A, B). Extracellular GLU levels were shown to be decreased by both concentrations (see figure 3.5 C).

NMDA at 25μM was observed to cause a rapid, statistically significant increase (p<0.05) in extracellular 5-HT levels to 170 ± 15% of basal. However at 100μM NMDA, the opposite effect was observed: levels of 5-HT rapidly fell to 35 ± 5% of basal. In both cases the effect lasted between 120 and 180mins, with levels returning to basal by the end of the experiment.

The effect of NMDA on DA release was also shown to be biphasic in this region. However the higher concentration of NMDA elicited an increase in DA (206 ± 29%), whilst 25μM NMDA infusion decreased DA levels to 25 ± 5% of basal. The time period of the effect was similar to that observed for 5-HT and levels of DA were observed to have returned to basal by the end of the experiment.

FC GLU release was decreased by the infusion of NMDA into the RN. It is interesting to note that the lower concentration of NMDA (25μM) elicited a greater and more consistent decrease (27 ± 3%), than that observed with 100μM (49 ± 22%). The onset of the effect was delayed until 120mins after infusion in both cases and was maintained until the end of the experiment (figure 3.5 C).

Previous studies carried out in our laboratory have shown that these effects of NMDA are specific for amino acids and monoamines considered to be neurotransmitters (J.Segieth, Personal Communication). For example, the levels of the amino acid serine in the extracellular space are unaltered by the infusion of NMDA.
Figure 3.5 The effect of NMDA infusion into the raphe nuclei, via the dialysis probe, on extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different from control (p<0.05) and *** indicates data points significantly different from control (p<0.001).
3.2.5 Effect of NMDA infusion into the raphe nuclei on 5-HT, DA and GLU release in the raphe nuclei

The 30min infusion of two different concentrations of NMDA into the RN was observed to have a biphasic effect on extracellular levels of 5-HT and GLU (see figure 3.6 A, C). Levels of DA in the RN were observed to be decreased by infusion of NMDA in a concentration-dependent manner (see figure 3.6 B).

In the RN, 5-HT levels fell following the 30min infusion of 25μM NMDA: the opposite effect to that observed in the FC. The effect was rapid in onset and again persisted for 180mins before returning to basal by the end of the experiment. 5-HT levels were increased significantly (p<0.05) to 427 ± 55% in response to the infusion of 100μM NMDA but had also returned to basal by the end of the experiment.

The effect of NMDA on RN DA levels was shown to be a concentration-dependent decrease. Levels were decreased to 24 ± 8% after 100μM NMDA and similarly to 20 ± 5% after 25μM NMDA. However the onset of the effect was quicker at the higher NMDA concentration. At both concentrations the trend suggested a return to basal, although this did not occur during the period of the experiment.

There was a brief, but significant (p<0.01), increase in RN GLU levels following the 30min infusion of 100μM NMDA. However this was rapidly reversed and the overall effect was a decrease in GLU levels: although the effect was similar in the FC (see figure 3.5 C), the magnitude was greater in the RN (46 ± 10%). The effect of 100μM NMDA persisted until the end of the experiment. Extracellular GLU levels were significantly and rapidly increased (p<0.0001) in response to 25μM NMDA. This was the opposite effect to that observed in the FC with levels peaking at 159 ± 42% before returning to basal at the end of the experiment.
Figure 3.6 The effect of NMDA infusion into the raphe nuclei, via the dialysis probe, on extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei. The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different from control (p<0.0001).
3.2.6 The effect of NMDA infusion into the raphe nuclei on the clomipramine-induced changes in 5-HT and DA levels in the frontal cortex

A 30min infusion of 25μM NMDA into the RN 30mins after an i.p. injection of CIM was observed to cause an increase in 5-HT and a decrease in DA levels in the FC (see figure 3.7 A, B).

The infusion of NMDA into the RN reversed the CIM-induced effects of 5-HT release in the FC. At both 10 and 20mg/kg CIM, a substantial and significant increase was observed, peaking at 500 ± 20% and 587 ± 25% of basal respectively. The effect was sustained for the duration of the effect of the NMDA, and levels returned towards basal by the end of the experiment (figure 3.7 A).

The CIM-induced decrease in DA levels in the FC was potentiated by the infusion of NMDA into the RN. Levels fell to 27 ± 5% of basal (20mg/kg CIM + NMDA, compared to 45 ± 15% with 20mg/kg CIM alone, see fig 3.3 B) and the effect was sustained for the duration of the effect of the NMDA. As with 5-HT the levels of DA returned towards basal by the end of the experiment (figure 3.7 B).

3.2.7 The effect of NMDA infusion on the clomipramine-induced changes in 5-HT and DA levels in the raphe nuclei

NMDA infusion into the RN results in a reversal of the CIM-induced increase in 5-HT in the RN, whilst the decrease in DA is potentiated (figure 3.8 A, B).

The infusion of 25μM NMDA into the RN reversed the observed CIM-induced increase in 5-HT levels (figure 3.8 A). Levels fell to 48 ± 10% of basal by the end of the experiment, mirroring the effect of NMDA alone in both duration and effect.

The decrease in RN DA in response to CIM seemed to be potentiated by the infusion of NMDA into the RN (figure 3.8 B), although the overall effect was the same. As with 5-HT, the effect of NMDA on CIM-induced changes in DA mirrored those seen with NMDA alone.
Figure 3.7 The effect of NMDA infusion into the raphe nuclei, via the dialysis probe, on clomipramine-induced changes in the extracellular levels of A) 5-HT and B) DA in the frontal cortex

The arrow indicates the time of the i.p. injection of clomipramine and the bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control (p<0.05).
Figure 3.8 The effect of NMDA infusion into the raphe nuclei, via the dialysis probe, on clomipramine-induced changes in the extracellular levels of A) 5-HT and B) DA in the raphe nuclei

The arrow indicates the time of the i.p. injection of clomipramine and the bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control (p<0.05).
3.2.8 The effect of CGP40116 on the clomipramine-induced changes in 5-HT, DA and GLU levels in the frontal cortex

The administration of CGP40116 was observed to cause a decrease in the extracellular levels of 5-HT, DA and GLU in the FC (see figure 3.9 A, B, C).

The effect of CGP40116 on 5-HT release in the FC only reached statistical significance towards the end of the experiment (figure 3.9 A). However a clear downward trend can be clearly identified immediately following CGP40116 injection, with levels falling to 45 ± 12% by the end of the experiment. The effect of CGP40116 was shown to be similar to that observed with CIM in terms of both magnitude and duration. CIM-induced 5-HT release was significantly decreased following CGP40116 administration in comparison to both basal and CIM-induced levels. The onset was rapid and maintained for the duration of the experiment. The overall decrease in 5-HT was greater and more persistent following CIM + CGP40116 treatment than following CGP40116 alone.

Administration of either CIM or CGP40116 resulted in a decrease in DA levels of similar magnitude and duration (figure 3.9 B). Levels fell to 45 ± 15% of basal following CIM treatment and remained low throughout the experiment. The CIM-induced decrease in extracellular DA was unaffected by the administration of CGP40116 and was observed to mirror the effects of CIM and CGP40116 alone in both duration and magnitude.

CGP40116 was observed to elicit a decrease in FC GLU that was more rapid than that observed with CIM alone (figure 3.9 C). By the end of the experiment the level of GLU following the administration of CGP40116 was 60 ± 25% of basal, which was not significantly different from that observed with CIM alone. Interestingly the administration of CGP40116 was shown to abolish the CIM-induced decrease in extracellular GLU, resulting in no significant change from basal levels (figure 3.9 C).
Figure 3.9 The effect of CGP40116 on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex

The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the CGP40116 injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different from control (p<0.001).
3.2.9 The effect of CGP40116 on the clomipramine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

In the RN, CGP40116 was observed to decrease both 5-HT and GLU levels and to have no significant effect on DA release (see figure 3.10 A, B, C).

CGP40116 alone significantly decreased extracellular 5-HT levels to 10 ± 35% of basal levels. A rapid onset of action was demonstrated and levels remained low for the duration of the experiment (figure 3.10 A). The CIM-induced increase in 5-HT was unaffected by CGP40116 administration and the observed effect was similar in terms of magnitude and duration.

DA levels were unaffected by CGP40116, which also had no significant effect on the CIM-induced decrease (figure 3.10 B). As with 5-HT, the observed effect was of similar magnitude and duration to that observed with CIM alone.

CGP40116 did not show a significant effect on RN GLU release, however a downward trend can be clearly recognised (figure 3.10 C). CIM has been previously shown to have no effect on GLU in this region (figure 3.4 C), so it was interesting to observe that the effect of CGP40116 on CIM-induced GLU release was a significant decrease that persisted to the end of the experiment.

3.2.10 The effect of amantadine on the clomipramine-induced changes in 5-HT, DA and GLU levels in the frontal cortex

Amantadine was observed to have no significant effect on 5-HT release but increased DA and decreased GLU release in the FC when administered alone (see figure 3.11 A, B, C).

Although amantadine did not affect 5-HT levels in the FC when administered alone, it was observed to reverse the CIM-induced changes. Levels of 5-HT were significantly increased to 211 ± 97% of basal by the end of the experiment, although the onset of the effect was delayed until 150mins after the administration of amantadine (figure 3.11 A).
Figure 3.10 The effect of CGP40116 on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei

The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the CGP40116 injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different from control (p<0.001).
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A)

Figure 3.11 The effect of amantadine on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex.

The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the amantadine injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different from control (p<0.001).
The effect of the application of amantadine on DA was rapid in onset and was maintained for 180mins before returning to basal at the end of the experiment (figure 3.11 B). The increase in DA release was only statistically significant at two time points, but, as figure 3.11 B clearly shows, a trend of increased DA release was observed. Amantadine was also observed to reduce the effect of CIM on DA release in the FC (figure 3.11 B). A clear decreasing trend was observed almost immediately but unlike CIM alone, statistical significance was only achieved towards the end of the experiment.

Similarly the effect of amantadine on GLU release in the FC also did not reach statistical significance. As with DA, a clear trend could be observed (figure 3.11 C). An overall decrease in extracellular levels of GLU was observed in response to the administration of amantadine, remaining at 50 ± 29% of basal at the end of the experiment. The CIM-induced effect on FC GLU was not significantly affected by amantadine (figure 3.11 B) and the overall effect was of similar magnitude to both CIM and amantadine alone.

3.2.11 The effect of amantadine on the clomipramine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

In the RN, the administration of amantadine had no significant effect on extracellular 5-HT, DA or GLU levels (see figure 3.12 A, B, C).

Amantadine was also observed to have no significant effect on CIM-induced changes in 5-HT release (figure 3.12 A).

CIM-induced DA release was significantly decreased in comparison to basal RN levels following the administration of amantadine (figure 3.12 B). Levels fell to 47 ± 9% of basal, but this was shown to be of similar magnitude to the decrease observed with CIM alone (see figure 3.4 B). Therefore no additional effect on CIM-induced DA release was observed following the administration of amantadine.

However, an interesting change in CIM-induced GLU was observed. Although CIM and amantadine had no effect on extracellular RN GLU levels when administered alone, the
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A) 

![Graph showing the effect of amantadine on clomipramine-induced changes in the extracellular levels of 5-HT](chart)

Figure 3.12 The effect of amantadine on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.

The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the amantadine injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.001).
administration of both drugs resulted in a significant, steady increase which peaked at 448 ± 6% of basal by the end of the experiment (figure 3.12 C).

3.2.12 The effect of D-cycloserine on the clomipramine-induced changes in 5-HT, DA and GLU levels in the frontal cortex

D-cycloserine was observed to decrease 5-HT, increase DA and have no significant effect on GLU release in the FC (see figure 3.13 A, B, C).

The significant decrease in 5-HT in response to D-cycloserine treatment was maintained for the duration of the experiment. The extracellular level of 5-HT fell to 55 ± 8% of basal (figure 3.13 A). Administration of D-cycloserine potentiated the overall effect of CIM, levels of 5-HT further were decreased to 47 ± 12% (compared to 57 ± 5% for CIM). Although not significant it suggests a trend and was maintained until the end of the experiment. It is worth noting that following the administration of CIM, levels of 5-HT were observed to return to basal by the end of the experiment (see figure 3.3 A) and that this effect seems to have been abolished by D-cycloserine.

D-cycloserine rapidly and significantly increased extracellular levels of DA in the FC (figure 3.13 B). Levels peaked at 293 ± 62% of basal and remained consistently high for the duration of the experiment. D-cycloserine was observed to abolish the CIM-induced decrease in DA release, with levels not differing significantly from control. However no increase in DA release, as had been seen with D-cycloserine alone, was observed.

Although D-cycloserine had no effect on extracellular GLU when administered alone, it was observed to abolish the CIM-induced decrease (figure 3.13 C) to such a degree that levels were no longer significantly different from control.
Figure 3.13 The effect of D-cycloserine on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex
The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the D-cycloserine injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.001).
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3.2.13 The effect of D-cycloserine on the clomipramine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

In the RN, D-cycloserine was observed to cause an upwards trend in 5-HT release, but had no significant effect on the basal or CIM-induced release of DA or GLU (see figure 3.14 A, B, C).

In response to D-cycloserine levels of RN 5-HT rose to 155 ± 27% of basal and the effect was shown to be both rapid and persistent. Interestingly the CIM-induced increase in extracellular 5-HT release was reversed and a significant and persistent decrease was recorded (64 ± 22% of basal).

3.2.14 The effect of ifenprodil on the clomipramine-induced changes in 5-HT, DA and GLU levels in the frontal cortex

Ifenprodil was observed to decrease extracellular levels of 5-HT and GLU in the FC, no significant effect on DA release was recorded (see figure 3.15 A, B, C).

Extracellular 5-HT was shown to decrease following the administration of ifenprodil. Levels fell to 64 ± 9% of basal before returning to the level of control by the end of the experiment (figure 3.15 A). The overall effect of ifenprodil on the CIM-induced decrease in 5-HT was a potentiation of this effect. However an initial, significant increase (226 ± 54% of basal) was observed and this persisted for 60mins before levels rapidly decreased to 46 ± 16% of basal, remaining low at the end of the experiment (figure 3.15 A).

Although ifenprodil did not have a significant effect on DA release when administered alone, it did have a significant effect on the CIM-induced decrease seen in the FC. The decrease observed in response to CIM was reversed and a significant increase in extracellular DA was observed (325 ± 25% of basal). The effect was rapid in onset and maintained for 150mins, returning to basal by the end of the experiment (figure 3.15 B).
Figure 3.14 The effect of D-cycloserine on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.

The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the D-cycloserine injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.001).
Figure 3.15 The effect of ifenprodil on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex
The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the ifenprodil injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.001).
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FC GLU levels were significantly decreased in response to ifenprodil. The effect was rapid in onset and persisted for most of the experimental period before returning to basal levels (figure 3.15 C). The effect of ifenprodil on the CIM-induced decrease in GLU release was initially observed as a rapid decrease to 57 ± 16% of basal. However around the time that the ifenprodil effect had been shown to be reduced, this effect was reversed and GLU release was significantly increased to 157 ± 7% of basal (figure 3.15 C).

3.2.15 The effect of ifenprodil on the clomipramine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

Ifenprodil was observed to increase extracellular 5-HT and DA in the RN. No significant effect of ifenprodil on GLU release was observed in this region (see figure 3.16 A, B, C).

The increase in 5-HT release (401± 89% of basal) in response to ifenprodil was rapid in onset and persisted for 150 mins before returning to basal level (figure 3.16 A). The CIM-induced increase in 5-HT release was unaffected by ifenprodil, although the increase was initially slower and the level at the end of the experiment peaked at 332 ± 71% of basal (compared to 210 ± 10% for CIM).

Although the effect on DA was not as impressive as that observed for 5-HT, ifenprodil was shown to cause a brief increase in extracellular DA (figure 3.16 B). Levels were only increased to 124 ± 3% but had returned to basal by the end of the experiment. The CIM-induced decrease in DA release was abolished by ifenprodil and a brief, but rapid, increase (214 ± 30%) was observed.

The application of ifenprodil did not significantly alter the RN levels of GLU (figure 3.16 C), however when administered in combination with CIM a small increase (128 ± 16%) was observed which lasted for the duration of the experiment (figure 3.16 C). It is worth remembering that CIM alone had no effect on GLU release in the RN (figure 3.4 C).
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![Graph A](image)

A) Control
- CIM 20mg/kg
- Ifenprodil 0.9mg/kg
- CIM 20mg/kg + Ifenprodil 0.9mg/kg

![Graph B](image)

B) Control
- CIM 20mg/kg
- Ifenprodil 0.9mg/kg
- CIM 20mg/kg + Ifenprodil 0.9mg/kg

![Graph C](image)

C) Control
- CIM 20mg/kg
- Ifenprodil 0.9mg/kg
- CIM 20mg/kg + Ifenprodil 0.9mg/kg

Figure 3.16 The effect of ifenprodil on clomipramine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei. The first arrow indicates the time of the i.p. injection of clomipramine and the second the time of the ifenprodil injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and ** indicates data points extremely significantly different from control (p<0.001).
3.2.16 The effect of sub-chronic dosing of amantadine and clomipramine on 5-HT, DA and GLU levels in the frontal cortex

10mg/kg/day i.p. CIM was administered for 4 days and resulted in an extremely significant fall in FC 5-HT levels, whilst levels of both DA and GLU were observed to increase (see figure 3.17 A, B, C). In all cases NMDA, infused via the RN probe for 30mins at a concentration of 100μM, had no significant effect on CIM-induced transmitter release.

40mg/kg/day i.p. amantadine administered in the same fashion resulted in decreases in extracellular levels of both 5-HT and DA, with no significant effect on GLU release (see figure 3.17 A, B, C). NMDA was infused to monitor the activity of the NMDA receptor and was observed to increase 5-HT and GLU release, but no significant effect on extracellular DA levels was recorded.

Sub-chronic dosing of CIM drastically reduced the levels of 5-HT in the FC (3.1 ± 0.7fmol/10μl compared to basal values of 19 ± 0.7fmol/10μl) and the effect, though not as large, was similar to that observed after acute dosing of CIM (see figure 3.3 A). NMDA infusion resulted in decreased levels of 5-HT, however sub-chronic dosing of CIM abolished this effect and therefore no significant change in FC CIM-induced 5-HT release was observed (figure 3.17 A). Amantadine administered sub-chronically was also shown to significantly decrease FC 5-HT to 7.7 ± 3fmol/10μl, but the infusion of NMDA was then shown to briefly increase 5-HT levels to 13.5 ± 2.25fmol/10μl. However the level of 5-HT was still significantly less than that of control (p<0.05). The effect of NMDA was delayed in comparison to controls and was much briefer in duration (60mins compared to 150+mins) with levels falling back to amantadine-basal by the end of the experiment (figure 3.17 A). Sub-chronic dosing of both amantadine and CIM also resulted in a significant decrease in extracellular levels of 5-HT (5.38 ± 1.79fmol/10μl) which, although significantly lower than control was significantly higher than observed with CIM alone. Infusion of NMDA had no significant effect on FC 5-HT release in this situation.
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Figure 3.17 The effect of sub-chronic dosing of amantadine and clomipramine on the extracellular levels of A) 5-HT, B) DA and C) GLU in the frontal cortex.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.001). # indicates significantly different from treatment basal and + indicates significantly different from CIM alone (p<0.05) and extremely significantly different from control (p<0.001).
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When dosed sub-chronically, CIM was observed to greatly increase the levels of DA in the FC (185 ± 19fmol/10μl as compared to basal values of 88 ± 6.3fmol/10μl), the completely opposite response to that seen after acute dosing (see figure 3.3 B). NMDA infusion significantly increases DA release in controls, however, as with 5-HT sub-chronic DA appears to abolish this effect and no significant change was observed (figure 3.17 B). Sub-chronic dosing of amantadine significantly decreases extracellular DA to 26.8 ± 4fmol/10μl and the subsequent infusion of NMDA was shown to have no significant effect (figure 3.17 B). Sub-chronic dosing of both amantadine and CIM resulted in a similar decrease in extracellular DA (29 ± 4.7fmol/10μl) to that seen after sub-chronic amantadine, but the subsequent infusion of NMDA was shown to increase DA. The effect of NMDA was similar to that observed for controls in terms of both onset and duration, however although the DA levels were significantly higher (p<0.0001) than treatment basal, they were not significantly altered from control basal levels (figure 3.17 B).

Extracellular FC levels of GLU were seen to double following sub-chronic dosing of CIM – the opposite effect to that observed following acute treatment (see figure 3.3 C). Levels were shown to have been increased to 29 ± 5pmol/10μl, compared to the control basal level of 15 ± 0.3pmol/10μl (figure 3.17 C). The infusion of NMDA showed a decreasing trend that was slow in onset, 120mins after infusion a significant decrease in GLU release was recorded and this was maintained until the end of the experiment (figure 3.17 C). Sub-chronic dosing of amantadine also had no significant effect on FC GLU (figure 3.17 C), although levels were significantly increased after infusion of NMDA. This was the opposite effect to that observed when NMDA was infused into controls (see figure 3.5 C). Sub-chronic dosing of both amantadine and CIM resulted in an extremely significant reduction in FC GLU levels to 1.6 ± 0.33pmol/10μl, compared to the control basal level of 15 ± 0.3pmol/10μl (figure 3.17 C). NMDA infusion was subsequently shown to have no significant effect on the changes in extracellular FC GLU induced by the combined treatment with amantadine and CIM.
3.2.17 The effect of sub-chronic dosing of amantadine and clomipramine on 5-HT, DA and GLU levels in the raphe nuclei

CIM administered for 4 days at a dose of 10mg/kg/day i.p. was observed to reduce DA and GLU but had no significant effect on 5-HT release in the RN (see figure 3.18 A, B, C). The infusion of NMDA for 30mins was shown to increase extracellular 5-HT levels (figure 3.18 A) following CIM-treatment, but had no effect on CIM-induced DA or GLU release (figure 3.18 B, C).

When amantadine (40mg/kg/day i.p.) was administered in the same way, extracellular levels of both 5-HT and GLU were increased, whilst that of DA was seen to fall (figure 3.18 A, B, C). The subsequent infusion of NMDA via the RN probe increased RN GLU, but had no effect on either 5-HT or DA release.

Sub-chronic dosing of CIM was observed to have no significant effect on extracellular levels of 5-HT in the RN, therefore the increase observed following acute CIM treatment was abolished (see figure 3.18 A). The subsequent infusion of NMDA via the RN probe resulted in a rapid increase in RN 5-HT levels to 73.4 ± 8.6fmol/10μl, which was significantly increased in comparison to CIM basal levels (p<0.05). The effect lasted for 90mins before returning to basal levels (figure 3.18 A) and was similar to that observed after NMDA infusion in controls, with the exception of earlier onset of the effect. 4 day dosing of amantadine was shown to significantly increase extracellular 5-HT to 77 ± 15fmol/10μl. The subsequent infusion of NMDA had no significant effect on 5-HT levels and levels remained high for the duration of the experiment (figure 3.18 A). The effect of dosing both amantadine and CIM together for 4 days was a significant increase in RN 5-HT levels when compared to both control basal and CIM basal values (p<0.0001). The level of 5-HT was 60 ± 7fmol/10μl and the subsequent infusion of NMDA was observed to briefly decrease this to 22 ± 6fmol/10μl before levels returned to treatment basal (figure 3.18 A). It is interesting that this was the opposite effect to that observed when NMDA was administered to controls and was in fact more like that seen following the infusion of 25μM NMDA (see figure 3.6 A).
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Figure 3.18 The effect of sub-chronic dosing of amantadine and clomipramine on the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different from control (p<0.001). # indicates significantly different (p<0.05), ## indicates very significantly different (p<0.001) and ### indicates extremely significantly different (p<0.0001) from treatment basal. ++ indicates very significantly different from CIM alone (p<0.005) and extremely significantly different from control (p<0.001) and +++ indicates extremely significantly different from basal and CIM alone (p<0.0001).
Sub-chronic dosing of CIM was observed to decrease RN DA release to 20 ± 2fmol/10µl, which was significantly lower than control basal (figure 3.18 B). This was similar to the effect recorded following acute administration of CIM (figure 3.4 B). The subsequent infusion of NMDA had no significant effect on extracellular DA levels and the NMDA-induced decrease observed in controls was shown to be blocked. Following 4 days of amantadine treatment, extracellular DA levels were seen to be decreased to a similar level to those observed following sub-chronic CIM treatment (figure 3.18 B), the subsequent infusion of NMDA also had no significant effect. Sub-chronic dosing of both amantadine and CIM was also observed to significantly decrease (p<0.0001) RN DA levels (32 ± 5fmol/10µl), however this was significantly higher (p<0.005) than the levels recorded for CIM alone. The subsequent infusion of NMDA again had no significant effect (figure 3.18 B).

RN GLU levels were significantly decreased in response to sub-chronic CIM treatment. Extracellular release fell to 6.9 ± 1.3fmol/10µl and this was unaffected by the subsequent infusion of NMDA via the RN probe (figure 3.8 C). NMDA was shown to decrease GLU levels in the RN, therefore sub-chronic CIM blocked this effect. After 4 days of amantadine treatment extracellular GLU had risen to 20.8 ± 0.92pmol/10µl, compared to 8 ± 2pmol/10µl in control. Following NMDA infusion a steady increase was observed, peaking at 35.5 ± 4pmol/10µl at the end of the experiment. Interestingly, as with 5-HT (see above), this effect of NMDA was more like that observed following 25µM NMDA infusion (see figure 3.6 C). Following sub-chronic dosing of both amantadine and CIM, a decrease in GLU, similar to that observed with CIM alone, was observed. NMDA infusion had no significant effect on RN GLU levels and the treatment therefore seemed to have blocked the NMDA-induced decrease observed in controls (figure 3.18 C).

3.2.18 The effect of 25µM NMDA infusion into the raphe nuclei on the chronic clomipramine-induced changes in 5-HT and DA levels in the frontal cortex

Chronic dosing of CIM for 14 days resulted in a concentration-dependent increase in FC levels of both 5-HT and DA (see figure 3.19 A, B). This is the opposite effect to that observed following both acute (see figure 3.3 A, B) and sub-chronic (see figure 3.17 A,
Figure 3.19 The effect of 25μM NMDA infusion into the raphe nuclei, via the dialysis probe, on clomipramine-induced changes in the extracellular levels of A) 5-HT and B) DA in the frontal cortex.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control (p<0.05) and # indicates significantly different from treatment basal (p<0.05).
B) dosing (with the exception of DA following 4 day CIM). The subsequent infusion of NMDA had a potentiating effect on both transmitters and levels returned to treatment basal by the end of the experiment. In the FC, 25μM NMDA was observed to increase 5-HT and decrease DA release in controls.

Following the chronic administration of CIM, a significant, dose-dependent increase in 5-HT release was observed (figure 3.19 A). At the lower dose of CIM, levels were quadrupled to 87 ± 10fmol/10μl and at the higher dose extracellular 5-HT was recorded at 233 ± 20fmol/10μl. The subsequent infusion of NMDA for 30mins further increased extracellular 5-HT in 10mg/kg CIM-treated animals, as seen in controls, but had no significant effect on the 20mg/kg CIM-induced increase (figure 3.19 A). The levels of 5-HT reached 320 ± 40fmol/10μl in the 10mg/kg CIM-treated animals, which is similar to that achieved following chronic 20mg/kg CIM treatment.

Chronic CIM treatment also increased FC DA in a concentration-dependent manner (figure 3.19 B). Levels of DA were doubled (192 ± 10fmol/10μl) and tripled (324 ± 20fmol/10μl) following 10mg/kg and 20mg/kg CIM respectively. The subsequent infusion of NMDA had no significant effect on 10mg/kg CIM-induced release, although a slight upward trend could be clearly observed (figure 3.19 B). However infusion of NMDA caused a potentiation of 20mg/kg CIM-induced release which was immediate in onset and was maintained for 120mins before returning to basal. It is worth noting here that 25μM NMDA infusion into the RN results in decreased FC DA levels in controls, suggesting that chronic CIM is somehow blocking the effects of NMDA.

3.2.19 The effect of 25μM NMDA infusion into the raphe nuclei on the chronic clomipramine-induced changes in 5-HT and DA levels in the raphe nuclei

Chronic dosing of CIM for 14 days resulted in a concentration-dependent increase in RN levels of both 5-HT and DA (see figure 3.20 A, B). The subsequent infusion of NMDA had no effect on either transmitter following 20mg/kg CIM-treatment and only affected 10mg/kg CIM-induced 5-HT release. In the RN, 25μM NMDA was observed to decrease extracellular 5-HT and DA in controls.
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Figure 3.20 The effect of 25\mu M NMDA infusion into the raphe nuclei, via the dialysis probe, on clomipramine-induced changes in the extracellular levels of A) 5-HT and B) DA in the raphe nuclei.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control (p<0.05) and # indicates significantly different from treatment basal (p<0.05).
Following the chronic administration of CIM, a significant, dose-dependent increase in 5-HT release was observed (figure 3.20 A). At the lower dose of CIM, levels were increased to 80 ± 20fmol/10μl and at the higher dose extracellular 5-HT levels were five times those of control (160 ± 20fmol/10μl compared to 30 ± 5fmol/10μl). The subsequent infusion of NMDA for 30mins significantly decreased extracellular 5-HT in 10mg/kg CIM-treated animals, as seen in controls, but had no significant effect on the 20mg/kg CIM-induced increase (figure 3.20 A). The levels of 5-HT fell to 38 ± 6fmol/10μl in the 10mg/kg CIM-treated animals, which was not significantly different from control basal. The onset, duration and magnitude of the effect followed the pattern observed after NMDA infusion in control.

Chronic CIM treatment also significantly increased RN DA in a concentration-dependent manner (figure 3.20 B). Levels of DA were increased to 155 ± 15fmol/10μl and 245 ± 10fmol/10μl following 10mg/kg and 20mg/kg CIM respectively. The subsequent infusion of NMDA had no overall significant effect on CIM-induced release, although following 10mg/kg CIM a significant decrease was recorded at one time point (figure 3.20 B). In controls 25μM NMDA infusion into the RN results in decreased RN DA levels, suggesting that chronic CIM is somehow abolishing the effects of NMDA.

3.2.20 The effect of 100μM NMDA infusion into the raphe nuclei on the chronic clomipramine-induced changes in 5-HT in the frontal cortex

Chronic dosing of CIM for 14 days resulted in a significant dose-dependent increase in FC levels of 5-HT (see figure 3.21 A) with levels ten to fifteen times those observed in controls (120–240 ± 10fmol/10μl compared to 19 ± 3fmol/10μl). The subsequent infusion of NMDA had no significant effect following 20mg/kg CIM treatment, but a significant decrease was observed in the 10mg/kg CIM-treated animals and levels remained low at the end of the experiment. In the FC, 100μM NMDA was also observed to decrease control extracellular 5-HT levels, but the magnitude of the effect was much greater than observed following infusion into CIM-treated animals.
Figure 3.21 The effect of 100μM NMDA infusion into the raphe nuclei, via the dialysis probe, on clomipramine-induced changes in the extracellular levels of 5-HT in the A) frontal cortex and B) raphe nuclei.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates data points significantly different from control (p<0.05) and # indicates significantly different from treatment basal (p<0.05).
3.2.21 The effect of 100μM NMDA infusion into the raphe nuclei on the chronic clomipramine-induced changes in 5-HT in the raphe nuclei

In the RN, chronic CIM treatment also results in a significant, dose-dependent increase in extracellular 5-HT release (see figure 3.21 B) and levels were increased to between three and five times those of control. The subsequent infusion of 100μM NMDA for 30mins was observed to potentiate the CIM-induced increase, with levels returning to basal by the end of the experiment (figure 3.21 B). In the CIM-treated animals the onset of the NMDA effect was delayed by 60mins and the magnitude was much smaller than that of control.

3.2.22 The effect of chronic clomipramine dosing on body weight

Over a period of 14days, control animals were observed to steadily gain weight at a rate of approximately 4g/day (see figure 3.22), increasing in weight from 205 to 258g. Animals, of a similar starting weight, chronically dosed with CIM were also observed to gain weight, but much less rapidly than controls. During the dosing period, CIM-treated animals gained weight at an overall rate of approximately 2g/day (figure 3.22), although the increase in weight was less towards the end of the experiment than at the start. CIM-treated animals weighed an average of 225g at the end of the experiment, which was significantly lower (p<0.0001) than that of controls at the same time point.
Figure 3.22 The effect of chronic clomipramine dosing on body weight

All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates extremely data points significantly different from control (p<0.0001).
3.3 Discussion

3.3.1 Summary

The data presented in this chapter (see table 3.2 for summary) support the involvement of the NMDA receptor in the action of the TCA CIM. They also suggest that the manipulation of NMDA receptor activity by one class of NMDA receptor antagonists may reduce the ‘lag’ period associated with current antidepressant treatment, as indicated by increased cortical levels of the monoamine 5-HT. Levels of GLU in the RN and FC suggest that this transmitter too may be linked to NMDA-evoked release of monoamines in these brain structures. Collectively the data suggest that a complex series of adaptive changes in the regulation of transmitter release in the RN and FC are induced by treatment with the antidepressant CIM. Differences in effects in the two regions may be explained by differences in NMDA receptor subunits or by transmitter interactions resulting in modulated release.

3.3.2 Effect of clomipramine on neurotransmitter release in the frontal cortex and raphe nuclei

Acute CIM was shown to increase RN 5-HT release in a dose-dependent manner and was accompanied by a dose-dependent decrease in FC 5-HT levels, confirming earlier studies (Adell & Artigas 1991, Pallotta et al. 2001). However Adell & Artigas (1991) also suggested that CIM has a differential effect on dialysate 5-HT release dependent on the route of administration: local infusion into the FC resulted in increased extracellular 5-HT whilst systemic administration had no significant effect. 20mg/kg CIM s.c. was observed to result in a 100% increase in FC 5-HT (Carboni & Di Chiara 1989), possibly by avoiding the passage of CIM through the liver (Adell & Artigas 1991). Thus the differential effect of 5-HT is not believed to be due to pharmacodynamic reasons and may instead be explained by differences in brain region, type of probe and composition of perfusion fluid (Di Chiara 1990). Studies of FC 5-HT levels following acute venlafaxine (5-20mg/kg i.p.), a novel SSRI and SNRI, showed increased levels of 5-HT in both the FC and hippocampus (Gur et al. 1999a), as did fluvoxamine (1-10mg/kg i.p.) in the FC (Bel & Artigas 1992). It is worth noting that TCA administration (e.g. CIM, imipramine) only results in large increases in terminal areas,
Table 3.2 Summary of results compared to basal or treatment-basal

Effects of drug treatments are expressed in comparison to basal or treatment-basal as appropriate. nd indicates transmitter not measured in this study.

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including the FC, when doses of 10mg/kg or higher are used (Bel & Artigas 1996, Romero et al. 1996). The RN are known to be enriched with 5-HT$_{1A}$ receptors displaying an affinity for 5-HT in the low nanomolar range (Pazos & Palacios 1985, Pedigo et al. 1981). Increased RN 5-HT would therefore activate 5-HT$_{1A}$ receptors in cell bodies and dendrites, resulting in inhibition of cell firing and reduced release of 5-HT in the FC. This view is supported by Hutson et al. (1989) who showed that the specific 5-HT$_{1A}$ agonist 8-OH-DPAT decreased hippocampal 5-HT release when applied locally to the dorsal RN. A study by Bel & Artigas (1992) observed that fluvoxamine, an antidepressant devoid of noradrenergic activity, preferentially increased RN 5-HT. Extracellular 5-HT was significantly increased in both the RN and FC, but the increase in the RN was several-fold that in the FC (Bel & Artigas 1992).

Sub-chronic (4day) CIM administration had no effect on RN 5-HT release and was therefore observed to block the increase resulting from acute administration. When compared to levels following acute CIM treatment, sub-chronic CIM was observed to decrease FC 5-HT by a much greater degree. Finally, chronic (14day) CIM was observed to dose-dependently increase extracellular 5-HT in both regions, confirming previous studies (Pallotta et al. 2001). A study of the effects of chronic fluvoxamine (1mg/kg/day for 2 weeks) observed increased extracellular 5-HT in the FC and levels in the RN similar to those of controls (Bel & Artigas 1993). Chronic CIM and imipramine have also been shown to initiate larger increases in terminal regions, with lower doses than those used in acute studies (Bel & Artigas 1993, 1996). Chronic treatment therefore reverses the decrease in FC levels observed following acute and sub-chronic treatment, suggesting that some form of adaptation within the system is occurring.

It is interesting to note that changes in 5-HT occur after as little as 4days of CIM treatment. Together with the chronic data this would seem to confirm the theory that adaptive changes (e.g. desensitisation of the 5-HT$_{1A}$ receptor) must occur before the clinical benefit of antidepressants can be observed (Artigas et al. 1996, Blier & de Montigny 1994). The increases in terminal 5-HT levels initiated by 5-HT uptake blocking drugs has been shown to be potentiated by co-administration with a 5-HT$_{1A}$ autoreceptor antagonist in a number of studies (Artigas et al. 1994, Blier & Bergeron 1995). The effect of co-administration of a 5-HT$_{1A}$ antagonist is analogous to the desensitisation of RN 5-HT$_{1A}$ autoreceptors by chronic administration of SSRIs (Blier
This process is thought to underlie the therapeutic effect of pindolol as an adjunct to antidepressant therapy (Artigas et al. 1994, Blier & Bergeron 1995, Blier et al. 1997). However there are few reports of the effects of co-administration of 5-HT1A antagonists with 5-HT uptake blockers which are not SSRIs (Gur et al. 1999a, b). The β-blocker pindolol is not, as originally thought, a pure antagonist of the 5-HT1A receptors and instead is believed to act as a partial agonist at 5-HT1A and as an antagonist at 5-HT1B receptors (Newman-Tancredi et al. 1998). Studies with WAY100635, a full antagonist at the 5-HT1A receptor, have also shown that co-administration with an antidepressant (e.g. citalopram, CIM, duloxetine, fluoxetine, paroxetine) potentiates the effect of the antidepressant (Cryan et al. 1999, Gartside et al. 1995, Millan et al. 1998, Romero & Artigas 1997, Romero et al. 1996). This indicates that the effects of serotonergic antidepressant drugs can be potentiated by 5-HT1A autoreceptor blockade and therefore emphasises the role of this receptor in the mechanism of action of antidepressants. SSRIs including fluoxetine and paroxetine preferentially reduce output in the FC, compared to the hippocampus (Hervás & Artigas 1998, Romero & Artigas 1997, Romero et al. 1997). Hervás et al. (2000) have suggested that this is due to a greater effect of reuptake blockade in the FC that is offset by a greater autoreceptor-mediated inhibition of 5-HT release. A study by Newman et al. (2000) demonstrated that following chronic (28day) CIM (10mg/kg/day) administration, presynaptic 5-HT1A and post-synaptic 5-HT1B receptor sensitivity were altered in the rat hypothalamus and hippocampus respectively. Serotonergic afferents from the RN are known to innervate both of these regions (Jacobs & Azmitia 1992), which are also believed to be important in the aetiology of depression (Nemeroff 1999, Marano 1999, Newman et al. 2000). Taken together with earlier experiments involving the cerebral cortex (Gur et al. 1999b) and electrophysiological experiments (de Montigny & Aghajanian 1978), these in vivo results indicate that chronic CIM exerts highly regional-specific effects on both pre- and post-synaptic 5-HT receptors. The finding by Bel & Artigas (1993) that an acute dose of fluvoxamine did not further increase 5-HT output in the FC of chronically fluvoxamine-treated rats (although it did in the RN) indicates changes in the function of the 5-HT transporter in the FC of these animals. This theory is supported by the observation that chronic paroxetine treatment results in decreased efficacy of the hippocampal 5-HT transporter (De Montigny et al. 1992).
Although DA has not always been thought of in conjunction with the depressive condition, evidence now exists that the transmitter plays an important role in its pathogenesis (Willner 1983). For example, chronic desipramine treatment enhances the ability of amphetamine to increase DA in the nucleus accumbens (Brown et al. 1991) and chronic antidepressant treatment increases behavioural responses to apomorphine, a DA agonist (Maj et al. 1984). It is demonstrated here that acute treatment with CIM results in a dose-dependent decrease in both the RN and FC, confirming earlier studies (Pallotta et al. 1999a,b). However acute administration of fluoxetine, CIM, imipramine, desipramine, mianserin, nortryptiline and paroxetine have all reportedly increased extracellular concentrations of FC DA (Carlson et al. 1996, Tanda et al. 1994, 1995, 1996). A number of studies of acute CIM treatment (Ichikawa & Meltzer 1995, Pallotta et al. 1999a,b), when considered collectively, suggest that the effects observed following acute treatment are probably not due to an inhibitory interaction with the DA transporter. Instead it is thought to be the result of secondary changes in the reuptake of other transmitters (e.g. 5-HT) more potently affected by CIM (Pallotta et al. 1999b). 5-HT has been shown to decrease DA release in a number of brain regions (Westfall & Tittermay 1982), but acute SSRI treatment has been shown to increase FC DA (Gobert et al. 1997), suggesting the involvement of other factors in the acute effect of CIM on DA release (Pallotta et al. 1999b).

Sub-chronic (4day) CIM still results in decreased RN DA, but the effect in the FC is reversed and an increase is observed. Following chronic treatment (14days), the acute effects are reversed in both brain regions and levels of DA are dose-dependently increased. FC DA release is considerably increased in comparison to that observed following sub-chronic treatment. The results of the chronic studies confirmed those of Pallotta et al. (1999a,b). The contrast between acute and chronic CIM treatment is therefore a complete reversal of the effect of the antidepressant on FC and RN DA release. The observation that after sub-chronic CIM treatment DA is increased in the FC suggests that adaptations are occurring after as little as 4days of antidepressant treatment and therefore follow the pattern of changes exhibited by 5-HT (see above). Adaptive changes in serotonergic transmission to the cortex during chronic treatment may lead to increased cortical 5-HT which, in turn may directly increase DA release. Similar changes in DA D₂ receptor density or function may occur instead of, or in addition to, the direct effects of 5-HT. Dopaminergic pathways are known to project...
from the ventral tegmental area and substantia nigra to the RN (Ferre & Artigas 1993) and D<sub>2</sub> receptors have been shown to mediate increased release of 5-HT (Kalen <i>et al.</i> 1988, Stern <i>et al.</i> 1981) in this region. Therefore changes in the RN concentration of DA may regulate RN 5-HT release, and in turn regulate FC 5-HT and DA. Finally changes in the FC basal dialysate DA concentration may be due to the biotransformation of CIM to its metabolite DCIM, which has been shown to have a higher affinity for the NA transporter (Benfield 1980, Thomas & Jones 1977). Pozzi <i>et al.</i> (1994) have provided evidence that extracellular concentrations of DA are regulated by noradrenergic neurones in the frontal cortex of rats. Studies have suggested that DA uptake blockers have no effect on DA release in the FC because noradrenergic terminals take up most of the DA (Carboni <i>et al.</i> 1990, Izenwasser <i>et al.</i> 1990). This hypothesis was further refined to suggest that DA uptake blockers do increase FC DA concentrations but that the majority of the DA is taken up into noradrenergic terminals by a high-affinity uptake carrier (Pozzi <i>et al.</i> 1994). It was then shown that the selective NA uptake blocker desipramine significantly increased output selectively in the FC (Pozzi <i>et al.</i> 1994), it did not modify extracellular DA concentrations in the nucleus accumbens (Nomikos <i>et al.</i> 1991). Taken together these observations suggest a different organisation and regulation of DA and NA terminals in the two brain regions (Pozzi <i>et al.</i> 1994) and it may be this interaction which explains the action of CIM on DA release in the FC.

The role of GLU in the aetiology of depression has become more accepted following studies showing that a dysfunction of NMDA-glutamatergic receptors may be involved (Cappello <i>et al.</i> 1997, Layer <i>et al.</i> 1998). Acute CIM treatment is shown here to increase FC GLU release and have no effect on levels in the RN. A number of studies confirm these findings, although none have involved CIM, other members of the TCA class (e.g. imipramine) have been studied. Golembiowska & Zylewska (1999) infused a number of different antidepressants directly into the pre-FC and observed significantly decreased levels of GLU and aspartate. More recently a study of imipramine and phenelzine, two antidepressants with different modes of action, showed that rapid, significantly decreased stimulated GLU outflow in brain slices was only observed in the pre-FC (Michael-Titus <i>et al.</i> 2000). However a study using cultured hippocampal neurones demonstrated that the acute administration of the TCA desipramine by superfusion stimulated the excytosis of GLU (Bouron & Chatton 1999). Desipramine is
believed to exert its therapeutic effects by interfering with serotonergic and/or noradrenergic cells (Blier & de Montigny 1994, Potter et al. 1995). Bouron & Chatton (1999) have therefore demonstrated that it may also modulate the glutamatergic system. Therapeutic concentrations of desipramine rapidly enhanced the release of hippocampal GLU presynaptically by the activation of a protein kinase (Bouron & Chatton 1999). They go on to speculate that TCA drugs may act to modify glutamatergic neurone activity, as well as acting on serotonergic and noradrenergic cells and that these glutamatergic neurones of the limbic system could also be important in the treatment of depression by TCAs (Bouron & Chatton 1999).

Following subchronic dosing (4day) of CIM, GLU levels in the RN were observed to decrease, whilst the acute CIM-induced increase in FC GLU release was reversed and a significant increase was observed. A review of the literature revealed few studies (in vivo or in vitro) of the effect of chronic antidepressants on excitatory amino acid release. The evidence was inconsistent - the findings of Michael-Titus et al. (2000) demonstrated that pre-FC GLU outflow was rapidly decreased following the chronic (21days) administration of imipramine or phenelzine, whilst Nowak et al. (1996) showed that chronic (14days) citalopram increased FC and hippocampal aspartate levels in mice. Regulation of GLU release in the FC may be achieved by the direct effect of antidepressants on Na+ channels (Golembiowska & Zylewska 1999). Antidepressants may also indirectly regulate FC GLU levels by the involvement of D2/D3, α2 or 5-HT1B heteroreceptors activated by the increased levels of monoamines in response to the blockade of their respective transporters (Golembiowska & Zylewska 1999). Electrophysiological studies have shown that 5-HT transmission in ascending pathways is partly regulated by glutamatergic inputs acting at the levels of the RN, the application of GLU to the RN activates 5-HT neurones in this region (Vandermaelen et al. 1986). Maura et al. (1998) have shown that GLU release in cerebral cortex synaptosomes to be inhibited by 5-HT acting at 5-HT1D receptors, whilst Crowder & Bradford (1987) observed NA inhibiting veratrine-induced GLU release from cortical slices. Dopamine has also been shown in vitro to facilitate the long-term depression of glutamatergic transmission in the rat pre-FC (Otani et al. 1998). Although this conflicts with the results presented here, it is worth noting that Otani et al. (1998) carried out an in vitro study that may not have been subjected to the conditions demonstrated in vivo. Following sub-chronic and chronic treatment (4 and 14days) with CIM, FC DA levels
are significantly increased and by the hypothesis of Otani et al. (1998), transmission of GLU in the FC should have been decreased – an increase in GLU transmission was recorded. These results suggest that receptor alterations are taking place during CIM treatment, but that any interaction between DA and GLU in the FC does not play a vital role. It remains to be confirmed whether the effect of antidepressants on GLU outflow in the FC are a consequence of their effects on amine levels or are also due to intrinsic effects of the drugs on the cellular uptake or release mechanisms (Bouron & Chatton 1999, Michael-Titus et al. 2000, Popoli et al. 2000).

In summary it has been shown that chronic treatment with CIM induces changes in the release of the neurotransmitters 5-HT, DA and GLU and that these changes require adaptation within the systems involved in order for the effects of the antidepressant to be observed. The effects of these adaptive changes can be observed as early as 4 days after the onset of treatment, although the presumed therapeutic effects (i.e. increased cortical 5-HT and DA) are not fully realised until later.

3.3.3 Effect of NMDA on basal and clomipramine-induced neurotransmitter release in the frontal cortex and raphe nuclei

NMDA has been shown to have a biphasic effect on 5-HT release in the RN and FC, confirming previous studies carried out by Pallotta et al. (1998). At lower concentrations, NMDA infusion into the RN leads to a substantial decrease in local dialysate 5-HT and a prolonged increase in terminal FC 5-HT, whilst at the higher concentration of NMDA the effects can be seen to be reversed. Pallotta et al. (1998) used a selective 5-HT1A receptor antagonist (WAY100635) and a competitive NMDA receptor antagonist (D-AP5) to study the relationship between NMDA-evoked changes in the RN and serotonergic transmission to the FC. Their data suggests that the degree of NMDA receptor activation results in dramatically different outcomes with regard to serotonergic transmission to the FC and that there appears to be a differential role for the 5-HT1A receptor in regulating these effects.

Infusion of 25μM NMDA into the RN following acute CIM administration reversed the increase in 5-HT observed in this region and was accompanied by an increase in the extracellular levels of FC 5-HT. These results confirmed those of Pallotta et al. (1999a).
However a similar infusion following sub-chronic CIM administration (4 days) resulted in entirely different effects. In the RN no effect of the treatment was observed on extracellular 5-HT, therefore abolishing the acute effect of CIM in this region. The infusion of 100 μM NMDA into the RN of these sub-chronically dosed animals was observed to increase RN 5-HT release and was similar to the effect seen following the administration of 100 μM NMDA to controls. In the FC levels of 5-HT remained decreased in response to CIM treatment but the infusion of NMDA was seen to have no significant effect, therefore the acute effect of NMDA was shown to have been abolished by sub-chronic CIM treatment. After chronic (14 day) CIM treatment, levels of 5-HT are dose-dependently increased in both the RN and FC. An infusion of 100 μM NMDA was observed to have no effect on extracellular 5-HT levels in either region, the effects of NMDA were therefore abolished. Pallotta et al. (1999a) showed similar results and also that the effects of infusing 25 μM NMDA were greatly attenuated following chronic treatment with CIM.

The role of NMDA receptors in regulating serotonergic transmission is therefore complex and the data presented here and by Pallotta et al. (2001) confirms that antidepressant treatment may play a role in smoothing NMDA receptor-mediated fluctuations in serotonergic transmission to the FC. A clear link between NMDA and nitric oxide (NO) has been established (Garthwaite & Boulton, 1995), whereby activation of NMDA receptors increases cytosolic Ca^{2+}, stimulating nitric oxide synthase (NOS) to produce NO (Dawson et al., 1991). Several studies have implicated NO in the modulation of NMDA-evoked release of neurotransmitters within the brain (Garthwaite & Boulton, 1995, Getting et al., 1996, Kaehler et al., 1999, Segeith et al., 1995, Silva et al., 1995) and Smith & Whitton (2000) extended these studies to show that NO plays a role in mediating both basal and NMDA-evoked changes in serotonergic transmission between the RN and FC. Studies by Adell & Artigas (1991) showed that systemic administration of CIM resulted in increased dialysate 5-HT in the RN, but not the FC. However infusion of CIM directly into the FC resulted in an increased extracellular release of 5-HT in this region. Similarly infusion of CIM into the RN increased 5-HT in this region and was accompanied by a decrease in 5-HT release in the FC (Adell & Artigas, 1991). The data of Adell & Artigas (1991), Pallotta et al. (1999a, 2001) and the observations presented here are therefore almost certainly the result of the action of 5-HT at 5-HT_{1A} somatodendritic autoreceptors. Trullas &
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Skolnick (1990) advocated the potential role of NMDA receptors in the pathology of depression and there is now a considerable body of evidence indicating a probable role for the NMDA receptor (see 1.8.6). Chronic antidepressant treatment has been shown to reduce the potency of GLY to inhibit 5,7-DCKA binding to the NMDA receptor-associated GLY sites in neocortical membrane (Nowak et al. 1993, Paul et al. 1993, 1994). Porter & Greenamyre (1995) used quantitative autoradiography to study the regional binding characteristics of a number of NMDA receptor antagonists (e.g. MK801, amantadine). They observed that there were regional variations in the pharmacology of NMDA receptor channel blockers, suggesting that pharmacological properties of NMDA receptors are region-specific (Porter & Greenamyre 1995).

However, to date, only one study has published results of the effects of chronic antidepressant treatment on the expression of NMDA receptor subunit mRNAs in brain. Boyer et al. (1998) carried out a quantitative in situ hybridisation study to investigate the effects of chronic administration of imipramine and citalopram in mouse brain. These antidepressants were observed to alter the levels of mRNA encoding the ζ-subunit in a parallel fashion. Both drugs reduced transcript levels in regions including the cortex, cerebellum, thalamus and striatum and had no significant effect in the hippocampus. Distinct, region-specific effects of citalopram and imipramine on the mRNA encoding for the ε-family of subunits was observed. Imipramine produced widespread reductions in ε2 subunit mRNA levels in the cortex, hippocampus and amygdala, whilst citalopram reduced ε1 mRNA levels in the same areas (Boyer et al. 1998). The mouse ζ subunit is thought to be equivalent to the rat NR1 subunit, ε1 to NR2A and ε2 to NR2B. A recent unpublished study (F. Murray, Personal Communication) looked at the effect of chronic CIM (10mg/kg/day, 14day) treatment on NR1 protein levels in three brain regions – the amygdala, FC and hippocampus. Other NMDA subunit protein levels have still to be determined but their results suggest that chronic treatment with CIM significantly reduces NR1 protein levels in FC, amygdala and hippocampus, with the greatest effect recorded in the FC. These three regions are believed to be important in the processing of emotions and therefore may be important in depression (Marano 1999). The observations of Murray confirm and extend the study of Boyer et al. (1998), suggesting that chronic antidepressant treatment produces region-specific changes in NMDA receptor subunit mRNA expression. It is therefore logical to suggest that the composition of the NMDA receptor is altered by
chronic antidepressant treatment and that this, in turn, determines the physiological and pharmacological properties of NMDA receptors and therefore the therapeutic actions of antidepressants (Boyer et al. 1998). A study of NMDA receptor subunit mRNA levels in rat brain following acute and chronic exposure to antipsychotic drugs (e.g. haloperidol, clozapine) also indicated significant differences in the regulatory pattern of NMDA receptor subunits (Riva et al. 1997), suggesting that this form of regulation may be critical in more than one type of disorder. The idea of different subtypes of a receptor exhibiting different, distinct functions is not a new one, Gallo & Russell (1995) suggested that different subtypes of excitatory amino acid receptors on glia may have distinct functions. The findings presented here suggest a clear association between chronic CIM treatment and regulation of serotonergic transmission by NMDA receptors located within the RN. However from the sub-chronic data it can also be hypothesised that CIM treatment results in adaptive changes in the NMDA receptor and that such changes are starting to occur as early as 4 days after the start of CIM treatment. The relationship between serotonergic and glutamatergic transmission in this situation will be discussed below.

NMDA infused via the RN probe is observed to dose-dependently decrease RN DA release. In the FC a biphasic effect is observed with higher NMDA concentrations, resulting in an increased release of FC DA. The dose-dependent decrease in RN DA observed following the infusion of NMDA via the RN probe confirmed and extended the study of Pallotta et al. (1999a). The same group studied the effect of infusing NMDA into the FC on FC DA release and observed a concentration-dependent decrease Pallotta et al. 1999b), which is different to the biphasic effect on FC DA seen when NMDA is infused into the RN. Another study showed that directly infused NMDA into the pre-FC resulted a concentration-dependent dual action on local DA release (Feenstra et al. 1995). The concentration of 1mM NMDA is substantially higher than those employed in other studies and therefore the results observed may be due to non-specific interactions. A lower dose of 100μM was also studied and shown to have the opposite effect to that seen by Pallotta et al. (1999b). This suggests that modulation of NMDA-evoked DA release in the FC is affected by an external factor and lends support to the involvement of other transmitter systems in regulating NMDA-evoked DA release in the FC. Indeed a study by Del Arco & Mora (1999), which looked at the effects of endogenous GLU on extracellular concentrations of GABA and DA in the FC,
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suggested that endogenous GLU preferentially acts through NMDA receptors to
decrease DA metabolism in the rat FC. A similar theory was suggested by Feenstra et al. (1995), who explained NMDA-induced decreases in FC DA as a result of an indirect action via an inhibitory interneurone or polysynaptic circuit. Infusion of the NMDA antagonist cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS-19755) into the FC resulted in a concentration-dependent increase in DA, which could be completely inhibited by the sodium channel blocker tetrodotoxin (Nishijima et al. 1994). These findings suggest that DA neurones projecting to the FC may be under tonic trans-synaptic inhibition exerted by excitatory amino acid neurotransmission via the NMDA receptor at the level of dopamine terminal fields (Nishijima et al. 1994).

As with 5-HT, NMDA-evoked DA release has been shown to be regulated by NO in the RN and FC (Smith & Whitton 2001). NO is observed to have a biphasic effect on DA release and although an explanation for these results is unclear it has become apparent that the NMDA receptor exists in a number of potential subtypes. The molecular basis of differing NMDA receptor populations is believed to be due to the presence of different receptor subunits in a hetero-oligomeric receptor complex (Monaghan & Buller 1994). Although the role of individual subunits in generating physiological and pharmacological properties is currently unknown (Monaghan & Buller 1994), some, but not all, nNOS within spiny neurones coexist with the NR1 subunit of the NMDA receptor in the cerebral cortex (Aoki et al 1998). Choi et al. (2000) have recently identified a cysteine residue (cys399) on the NR2A subunit that is essential for s-nitrosylation (NO\(^+\) transfer) and allows modulation of the NMDA receptor ion channel by NO. It is therefore possible that the biphasic modulation of DA release could be a result of the redox state of the subtype of NMDA receptor present and the degree of sensitivity of different subtypes to NO (Jones et al. 1994).

A number of studies support the modulation of DA release by NMDA (Feenstra et al. 1995, Kretschmer 2000, Kretschmer et al. 2000, Pallotta et al. 1999a, b, Smith & Whitton 2001, Whitton 1997, Whitton et al. 1992b) and it is therefore a logical progression to study the roles of NMDA receptors in the development of DA receptor sensitivity induced by the chronic administration of antidepressants.
The infusion of 25\(\mu\)M NMDA into the RN of animals that had been acutely dosed with CIM potentiated the decrease in extracellular DA levels in both the RN and FC, confirming the studies of Pallotta et al. (1999a) in the RN. However this effect was abolished following sub-chronic (4days) CIM treatment, levels of DA remained decreased in the RN, but were increased in the FC. The infusion of 100\(\mu\)M NMDA had no significant effect on DA release in either region and the effect of NMDA was therefore abolished. It is possible that the action of CIM had exhausted stores of DA in these regions, as the effect of sub-chronic CIM was similar to that of 100\(\mu\)M NMDA on controls (Pallotta et al. 1999a). However the mechanisms by which pre-FC DA terminals release a larger proportion of their storage pool compared to other meotelencephalic DA terminals is unknown (Cubeddu et al. 1990). A study of three functionally distinct regions rich in DA axon terminals were studied using superfusion (Cubeddu et al. 1990). They showed that DA release from the medial pre-FC may be modulated by presynaptic receptors (D\(_2\)) and this effect was not observed in nigrostriatal or mesolimbic terminal regions (Cubeddu et al. 1990). Therefore it was concluded that as drug effects on autoreceptors are highly dependent on the rate and duration of stimulation applied to a specific neuronal group, this form of modulation may affect the type and magnitude of effect produced by a therapeutic agent (Cubeddu et al. 1990). Following chronic treatment (14days), levels of DA had dose-dependently increased in the RN and FC and the effects of 100\(\mu\)M NMDA infusion into the RN were abolished in this region. However in the FC, extracellular DA release was further potentiated in a manner similar to that observed following 100\(\mu\)M NMDA infusion into controls. It is therefore clear that during chronic CIM treatment, marked and qualitative adaptive changes are occurring in the manner in which NMDA receptors regulate the release of DA and this may also explain the effect of chronic CIM on basal DA release in the RN and FC (see above and Del Arco & Mora 1999, Feenstra et al. 1995, Nishijima et al. 1994). A large number of studies suggest that antidepressant-induced changes in the mesolimbic DA system depend on the stimulation of NMDA receptors. Studies with MK801 have shown that chronic but not acute treatment prevents the development of the behavioural supersensitivity to DA agonists induced by chronic imipramine treatment (D'Aquila et al. 1992, De Montis et al. 1993) and by repeated ECT (D'Aquila et al. 1997, Nomikos et al. 1992). De Monitis et al. (1993) also showed that the chronic administration of MK801 blocked changes in D\(_1\) receptor number and
adenylyl cyclase response to DA stimulation induced by chronic imipramine treatment. D'Aquila et al. (2000) have speculated that a "learning process" may be involved in both the pathogenesis of mood disorders and also the mechanism of action of antidepressants. This is primarily based on the antagonism of the effect of imipramine in the rat learned helplessness model by MK801 (Meloni et al. 1993). However, in contrast, other studies have shown that NMDA receptor antagonists display antidepressant-like activity in a number of animal models of depression (Maj et al. 1992a, Papp & Moryl 1994, Trullas & Skolnick 1990). These results are confirmed by a study of acute administration of MK801 and desipramine that showed increased extracellular DA levels in the pre-FC (Wedzony & Golembiowska 1993).

By observing the effects of NMDA and CIM treatment on GLU release in the RN and FC, the degree of NMDA receptor involvement in antidepressant action can be studied. It should however be remembered that glutamatergic transmission includes other receptors as well as the NMDA receptor and although the evidence supporting the involvement of the NMDA receptor in the aetiology of depression is strong, contributions from other glutamatergic receptors, e.g. AMPA/Kainate, may also play a role. It has been shown here that infusion of 100μM NMDA into the RN leads to a decreased extracellular release of GLU in the RN and FC. At lower concentrations of NMDA (25μM) levels in the FC are still decreased but to a greater degree than after 100μM NMDA. In the RN the levels of GLU are increased following 25μM NMDA infusion and this is a complete reversal of the effects of 100μM NMDA in this region. This suggests differences in NMDA receptor sensitivity in the brain regions studied and this may be due to the individual subunits making up the receptor. A review of the literature failed to find any similar studies but Palmer et al. (1989) showed that infusion of NMDA into the FC caused a substantial increase in aspartate release in the neostriatum of the rat. The administration of GABA antagonists to the neocortex evoked similar selectivity resulting in an increase in extracellular aspartate, but not GLU (Palmer et al. 1989). Co-administration of NMDA with GABA antagonists did increase the extracellular concentrations of both aspartate and GLU (Palmer et al. 1989), suggesting that a complex system of modulation exists in this region. A study by Dijk et al. (1995) also reported dose-dependent increases in striatal excitatory amino acids following topical application of NMDA over the frontal cortex, but due to the nature of the study the concentrations of NMDA (2 and 20mM) involved were substantially...
higher than those employed in the current study. Co-application of tetrodotoxin was shown to block the increase, suggesting that it was due to an exocytotic mechanism in the striatum (Dijk et al. 1995). The increase was hypothesised to be due to an increase in the activity of the corticostriatal pathway (Dijk et al. 1995) and this was tested by the co-application of the 5-HT$_{1A}$ antagonist, WAY100135 with the lower dose of NMDA. The cell bodies of corticostriatal neurones are known to be enriched with these receptors and the effects of the 5-HT$_{1A}$ agonist 8-OH-DPAT were also tested. WAY100135 was observed to block the effects of NMDA on GLU release, whilst co-application of 8-OH-DPAT was observed to have no significant effect on NMDA-evoked release of aspartate or GLU (Dijk et al. 1989). It was therefore concluded that a selective 5-HT$_{1A}$ antagonist can increase the activity of corticostriatal pyramidal neurones and this would explain the increased levels of GLU in response to the infusion of NMDA (Dijk et al. 1995).

Following the sub-chronic (4 days) administration of CIM, the effects of infusing NMDA into the RN were abolished in both brain regions studied. Levels of GLU were increased and decreased in the FC and RN respectively following CIM treatment and the abolition of the NMDA effect on GLU suggests that changes in NMDA-modulated transmitter release are occurring, confirming studies that show that antidepressants affect NMDA receptor function (Paul et al. 1994). It would have been interesting to see if these effects changed over the time course of a chronic study but the data presented here represents a potential argument for an interactive involvement between GLU and monoamines in the aetiology and treatment of depression. The interaction between GLU and DA is well recognised (Biggs & Starr 1997, Hu et al. 1999, Wu et al. 2000) and studies have shown DA release to be modulated by GLU (Hu et al. 1999, Kretschmer 2000, Wu et al. 2000). Studies have also shown GLU release to be regulated via 5-HT$_{1A}$ receptors in guinea pig dentate gyrus (Matsuyama et al. 1996), supporting the suggestion that a dysfunctional interaction between glutamatergic and serotonergic transmission, possibly at the level of the RN, may be significant in the aetiology of depression (Pallotta et al. 2001).

In summary these results have shown that the release of 5-HT, DA and GLU can be modulated by NMDA receptors and that some of the effects observed may be due to interactions between transmitter systems. The effects of NMDA are altered over the period of chronic administration of CIM and this suggests that the antidepressant is
inducing adaptive changes within the system, as well as supporting the hypothesis of NMDA receptor involvement in depression.

3.3.4 Effects of NMDA antagonists on basal and clomipramine-induced neurotransmitter release in the frontal cortex and raphe nuclei

Based upon previous studies and the literature a representative from each of the major classes of NMDA receptor antagonist was used to study the effects of NMDA receptor antagonists on neurotransmitter release.

Trullas & Skolnick (1990) suggested that NMDA receptors may be involved in the behavioural deficits induced by inescapable stress. This led to the suggestion that pathways subserved by the NMDA receptor may be involved in the pathophysiology of depression and that substances which reduce transmission at these receptors may represent a new class of antidepressants (Trullas & Skolnick 1990). Studies of the effects of NMDA antagonists on transmitter release have mainly been confined to the study of MK801 (Callado et al. 2000, Löschner et al. 1991, Wedzony & Golembiowska 1993, Wedzony et al. 1997, Whitton et al. 1992a,b, Yan et al. 1997). Other NMDA receptor antagonist studies include ketamine (Lindefors et al. 1997), CGP40116 (Wedzony et al. 1996), (+)-HA-966 (Goldstein et al. 1994), amantadine (Gianutos et al. 1985, Quack et al. 1995) and memantine (Quack et al. 1995, Spanagel et al. 1994). MK801 has been shown to increase extracellular 5-HT in both the hippocampus, striatum (Whitton et al. 1992a), nucleus accumbens (Yan et al. 1997) and RN (Callado et al. 2000) and also to increase the number of 5-HT\textsubscript{1A} receptors in the whole brain (Wedzony et al. 1997). A study of the effect of amantadine on the metabolism of 5-HT in rat brain found no significant effect on 5-HT levels or 5HTP decarboxylase activity (Tanaka et al. 1973). However 1 hour after 100mg/kg amantadine, 5HIAA and MAO levels were reduced (Tanaka et al. 1973). As MAO inhibition induced 5-HT accumulation in the brain was enhanced by amantadine treatment it was concluded that amantadine affects the serotonergic system (Tanaka et al. 1973). Extracellular levels of DA have also been shown to be affected by NMDA receptor antagonists. MK801 has been observed to increase DA metabolism in several brain regions including FC, nucleus accumbens and striatum (Callado et al. 2000, Löschner et al. 1991, Wedzony et al. 1994, Yan et al. 1997), although Whitton et al. (1992b) noted a decrease in striatal
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DA release, despite MK801 inducing the intense circling behaviour often associated with increased DA. In the substantia nigra neither MK801 nor CGP40116 had any effect on DA release in intact rats (Biggs et al. 1996). Ketamine has been observed to increase DA levels in the FC (Lindefors et al. 1997), as have amantadine and memantine in the FC (Spanagel et al. 1994) and striatum (Quack et al. 1995, Spanagel et al. 1994). These studies suggest that the effects of NMDA antagonists may be region-specific and this may be important in developing new antidepressant therapies.

A quantitative autoradiographic study was used to examine the regional binding characteristics of a diverse group of NMDA receptor channel blockers including MK801, amantadine and budipine (Porter & Greenamyre 1995). The results provided further support for the theory that cerebellar and forebrain NMDA receptors have different pharmacological properties.

It is interesting to note that Vasiliadis et al. (1999) suggested that an interaction between DA and GLU receptors occurs following treatment with NMDA antagonists, including MK801. Using quantitative ligand binding autoradiography they observed that MK801 and 3-carboxy-piperazin-propyl phosphonic acid (CPP) did not have different profiles of action. A significant negative relationship between NMDA receptors and DA (D₁ and D₂) receptors was recorded in the neostriatum, suggesting an interrelationship between DA and NMDA receptors is highly controlled (Vasiliadis et al. 1999).

Van Lookeren Campagne et al. (1995) studied the effect of acute and long-term treatment of neonatal rats with NMDA receptor antagonists (e.g. MK801) on changes in NMDA receptor properties. They showed that neither the single or subchronic treatments exerted a significant influence on the density of antagonist binding sites or on the modulation of [³H] MK801 binding by GLU, Mg²⁺ or D-CPPe (Van Lookeren Campagne et al. 1995). From these data they concluded that in vivo neonatal treatment (i.e. during the period when expression of these receptors is subject to developmental regulation) with NMDA receptor antagonists does not significantly alter the properties and densities of NMDA receptors in the cerebral cortex and CA1 region of the hippocampus (Van Lookeren Campagne et al. 1995). This may be important in understanding the involvement of NMDA receptors in the pathophysiology of depression.
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CGP40116 is a competitive antagonist of the NMDA receptor and was observed to decrease dialysate FC levels of 5-HT, DA and GLU. In the RN a decrease in extracellular levels of 5-HT and GLU was also observed, but CGP40116 had no significant effect on DA in this region. On the basis of the results of this acute experiment, administration of CGP40116 alone does not seem to increase the levels of 5-HT and DA in the FC, as would be expected for antidepressant effects to occur. The observations are similar in some ways to those observed with NMDA and CIM alone, as may be expected from evidence already presented. The effect of CGP40116 on GLU release was unexpectedly similar to that observed after 100µM NMDA infusion and suggests that interactions are occurring between the systems regulating GLU release. Biggs et al. (1996) showed that NMDA receptor antagonists, including CGP40116 (10µM, via the dialysis probe), increased the release of dopamine in the substantia nigra of reserpine-treated rats, but did not affect the release of DA or its metabolites in intact rats. They suggested that the facilitation of DA formation from L-3,4-dihydroxyphenylalanine (L-DOPA) by the NMDA antagonists in the substantia nigra might explain the enhancement of L-DOPA’s antiparkinsonian activity by these compounds in behavioural experiments. In 1994, Papp & Moryl studied the antidepressant activity of NMDA receptor antagonists in a chronic mild stress model of depression. Using CGP40116 as an example of a competitive NMDA receptor antagonist, they showed that chronic treatment (5 weeks, 25mg/kg p.o., twice daily) reversed chronic mild stress-induced anhedonia in a manner similar to imipramine (10mg/kg i.p. or p.o.) in terms of time course and magnitude. This confirmed the results of previous studies on ‘normal’ animals that suggested that NMDA receptor antagonists may exhibit antidepressant properties (Leander 1989, Sills & Loos 1989, Reynolds & Miller 1988, White et al. 1990). It was therefore interesting to study the effect of CGP40116 on CIM-induced changes in transmitter release to see if any positive correlation could be found. CGP40116 administered in this way was observed to decrease FC levels of both 5-HT and DA, which was the same effect observed following CIM and CGP40116 alone. However the CIM-induced decrease in GLU was abolished following the injection of CGP40116 and FC GLU levels were not significantly changed from those of basal. It is also interesting to note that co-administration of CGP40116 and CIM abolishes the CGP40116-induced decrease in GLU in this region. This may suggest that CIM is interfering with the action of CGP40116, possibly by inducing other cellular events, e.g. second messenger.
formation, which, in turn, block the action of CGP40116. In the RN extracellular concentrations of DA and GLU were decreased following CIM and CGP40116 administration, an effect observed for DA after CIM alone but not for GLU. RN 5-HT was increased by the treatment and this reversed the effect of CGP40116 alone, although it had no significant effect on the CIM-induced release. Due to serotonergic transmission between the RN and FC, any increase in the RN would be expected to result in decreased FC 5-HT levels and it is this effect that is believed to be overcome by chronic antidepressant treatment. As has been stated earlier, a reduction in terminal monoamine release may be responsible for the development of depression. These results therefore show that the addition of a competitive NMDA antagonist to CIM may not be a suitable treatment for depression and will be unlikely to increase the effectiveness of the antidepressant. However the results are interesting in terms of the involvement of the NMDA receptor in the mechanism of action of antidepressants and also in suggesting that a subtle change in NMDA receptor function may be required for clinical benefit. As studies outlined above have shown, CGP40116 has displayed antidepressant activity in a number of animal models of depression, however this does not seem to be due to increased FC levels of monoamines which are widely believed to be necessary for increased mood. On the basis of the results observed in this study I would therefore suggest that this type of NMDA antagonist would be unsuitable as an antidepressant treatment and would not reduce the 'lag period' currently observed between the start of antidepressant treatment and onset of antidepressant action.

Non-competitive antagonists of the NMDA receptor can be further divided and the first group to be examined were the ion channel blockers. Amantadine is believed to interact with the MK801 binding site in the NMDA receptor channel, thus blocking the flow of ions through the receptor. When administered alone it was observed to have no significant effect on 5-HT release in either the FC or RN, or on RN levels of DA and GLU. Although no definite effects were noted in the FC, there were trends of an increase and decrease in levels of DA and GLU respectively. When compared to the effects of CGP40116 no real similarities can be observed. Vale et al. (1971) suggested in a letter to the Lancet that amantadine may be useful in the treatment of depression, but it was not until more recently that its potential antidepressant properties were put to the test (Huber et al. 1999, Moryl et al. 1993). Adequately performed clinical studies on the use of amantadine as an antidepressant are small in number, however indirect
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evidence has shown that amantadine may be useful in the treatment of depressive symptoms. The effect of amantadine on CIM-induced neurotransmitter changes proved to be interesting, especially in terms of 5-HT. Amantadine was observed to abolish the CIM-induced increase in RN 5-HT release and also reverse the consequent decrease in FC 5-HT. Levels of FC 5-HT were seen to be significantly increased following acute treatment with CIM and amantadine, without the usual ‘lag period’ associated with CIM treatment. However a similar effect on DA was not observed, CIM-induced changes in DA levels were unaffected by amantadine treatment, although the magnitude of the decrease in the FC was significantly reduced, suggesting that changes in DA release may take longer and be the result of adaptive changes in other transmitter systems. CIM-induced GLU levels were unaffected in the FC but an increase in RN levels was recorded, similar to that observed following the local infusion of NMDA (25μM). These results support the hypothesis of an interaction between serotonergic, dopaminergic and glutamatergic transmitter systems playing a role in the regulation of 5-HT and DA transmission. The principal observation of this study is that prior treatment with amantadine altered the effect of CIM on RN and FC 5-HT release and also that of GLU in the RN. By combining these drugs, the latent period typically required before increased cortical 5-HT release is observed following treatment with CIM is abolished. This may suggest that amantadine, possibly by decreasing NMDA receptor function, prevents the CIM-induced increase in RN 5-HT and the consequent activation of 5-HT1A autoreceptors. As desensitisation of RN 5-HT1A receptors appears to be a requirement for antidepressant action (Artigas et al. 1996), therapies which decrease the latency of this process may be of future clinical benefit. Such therapies include the 5-HT1A receptor antagonists pindolol and WAY100635 (Artigas et al. 1994, Blier & Bergeron 1995, Blier et al. 1997, Cryan et al. 1999, Gartside et al. 1995, Millan et al. 1998, Romero & Artigas 1997, Romero et al. 1996). Both have been shown to potentiate antidepressant action, although pindolol is not, as originally thought, a pure antagonist of the 5-HT1A receptor (Newman-Tancredi et al. 1998). It is worth noting that a number of studies have suggested a number of dopaminergic effects of amantadine, e.g. inhibition of uptake, stimulation of uptake and altered receptor confirmation (Allen 1981, Gianutsos et al. 1985, Heikkila & Cohen 1972), and this may explain the effects of amantadine on dopaminergic transmission. A study of two of the enzymes important in monoamine biosynthesis, L-DOPA and 5-HTP decarboxylase (DDC and 5HTPDC), in the substantia nigra and corpus striatum of reserpine-treated
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Rats showed a strongly increased DDC activity in response to amantadine, whilst not affecting or decreasing 5HTPDC in the nigra and striatum (Fisher & Starr 2000). Fisher & Starr (2000) concluded that GLU exerts a differential physiological influence on the biosynthesis of DA and 5-HT in the brain, by tonically suppressing DDC and tonically stimulating 5HTPDC. From the results of this in vivo study I would suggest that the ion channel blocker class of NMDA receptor antagonists displays properties that may be important in understanding the aetiology of depression and of improving its treatment. These effects may be due to an indirect action of amantadine, e.g. intracellular signalling cascades leading to changes in protein phosphorylation or a direct consequence of its channel blocking action on levels of Ca$^{2+}$.

A second type of non-competitive NMDA receptor antagonist are the GLY site antagonists. D-cycloserine was shown to increase FC DA levels but to have no effect on RN DA, GLU or FC GLU levels. Extracellular RN 5-HT levels were observed to increase and a consequent decrease in FC release was noted. Antidepressant-like effects have been observed in an animal model of depression after chronic dosing with D-cycloserine (Papp & Moryl 1996). 10mg/kg i.p. was shown to increase sucrose intake in the chronic mild stress model, but the magnitude was less than that observed with imipramine (10mg/kg i.p.). The effects of D-cycloserine administration were variable and not believed to be dose-dependent as lower (2.5mg/kg i.p.) and higher (40, 100mg/kg i.p.) doses were ineffective (Papp & Moryl 1996). The effect of D-cycloserine on CIM-induced changes in transmitter release did not suggest an enhancement of antidepressant action for this combination. No change from basal levels was observed for either DA or GLU and levels of 5-HT in both the RN and FC were significantly decreased. The reduction in RN 5-HT is interesting as it the opposite of that observed after both CIM and D-cycloserine alone. D-cycloserine is known to have agonist properties at glycine$_B$ receptors but this would not explain the decrease as it was not observed when D-cycloserine was administered alone. Although not studied here CIM is known to have a noradrenergic effect, NA is known to regulate the serotonergic output from the RN (Haddjeri et al. 1996) via a presynaptic interaction and it may be the combination of D-cycloserine and CIM affecting noradrenergic transmission which results in the changed RN 5-HT levels. The abolition of effects of D-cycloserine or CIM on DA and GLU also raises interesting questions and suggests a complex system of interaction between transmitter systems. Although there is evidence for D-cycloserine
exhibiting antidepressant-like effects (Papp & Moryl 1996), on the basis of the results discussed here I would not consider the addition of a GLY site antagonist to CIM treatment of benefit in depression therapy. This may be due to the action of the antagonist at other sites besides the NMDA receptor, or more likely is due to its inability to modulate the intracellular cascades initiated by NMDA receptor activation.

Ifenprodil was originally developed as an anti-ischemic agent and is already in clinical use (Otomo et al. 1985). Its improved p.o. bioavailability allows more protection of the brain from neuronal loss after focal ischaemia in the cat and rat (Gotti et al. 1988). The effect on cerebral blood flow (Delage et al. 1985) is believed to be a result of interaction with α-adrenoceptors and voltage sensitive Ca^{2+} channels (Adeagbo & Magbagbeloa 1985, Honda & Sakai 1985). As well as its neuroprotective action, ifenprodil has been reported to act as an NMDA receptor antagonist. Although its precise mechanism of action is unclear (Gotti et al. 1988, Carter et al. 1988), it is thought to act at the polyamine site of the NMDA receptor (Carter et al. 1989). When given acutely, it induces an increase in the extracellular levels of 5-HT and DA in the RN. In the FC there is a consequent increase in 5-HT levels, but levels of DA do not change significantly from basal. GLU levels are only affected in the FC, where a decrease is observed. Although a review of the literature revealed no known antidepressant-like effects of ifenprodil, its profile as a NMDA receptor antagonist suggested that it might be of benefit in increasing the effectiveness of current antidepressant treatments. CIM-induced changes in transmitter release were observed following ifenprodil treatment. DA levels in both the RN and FC were significantly increased, reversing the effect of CIM, as were levels of GLU. It seems that the combination treatment has effects that are not seen following the administration of CIM or ifenprodil alone, suggesting that drug/transmitter interactions may be occurring. CIM-induced 5-HT release, however, was unaffected by ifenprodil administration and levels in the FC remained significantly decreased. The site of action of ifenprodil within the NMDA receptor complex remains obscure, but it has been suggested that antagonist activity is exerted at an allosteric polyamine site that positively modulates the NMDA receptor (Carter et al. 1989). A recent study suggests that zinc and ifenprodil allosterically inhibit two separate polyamine-sensitive sites at the NMDA receptor complex. It was concluded that zinc reduces NMDA receptor channel opening by allosteric inhibition of a polyamine-sensitive site different from that inhibited by ifenprodil and that the two sites influence
each other in a manner dependent on the brain region being investigated. Thus the
different percentages of zinc/ifenprodil inhibition in different regions may be explained
by the different percentages of NMDA receptor subtypes present (Berger & Rebernik

From these results I would not suggest the use of ifenprodil to potentiate the
effectiveness of CIM treatment. Although DA levels are significantly increased in the
FC, the degree of dopaminergic involvement in depression is still under discussion as
no hypothesis based upon a single transmitter system can be considered to fully explain
the therapeutic effects of antidepressants or the mechanisms underlying the
pathophysiology of the disorder (D’Aquila et al. 2000). Dopamine has been proposed to
play a role in depressive symptoms and in the antidepressive effect of drugs (D’Aquila
et al. 2000, Willner 1995b) and should therefore be considered in studies investigating
the aetiology of this disorder. This form of treatment (ifenprodil augmentation of CIM
therapy) resulted in decreased extracellular 5-HT in both of the regions studied and on
the basis of current evidence I would suggest that as 5-HT plays a major role in
depression aetiology these results do not suggest that ifenprodil would be of benefit in
this situation. Studies have shown ifenprodil to be highly selective for one particular
subunit of the NMDA receptor – NR2B (Williams 1993), and this property may be of
significance. It is also important to note that ifenprodil has been shown to alter cerebral
blood flow (Delage et al. 1985) by interaction with α-adrenoceptors and possibly
voltage-sensitive Ca^{2+} channels (Adeagbo & Magbagbeola 1985, Honda & Sakai 1985),
these properties too may explain the actions of ifenprodil on transmitter release. If
ifenprodil was shown to interact with voltage-sensitive Ca^{2+} channels then it may be a
common link between ifenprodil and amantadine, which is known to block the NMDA
receptor ion channel. It may well be that the selectivity of amantadine for the NMDA
receptor ion channel confers some advantage over ifenprodil, and this advantage is
displayed in the different overall effects of the two compounds.

In summary these results have shown that the addition of an NMDA receptor antagonist
will effect the release of transmitters induced by acute CIM treatment. However the
only compound to increase 5-HT release in the important area of the frontal cortex was
amantadine. This compound was not observed to increase DA at this stage, but the
effect of CIM was significantly reduced. This supports the study of Wedzony &
Golembiowska (1993). The interaction between the antidepressant desipramine and the non-competitive NMDA receptor antagonist MK801 was studied at the level of FC DA release. Although both compounds only elicited a weak enhancement of FC DA release when administered alone, co-administration resulted in a pronounced enhancement of extracellular DA (Wedzony & Golembiowska 1993). Of the other NMDA receptor antagonists tested here only ifenprodil increased FC DA levels, it had no positive effect on 5-HT levels however and therefore I do not believe its use to be of benefit. To my knowledge this study is the first to show NMDA receptor antagonist activity effecting antidepressant-induced release of 5-HT, DA and GLU in the FC and RN and also to suggest that only one type of NMDA receptor antagonist (i.e. amantadine) may be of benefit in the treatment of depression.

3.3.5 Effects of sub-chronic amantadine treatment on sub-chronic CIM-induced changes in transmitter release in the frontal cortex and raphe nuclei

From the results of the acute studies of the effects of NMDA antagonists on CIM-induced transmitter release, it was decided to see whether the increase observed with amantadine was still present following sub-chronic dosing and if so was the increase in FC 5-HT significantly higher than observed with CIM at the same time point. Studies of the pharmacological effects of 6weeks administration of amantadine were evaluated in the mouse by Gianutsos et al. (1985). They observed that motor stimulation was differentially affected – amphetamine and memantine-induced stimulation was reduced, whilst that of apomorphine was enhanced (Gianutsos et al. 1985). This was accompanied by increased binding of spiroperidol to striatal DA receptors, without affecting amantadine-induced inhibition of DA uptake in the striatum (Gianutsos et al. 1985). From this data it was suggested that amantadine acts at a postsynaptic site within the membrane adjacent to (or otherwise influencing) the actual recognition site (Gianutsos et al. 1985). If the chronic use of amantadine increases DA receptor number, then this would be expected to contribute to the therapeutic usefulness of amantadine by enhancing receptor activity produced by residual DA or other drugs (Gianutsos et al. 1985). Sub-chronic (4day) dosing of amantadine alone resulted in decreased FC 5-HT and DA and also RN DA. Levels of RN 5-HT and GLU were observed to increase following the treatment. These results do not correspond with those observed following the acute injection of amantadine, suggesting adaptive changes are occurring after as
little as 4 days. These changes may be within the NMDA receptor itself, in transmitter interactions or even changes in intracellular signalling cascades. Amantadine had no effect on 5-HT in either region when given acutely, following 4 days of dosing effects similar to those observed after acute CIM are recorded. These effects are not the same as those resulting following 4 days of CIM dosing, suggesting that the two drugs are exerting different effects on the system. NMDA infused via the RN probe was shown to increase 5-HT release in the FC suggesting that, in this region, the antagonism of the NMDA receptor may only be partial. Changes in DA levels resemble those observed after acute CIM treatment, but again are different to those observed after sub-chronic treatment, NMDA had no effect on DA release, suggesting that glutamatergic control of DA release is somehow blocked. Amantadine administered sub-chronically increases RN GLU, an effect not observed after acute treatment and this again suggests adaptive changes are occurring during the 4 days of treatment. This is backed up by the abolition of the decreasing trend in the FC observed following acute amantadine. GLU levels are increased by NMDA, again suggesting that full antagonism of the NMDA receptor has not occurred, there is however the possibility that the inhibition of the NMDA receptor transfers modulation of GLU release to other glutamatergic receptors, e.g. AMPA/Kainate, and increased release is the result.

The effect of sub-chronic dosing of amantadine and CIM was interesting. Levels of 5-HT in the RN remain increased and the consequent decrease in FC levels was still observed. However, although the levels of 5-HT are not in the region of those observed following 14 day CIM dosing, they are significantly higher than those observed following sub-chronic CIM treatment alone, suggesting that some changes in release are beginning to occur. Levels of GLU are reduced in both regions and the infusion of NMDA has no effect, suggesting that a reduction in glutamatergic transmission is occurring at this time point, presumably due to antagonism of the NMDA receptor. As the results do not reflect those seen with either CIM or amantadine alone, it raises the possibility of an interaction between the two drugs and/or the NMDA receptor as an explanation of these results. Disappointingly the levels of DA remain significantly decreased in both brain regions, as seen with sub-chronic amantadine treatment. This may be a reflection of how DA release is controlled by other transmitters and therefore any effect may be delayed. The effect of NMDA infusion is blocked in the RN but still occurs in the FC, possibly suggesting that the effects of amantadine may be dependent
on individual NMDA receptor subtypes present and that NMDA receptors present in the RN may be of different composition to those in the frontal cortex. This may affect their sensitivity to drugs such as amantadine. This theory was discussed briefly above and will be further discussed in chapter five, along with the reboxetine results.

In summary the present findings support the involvement of the NMDA receptor in the mechanism of action of the antidepressant CIM. Although the combined treatment with amantadine does not increase levels of 5-HT in the frontal cortex to above basal within 4 days, it does result in 5-HT levels significantly higher than those seen with CIM alone at the same time point. It is worth noting that amantadine is a weak NMDA receptor ion channel antagonist and therefore the development of more selective antagonists (without the side effects observed with MK801, for example) could result in a more substantial effect on both cortical 5-HT and DA.

### 3.3.6 Effects of chronic clomipramine treatment on body weight

Over 14 days of CIM treatment, average body weight was seen to increase at an overall rate of approximately 50% of that of control animals. Weight gain is a frequent and inconvenient side effect of TCA treatment (Berken et al. 1984) that is thought to be related to 5-HT2 and histamine receptor antagonism (Bernstein 1988, Fernstrom 1995, Garland et al. 1988). An attempt to model this effect in experimental animals used two commonly used TCAs, amitryptyline and desipramine (Nobrega & Coscina 1987). Chronic treatment failed to show an increase in food intake or rates of body weight gain, despite manipulations of drug doses, route of administration, diet composition and palatability, animal sex and housing conditions. Desipramine treatment was observed to cause decreased food intake and weight loss, similar to the CIM-treated rats in our study. Although a search of the literature failed to find any studies of the effect of chronic CIM treatment on animal body weight, a number of clinical studies are available (Beaumont 1977, Lacey & Crisp 1980, Shioiri et al. 1993). All failed to record an increase in weight gain following treatment with CIM, indeed Shioiri et al. (1993) failed to record a weight gain in response to any of the TCAs examined (imipramine, clomipramine and desipramine). A double-blind controlled study examined the impact of CIM when taken by an anorexia nervosa population and noted that CIM was significantly associated with increased hunger, appetite and energy intake (Lacey &
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Crisp 1980). However it tended to be associated with a reduced rate of weight gain, possibly due to an increase in activity. CIM is known to act at the hypothalamic level and is thought to influence hunger, appetite and dietary intake according to the body weight of the patient (Lacey & Crisp 1980). The results of these studies, indicating a reduction in weight gain following chronic CIM treatment confirm the results of the 14day study and may be of importance in studies of eating disorders, such as anorexia nervosa and bulimia. However a number of long-term studies of TCA treatment suggest an inducement of excessive body weight gain (Nobrega & Coscina 1987) and possible reasons for differences between animal and human data are discussed. Three possibilities account for these differences. One, a fundamental difference between the general pharmacology of antidepressants in humans and rats may be occurring. For example, amitryptiline would be expected to significantly change appetite and body weight in normal human volunteers, but not necessarily in rats (Nobrega & Coscina 1987). Two, the food intake and body weight effects of antidepressants in humans may result from some interaction between the drug effects and specific physiologic alterations associated with depression (Fernstrom et al. 1985, Nobrega & Coscina 1987). Finally food intake and body weight changes in humans may not reflect a direct effect on internal physiological controls of hunger. Nobrega & Coscina (1987) suggested that amitryptiline treatment may not elicit chronic hunger and/or decreased metabolic activity. Instead it may sensitise the organism towards acute stimuli capable of eliciting food intake, and is confirmed by their studies of increased caloric intake in response to a single systemic injection of the glucoprivic agent 2-deoxy-D-glucose after chronic antidepressant treatment (Nobrega & Coscina 1987).

3.4 Conclusions

1) CIM treatment modulates transmitter release in the FC and RN as measured by in vivo microdialysis in freely moving rats. Over a 14day period its effects can be observed to change suggesting adaptation is occurring within the system.

2) NMDA receptor activation also results in neurotransmitter release in these brain regions.

3) NMDA-evoked release of DA and 5-HT may be linked to the release of GLU in these structures.
4) The involvement of NMDA receptors in antidepressant action has been demonstrated.

5) Each class of NMDA receptor antagonist can be shown to effect transmitter release in the FC and RN.

6) Addition of amantadine to a CIM treatment regime may reduce the latent period observed before increased cortical monoamine levels are observed.

7) Antidepressant treatment may be dependent on NMDA receptor subtypes present in important brain areas.

8) Studies of weight gain following chronic CIM treatment showed a reduced rate of weight gain. This may be important in determining suitability of this drug for the treatment of depression in some categories of patients.
Chapter Four

Effects of the NMDA antagonist amantadine on reboxetine-induced monoamine and amino acid release in the raphe nuclei and frontal cortex as measured by in vivo microdialysis in the freely moving rat
4.1 Introduction

Prior to 1980 antidepressant medication consisted primarily of TCAs, MAOIs, and lithium (Kent 2000). These compounds possess a number of side-effects which are believed to be due to their binding to multiple unrelated receptors e.g. muscarinic cholinergic, H1-histaminergic, and α₁-adrenergic receptors. The introduction of the SSRIs in the late 1980s revolutionised the treatment of depression and drugs such as fluoxetine are now considered to be the mainstay of antidepressant therapy. SSRIs display reduced side-effects in comparison to TCAs and MAOIs, although they fail to elicit a response in many of the most severely effected (Anderson 1998).

Considerable research has been directed towards the discovery of new antidepressants with faster onset of action, fewer anticholinergic side-effects, less cardiotoxicity, and greater therapeutic efficacy. A series of α-ariloxy-benzyl derivatives were synthesised (Melloni et al. 1984) and the diastereo-isomer RS, RS 2 [α-(2-ethoxy-phenoxy)-benzyl]morpholine (reboxetine – figure 4.1) (Melloni et al. 1985) was shown to display potent activity in two of the classical laboratory tests used to predict potential antidepressant efficacy in man (anti-reserpine test and selective inhibition of NA reuptake in vivo and in vitro). Reboxetine shows structural similarities with other selective NA reuptake inhibitors (NRIs) including viloxazine, nisoxetine, and tenilozazine, as well as with the SSRI fluoxetine (Dostert et al. 1997). It displays two chiral centres, but, due to the regio- and stereospecificity of the key reactions in its synthesis (Melloni et al. 1985), only two enantiomers are present in reboxetine, which, like fluoxetine, exists as a racemic mixture (Fuller et al. 1991).

Metabolism of reboxetine is via three major pathways in humans: 2-O-dealkylation of the ethoxyphenoxy ring, hydroxylation, and oxidation of the morpholine ring (Cocchiara et al. 1991, Holm & Spencer 1999). Similar pathways are followed in rodents, however, substantial differences in the pharmacokinetic parameters and plasma levels normalised for the ratio dose/body weight have been observed between humans and animals (Dostert et al. 1997). In vitro metabolism studies suggest that reboxetine is metabolised by the CYP3A4 isoenzyme (Holm & Spencer 1999), but it is unclear whether the drug itself inhibits CYP3A4 (Holm & Spencer 1999, Rocchetti et al. 1995).
Figure 4.1 Chemical structure of reboxetine (methanesulphonate salt)
R/S indicates the chiral centres. Reproduced from Dostert et al. (1997) and Holm & Spencer (1999).

Studies in healthy volunteers show that 96 hours after single doses of reboxetine, 75% of the total administered drug has been excreted in urine, 6-15% has been excreted in faeces (Cocchiara et al. 1991), and only 9% of the administered dose is excreted unchanged in the urine (Edwards et al. 1995).

There are few available drug interaction studies for reboxetine (Holm & Spencer 1999), however, the extensive binding of reboxetine to α1-acid glycoprotein suggests that reboxetine may interact with drugs displaying high affinity for this plasma protein (Holm & Spencer 1999). Although yet to be determined, this includes drugs such as propanolol, methadone, dipyridamole, imipramine, chlorpromazine, lidocaine and other local anaesthetics (Pharmacia & Upjohn 1998).

The potential antidepressant effect of reboxetine was first demonstrated in two tests, carried out in mice, in which most of the currently used antidepressants are effective (Melloni et al. 1984): antagonism of reserpine-induced hypothermia and blepharospasm, and antagonism of clonidine-induced hypothermia. Riva et al. (1989) looked at the reuptake inhibition of NA, both in vivo and in vitro and showed that reboxetine was as potent as the relatively SNRI desipramine in inhibiting release from
Chapter Four: Reboxetine, amantadine and neurotransmitter release

rat cortical slices and synaptosomes. Acute administration (10mg/kg i.p.) significantly increased the levels of the O-methylated metabolite of NA, normetanephrine (NMN) in the hypothalamus (Riva et al. 1989) and this provided an index of the amount of neurotransmitter released into the synaptic cleft (Mocchetti et al 1981). The level of the other major metabolite of NA, 3-methoxy-4-hydroxy-phenylglycol-(MHPG)-sulphate, formed primarily by the action of MAO type A, was unaffected (Riva et al 1989). Studies after five days of reboxetine treatment (10mg/kg/twice daily i.p.) indicated that reboxetine induced a similar down-regulation of β-adrenergic receptors in cortical membranes to desipramine (7.5mg/kg/twice daily i.p.) and this was paralleled by a desensitisation of NA-dependent adenylate cyclase in cortical slices (Riva et al. 1989).

Connor et al. (1999) were the first to demonstrate that reboxetine attenuates forced swim test-induced behavioural and neurochemical alterations in the rat. They showed that the reboxetine-induced, dose-dependent decreases in immobility were accompanied by increases in the turnover of 5-HT in the FC and amygdala and of DA in the striatum.

A comprehensive study of the pharmacological properties of reboxetine was undertaken (Wong et al. 2000). Pharmacological selectivity for uptake systems was defined by uptake and binding assays for the three monoamine uptake sites, whilst specificity was determined in thirty-nine different receptor and six enzyme assays. Selectivity in vivo was measured by recording neuronal firing rates in discrete brain regions and in vivo pharmacology of reboxetine was defined using reserpine-induced blepharospasm and hypothermia, and clonidine-induced hypothermia. Finally the antidepressant potential of reboxetine was evaluated using three behavioural tests: the tail-suspension test, the forced swim test and the differential reinforcement of low rate 72 (DRL-72) operant responding test. Both the in vivo and in vitro results confirmed earlier studies that suggested reboxetine to be a potent, selective and specific NA reuptake inhibitor. Unlike the TCAs desipramine and imipramine, reboxetine was observed to display little affinity (Kᵢ>1000nmol/L) for muscarinic, histaminergic H₁, adrenergic α₁ and dopaminergic D₂ receptors. Its in vivo action was shown to be consistent with the pharmacological action of an antidepressant with preferential action at the NA reuptake site and has a superior pharmacological selectivity to existing TCAs and SSRIs. In the behavioural tests, reboxetine was observed to significantly decrease immobility in the tail suspension and forced swim tests, whilst increased efficiency in responding was observed in the DRL-72 test (Wong et al. 2000). Lucki et al. (2000) also looked at the effect of reboxetine in the
forced swim test, they confirmed and extended the results above (Wong et al. 2000) by demonstrating that reboxetine (5-20mg/kg) produced a dose-dependent reduction in immobility scores, which was coupled to increased climbing and decreased swimming behaviours. They also demonstrated that the infusion of chemical neurotoxins (DSP-4 and 6-OH-DA) differentially effected the dorsal and ventral noradrenergic pathways. Lucki et al. (2000) suggested that their results show that these two distinct noradrenergic pathways exert opposing influences on the behavioural responses to reboxetine and that the noradrenergic system must be intact to enable the effects of reboxetine to occur.

Evidence for the involvement of BDNF in the pathophysiology of depression has been accumulating (see 1.8.8.1) and a recent study has provided further reinforcement of this theory. The combination of reboxetine (20mg/kg i.p. for 2 or 7 days) with physical activity was shown to rapidly potentiate the expression of BDNF mRNA in rat hippocampus (Alejandre et al. 2000). Previous work by the same group has demonstrated that the combination of antidepressant treatment and physical activity, applied chronically, has an additive, potentiating effect on BDNF expression within several areas of the rat hippocampus (Russo-Neustadt et al. 1999).

Despite the specificity of reboxetine for the NA transporter, the substrates for its pre-clinical pharmacology have been largely unexamined (Lucki et al. 2000). Few studies have looked at the in vivo release of transmitters within the brain following administration of reboxetine and those that have mainly focused on the release of NA. Sacchetti et al. (1999) studied the effects of acute and chronic reboxetine on extracellular NA, 5-HT, and DA, suggesting that reboxetine significantly increased NA levels in the FC and dorsal hippocampus and that this effect was potentiated by the $\alpha_2$-adrenoceptor antagonist idazoxan. Surprisingly they did not record levels of 5-HT or DA in these regions, instead showing that extracellular striatal concentrations of DA and 5-HT were unaffected by reboxetine treatment and therefore that the effect of reboxetine was fairly selective for the noradrenergic system (Sacchetti et al. 1999). It was suggested that combining reboxetine with an $\alpha_2$-adrenoceptor antagonist may facilitate its antidepressant activity and recently the group has suggested that desensitisation of $\alpha_2$-adrenoceptors may be involved (Invernizzi et al. 2000, 2001). Comparing the inhibitory effect of clonidine on FC NA release in rats that had received
chronic reboxetine (10mg/kg/day for 14 days) assessed the activity of α2-adrenoceptors. It was observed that animals who had received vehicle showed decreased NA levels in response to an injection of clonidine, but that the increased NA levels in reboxetine-treated animals were unaffected (Invernizzi et al. 2000). A study of the activity and onset of action of reboxetine and the effect of combination with sertraline in the olfactory bullectomized rat model of depression suggested that both 7 and 14 days of administration initiated changes in 5-HT1A receptor and α2-adrenoceptor activity (Harkin et al. 1999). Lucki & Page (2000) have looked at the effects of reboxetine on both NA and 5-HT in the FC and have shown that a potent, dose-dependent increase in NA is observed in this region, but no significant change in 5-HT was recorded at any of the doses (1-20mg/kg) tested. The effects of reboxetine on DA in the pre-FC were studied by Linner et al. (2000) who observed that reboxetine increases neuronal burst activity in the ventral tegmental area and augments DA outflow in the pre-FC. They suggest that in view of the significance of phasic signalling in mesocorticolimbic DA neurones for accurate reward prediction and of pre-frontal DA for cognitive functioning, these effects of reboxetine may contribute to its clinical effectiveness (Linner et al. 2000). Taken together the results of Harkin et al. (1999), Linner et al. (2000) and Lucki & Page (2000) conflict with the suggestion that reboxetine is selective for the noradrenergic system (Sacchetti et al. 1999).

The effects of NMDA on 5-HT, DA, and GLU release were reviewed in 3.4.3. The NMDA receptor has also been shown to mediate a powerful stimulation of FC and hippocampal NA release in a large number of studies (Fink et al. 1989, 1990, 1992, Göthert & Fink 1991, Jones et al. 1987, Lehmann et al. 1992, Ransom & Deschenes 1988, Yoshida et al. 1997). A study of NA release from rat hippocampal brain slices suggested that this release is modulated by glycine (Ransom & Deschenes 1988). It was shown that the glycine regulatory site associated with the NMDA receptor can be demonstrated in whole brain slices by the use of an antagonist to attenuate the influences of endogenous glycine (Ransom & Deschenes 1988). In human cerebral cortex it has been demonstrated that as well as NMDA receptors, AMPA- and kainate-recognition non-NMDA receptors stimulate the release of NA, even in the presence of concentrations of Mg2+ ions which were observed to completely abolish NMDA-evoked release (Fink et al. 1992). Few studies of NMDA receptor involvement in NA release following antidepressant treatment are available. However Harkin et al. (2000b) have
demonstrated the first evidence for noradrenergic involvement in the adaptation of NMDA receptors following chronic treatment with the TCA and SNRI desipramine. Lesion of noradrenergic neurones was shown to block the increase in IC$_{50}$ of glycine to displace $[^{3}H]5,7$-DCKA binding to cortical membranes following chronic desipramine treatment. The lesion of noradrenergic neurones in the absence of desipramine treatment did not affect $[^{3}H]5,7$-DCKA binding or its ability to be displaced by glycine (Harkin et al. 2000b). As such, these results provide initial evidence that enables the connection of the recent studies relating antidepressant-induced NMDA receptor adaptation to a monoaminergic mechanism (Harkin et al. 2000b), but further studies into the direct effects of NMDA on NA release are required.

4.1.1 Drug doses used in this study

Reviews of the literature showed a range of doses (3-30mg/kg i.p.) of reboxetine in the study of transmitter release (Connor et al. 1999, Lucki et al. 2000, Page & Lucki 2000, Sacchetti et al. 1999). It was therefore decided to study the effects of two doses of reboxetine, 10 and 30mg/kg, on transmitter release in the RN and FC. 10mg/kg was chosen as it was the most commonly used dose and is also close to the ID$_{50}$ value of 7.51mg/kg demonstrated in an earlier study (Riva et al. 1989).

Yohimbine is an $\alpha_2$-adrenoceptor antagonist and a review of the literature showed the dose of 10$\mu$M to be suitable for the study of NA release in in vivo microdialysis (Drijfhout et al. 1996, Fernández-Galaz et al. 1993).

The NMDA receptor antagonists and the evidence supporting the doses used in this study have been described in 3.1.1.

The following questions were addressed:
1) Does reboxetine affect the release of 5-HT, DA, NA and GLU in the RN and FC?
2) How does NMDA affect the release of NA in the FC?
3) Do NMDA receptor antagonists modulate the release of NA in the FC?
4) Does acute or sub-chronic treatment with an NMDA receptor antagonist effect reboxetine-induced release of 5-HT, DA, NA, and GLU in the RN and FC?
Chapter Four: Reboxetine, amantadine and neurotransmitter release

4.2 Results

A summary of the results is presented in table 4.2 prior to the discussion.

4.2.1 Basal levels of NA measured in the frontal cortex dialysates

Basal levels of extracellular 5-HT, DA, and GLU are shown in table 3.1. The basal levels of NA were derived in a similar fashion and are expressed as mean ± s.e.mean, n=50, shown in table 4.1 below. A review of the literature showed this value to be within the acceptable range (Dalley et al. 1998, Gobert et al. 1998, Hatanaka et al. 2000, Kawahara et al. 2000). In the results, data are expressed as percentage of basal for acute studies and as absolute values for sub-chronic and chronic studies.

Table 4.1 Basal extracellular levels of NA in frontal cortex dialysates

<table>
<thead>
<tr>
<th>Frontal Cortex</th>
<th>14.91 ± 1.44 fmol/10μl</th>
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4.2.2 Effect of reboxetine on the release of 5-HT, DA, NA and GLU in the frontal cortex

The injection of reboxetine elicited a biphasic effect on 5-HT and DA release (see figure 4.2 A, B), whilst both NA and GLU were decreased (see figure 4.2 C, D).

The onset of the effect of reboxetine on FC 5-HT release was delayed by up to 90mins (figure 4.2 A). 10mg/kg reboxetine was observed to result in significantly increased levels of 5-HT, which remained high at the end of the experimental period (145 ± 34% of basal). However, the higher dose of 30mg/kg was observed to result in the opposite effect, a significant decrease in extracellular 5-HT (70 ± 23% of basal), which again was maintained until the end of the experiment.

A similar effect on DA release was observed following reboxetine administration (figure 4.2 B). However the onset of action was quicker, with clear effects observed
Figure 4.2 The effect of reboxetine on extracellular levels of A) 5-HT, B) DA, C) NA and D) GLU in the frontal cortex

The arrow indicates the time of the i.p. injection of reboxetine. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different (p<0.0001) from control.
within 30mins of injection. 10mg/kg resulted in a brief, but significant, increase in FC DA release which peaked at $175 \pm 27\%$ of basal before returning to basal levels. As with 5-HT, the higher dose of reboxetine resulted in a decrease in FC DA release. Levels fell over 90mins to a minimum of $53 \pm 16\%$ of basal, before returning to basal levels by the end of the experiment.

The effect of reboxetine on NA release in the FC was interesting, as it was the opposite of that expected (figure 4.2 C). Levels of NA were observed to decrease in a concentration-dependent manner when the overall effects of the two reboxetine doses were compared. After 10mg/kg reboxetine, extracellular NA fell to $62 \pm 13\%$ of basal, whilst the minimum level following 30mg/kg was $47 \pm 15\%$ of basal. Although these values are not statistically different, a clear trend can be observed. However FC NA release returned to that of basal at the end of the 30mg/kg reboxetine experiment, whilst animals which had received the lower dose still displayed significantly reduced FC NA levels at this stage.

GLU levels were significantly decreased following the administration of reboxetine (figure 4.2 D). There was no statistical difference between the effect of 10 and 30mg/kg, which was rapid in onset. Levels of FC GLU fell steadily, reaching $43 \pm 19\%$ of basal by the end of the experiment.

4.2.3 Effect of reboxetine on the release of 5-HT, DA and GLU in the raphe nuclei

Reboxetine was observed to elicit a dose-dependent increase in RN 5-HT and DA levels (see figure 4.3 A, B), whilst the opposite effect on RN GLU release was observed (see figure 4.3 C).

The effect of reboxetine on RN 5-HT was more rapid in onset compared to the FC effect (figure 4.3 A). 10mg/kg reboxetine was observed to result in a significant increase in RN 5-HT levels ($157 \pm 28\%$ of basal), and this was maintained for the duration of the experiment. Similarly 30mg/kg reboxetine also elicited a persistent increase in RN 5-HT ($231 \pm 80\%$ of basal), suggesting a dose-dependent effect of reboxetine in this brain region.
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Figure 4.3 The effect of reboxetine on extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei

The arrow indicates the time of the i.p. injection of reboxetine. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different (p<0.0001) from control.
Reboxetine was observed to have a similar dose-dependent effect on DA levels (figure 4.3 B). 10mg/kg reboxetine was shown to increase RN DA release, the onset was rapid but brief in duration and the levels had returned to that of control within 120mins of the injection. The effect of 30mg/kg reboxetine was more complex and appeared to be biphasic. Initially a rapid decrease in RN DA levels was observed, reaching a minimum of $32 \pm 18\%$ of basal 120mins after drug injection, however a sharp, brief increase in release was then observed, peaking at $268 \pm 66\%$ of basal. Levels of DA had returned to those of control by the end of the experiment.

The effect of acute reboxetine on GLU release in the RN was a decrease in extracellular levels (figure 4.3 C). At both doses, the onset of the effect was rapid and was maintained for the duration of the experiment. Although not statistically different, the results show a trend towards a dose-dependent decrease in RN GLU in response to reboxetine administration, with levels falling to a minimum of $21 \pm 3\%$ of basal.

4.2.4 Effect of reboxetine infused via the frontal cortex probe on the release of NA in the frontal cortex

Reboxetine was infused into the FC via the microdialysis probe and a concentration-dependent decrease in extracellular FC NA was observed (figure 4.4 A). The effect of 10μM reboxetine was not statistically different from basal levels for the duration of the experiment, but a decreasing trend can be clearly observed. However towards the end of the experimental period the decrease was significant ($75 \pm 16\%$ of basal) and remained low at the end of the experiment. The higher dose of 100μM elicited a more rapid response and a significant decrease was recorded within 30mins of infusion. Levels of NA were more consistently decreased by this concentration of reboxetine ($61 \pm 9\%$ of basal) and, as with 10μM, levels of NA remained low at the end of the experiment.
Figure 4.4 A) The effect of infusing reboxetine via the frontal cortex probe on extracellular levels of NA in the frontal cortex. B) The effect of infusing yohimbine via the frontal cortex probe on reboxetine-induced changes in extracellular levels of NA in the frontal cortex.

The open bar denotes the duration of infusion of reboxetine or yohimbine alone and the filled bar indicates the duration of the co-infusion of the two drugs. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates data points extremely significantly different (p<0.0001) from control. + indicates significantly different from yohimbine alone (p<0.05).
4.2.5 Effect of yohimbine on reboxetine-induced changes in extracellular NA in the frontal cortex

When infused alone, yohimbine infusion into the FC was observed to result in a rapid and consistent increase in NA release in the FC (figure 4.4 B). Extracellular levels of NA had risen to 179 ± 21% of basal by the end of the experiment. The co-infusion of yohimbine with reboxetine was then observed to reverse the decrease seen after the infusion of reboxetine alone. Within 90 mins of the addition of reboxetine to the infusion medium a second sharp increase in the level of FC NA was recorded. Although the effect was brief (60-90 mins) and the trend suggested that levels of NA were returning to basal at the end of the experiment, the increase following the co-infusion of yohimbine and reboxetine was greater than that observed following the infusion of yohimbine alone (p<0.05).

4.2.6 The effect of NMDA infusion on extracellular NA levels in the frontal cortex

NMDA was infused into the FC via the dialysis probe at two different concentrations (25 and 100 μM) and was observed to have a biphasic effect (figure 4.5). 25 μM NMDA was observed to cause a rapid and sustained decrease in FC NA levels (52 ± 12% of basal). This significant decrease was maintained for the duration of the experiment. However following infusion of 100 μM NMDA, the effect on NA was reversed. Levels were seen to sharply increase to 288 ± 64% of basal. The increase was relatively brief and levels had returned to basal well before the end of the experiment.

4.2.7 The effect of NMDA infusion on reboxetine-induced changes in 5-HT, DA, NA and GLU levels in the frontal cortex

NMDA infusion into the RN results in the abolition of the reboxetine-induced increase in FC 5-HT, a reversal of the reboxetine-induced increase in extracellular DA in the same region and an increase in RN GLU release (figure 4.6 A, B, D). Infusion of NMDA into the FC was not observed to have any significant effect on the reboxetine-induced decrease in FC NA levels (figure 4.6 C).
Figure 4.5 The effect of NMDA infusion into the frontal cortex, via the dialysis probe, on extracellular levels of NA in the frontal cortex

The bar denotes the duration of NMDA infusion. All values are represented as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different ($p<0.05$), ** indicates very significantly different and *** indicates data points extremely significantly different ($p<0.0001$) from control.
Figure 4.6 The effect of infusing NMDA, via the dialysis probe, on reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA, C) NA and D) GLU in the frontal cortex.

NMDA was infused via the RN probe for A) 5-HT, B) DA and D) GLU and via the FC probe for C) NA measurements. The arrow indicates the time of the i.p. injection of reboxetine and the bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates data points significantly different (p<0.05) and *** indicates extremely significantly different (p<0.001) from control.
Infusion of NMDA alone into the RN was observed to decrease extracellular levels of FC 5-HT to 35 ± 8% of basal. However when the infusion was carried out 30mins after reboxetine had been administered the effect of both reboxetine alone and NMDA alone was abolished and levels of FC 5-HT were not significantly different to those of control (figure 4.6 A).

A significant increase in FC DA levels was recorded when NMDA was infused via the RN probe and also following the injection of reboxetine. The reboxetine-induced increase was reversed by the subsequent infusion of NMDA and a highly significant decrease in FC DA was recorded (figure 4.6 B). Levels of DA rapidly fell to 59.5 ± 15.8% of basal and remained low for the duration of the experiment.

The decrease in FC NA levels in response to reboxetine administration was unaffected by the subsequent infusion of NMDA via the FC probe (figure 4.6 C). Levels of NA were still reduced in comparison to control (p<0.05), but were not statistically different to those observed following the administration of reboxetine alone. NMDA alone was shown to increase FC NA and therefore this effect seemed to have been blocked by the reboxetine treatment.

Reboxetine administration decreases levels of GLU in the FC and the subsequent infusion of NMDA, via the RN probe, was observed to reverse this (figure 4.6 D). The effect of NMDA on the reboxetine-induced RN GLU release was rapid in onset and resulted in a significant, persistent increase (p<0.0001, 169 ± 18% of basal). NMDA infusion into the RN normally reduces extracellular GLU, therefore reboxetine treatment seems to reverse the effects of NMDA on this transmitter in the FC.

4.2.8 The effect of NMDA infusion on reboxetine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

The reboxetine-induced increases in RN 5-HT and DA were differentially affected by the subsequent infusion of NMDA via the RN probe. Levels of 5-HT were decreased, whilst those of DA were increased (figure 4.7 A, B). RN GLU was decreased in response to reboxetine and this effect was reversed by the subsequent infusion of NMDA in to the RN (figure 4.7 C).
Figure 4.7 The effect of infusing NMDA into the raphe nuclei, via the dialysis probe, on reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.

The arrow indicates the time of the i.p. injection of reboxetine and the bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates data points significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates extremely significantly different (p<0.001) from control.
Both reboxetine (see figure 4.3A) and NMDA, when administered alone, result in increased levels of 5-HT in the RN. However the infusion of NMDA following the injection of reboxetine was observed to result in a rapid and highly significant decrease (figure 4.7 A). The extracellular levels of 5-HT were observed to decrease to 62.6 ± 10% of basal and remained low for the duration of the experiment. The effect of reboxetine and NMDA alone was therefore reversed.

The reboxetine-induced increase in RN DA was seen to be potentiated by the infusion of NMDA into the RN (figure 4.7 B). Although levels were not significantly higher than those seen after reboxetine alone, the effect was more robust. Extracellular DA rapidly increased to 202 ± 30% of basal and was still seen to be rising at the end of the experiment. The decrease seen following the infusion of NMDA alone was therefore reversed.

The effect of reboxetine on RN GLU release is a significant decrease and this was observed to be reversed by the infusion of NMDA into the RN (figure 4.7 C). Levels of GLU were considerably increased, peaking at 556 ± 67% of basal at the end of the experiment. The infusion of NMDA alone has been shown to decrease extracellular GLU in the RN, therefore, as in the FC, reboxetine treatment has reversed the effect of NMDA on GLU release.

4.2.9 The effect of amantadine on the reboxetine-induced changes in 5-HT, DA, NA and GLU levels in the frontal cortex

Amantadine was observed to have no significant effect on 5-HT release but increased DA and decreased NA and GLU release in the FC when administered alone (see figure 4.8 A, B, C, D).

Amantadine may have exerted no significant effect on FC 5-HT levels when administered alone, but it was seen to significantly potentiate the effects of reboxetine (figure 4.8 A). The extracellular level of FC 5-HT rose to 198 ± 20% of basal, and although onset of the effect was delayed slightly, levels remained high for the duration of the experiment.
Chapter Four: Reboxetine, amantadine and neurotransmitter release

A) Control
- Reboxetine 10mg/kg
- Amantadine 40mg/kg
- Reboxetine 10mg/kg + Amantadine 40mg/kg

B) 200 -
- 150 -
- 100 -
- 50 -
- 0 -
60 120 180 240 300 360
TIME (mins)

C) 150 -
- 125 -
- 100 -
- 75 -
- 50 -
- 25 -
0 60 120 180 240 300 360
TIME (mins)

D) 200 -
- 150 -
- 100 -
- 50 -
- 0 -
0 60 120 180 240 300 360
TIME (mins)

Figure 4.8 The effect of amantadine on reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA, C) NA and D) GLU in the frontal cortex

The first arrow indicates the time of the i.p. injection of reboxetine and the second the time of the reboxetine injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates extremely significantly different (p<0.001) from control. +++ and ### indicates extremely significantly different from amantadine and reboxetine alone respectively (p<0.0001).
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The effect of amantadine alone was a rapid increase in FC DA that was maintained for 180mins before returning to basal. Reboxetine-induced increases in extracellular levels of FC DA were reversed by the subsequent administration of amantadine (figure 4.8 B) and levels fell to 74.7 ± 8% of basal. In terms of onset and duration the effect was the same as that recorded for 5-HT in this region.

Amantadine results in a persistent, significant decrease in FC NA levels when administered alone (58 ± 5% of basal). The effect is similar to that of reboxetine in terms of onset, magnitude and duration (figure 4.8 C). The reboxetine-induced decrease in FC is briefly reversed by the subsequent injection of amantadine (138 ±16% of basal) and this is extremely significantly increased in comparison to both amantadine and reboxetine alone (p<0.0001). However levels rapidly decreased and by the end of the experiment had fallen to 55 ± 11% of basal, similar to that seen after reboxetine and amantadine alone.

FC GLU release is decreased by amantadine treatment and is similar to the effect of reboxetine in terms of onset, magnitude and duration (figure 4.8 D). Amantadine was further shown to have no significant effect on the reboxetine-induced decrease in FC GLU, and levels remained significantly lower than basal for the duration of the experiment (p<0.01).

4.2.10 The effect of amantadine on the reboxetine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

Amantadine had no significant effect on extracellular levels of 5-HT, DA or GLU in the RN (see figure 4.9 A, B, C).

The reboxetine-induced increase in 5-HT release was reversed by the subsequent administration of amantadine (figure 4.9 A). A steady decrease in the extracellular level of 5-HT was observed throughout the experiment and levels remained at 25 ± 9% of basal at the end of the experiment.
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A)

![Graph showing the effect of amantadine on reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.](image)

B)

C)

Figure 4.9 The effect of amantadine on reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei

The first arrow indicates the time if the i.p. injection of reboxetine and the second the time of the reboxetine injection. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates extremely significantly different (p<0.0001) from control.
Amantadine abolishes the brief increase in RN DA levels following reboxetine treatment and no significant change from control basal is seen until 150mins later when a significant decrease is observed (figure 4.9 B). Extracellular DA is significantly decreased to $79 \pm 4\%$ of basal and remains at this level for the final hour of the experiment.

Although amantadine has no significant effect on RN GLU release when administered alone it was observed to reverse the reboxetine-induced decrease (figure 4.9 C). The onset of the effect is slightly delayed but GLU levels increase steadily for the duration of the experiment to $196 \pm 28\%$ of basal ($p<0.0001$ compared to basal).

4.2.11 The effect of sub-chronic dosing of amantadine and reboxetine on 5-HT, DA, NA and GLU levels in the frontal cortex

10mg/kg i.p. reboxetine was administered twice daily for 4 days and resulted in significant decreases in the extracellular levels of 5-HT and NA (see figure 4.10 A, C). FC DA and GLU release were not significantly affected by this treatment (see figure 4.10 B, D). NMDA was infused via the RN probe and was observed to decrease 5-HT and GLU but have no effect on reboxetine-induced DA release. Reboxetine-induced decreases in FC NA were unaffected by the infusion of NMDA into the FC.

40mg/kg/day i.p. amantadine was observed to result in decreases in the extracellular levels of 5-HT, DA and NA, with no significant effect on GLU release (figure 4.10 A, B, C, D). The infusion of NMDA (as above) to monitor the activity of the NMDA receptor was observed to increase 5-HT and NA release, but no significant effect on extracellular DA and GLU levels were recorded.

Sub-chronic dosing of reboxetine reduced FC 5-HT levels to about 50% of controls ($11 \pm 3$ fmol/10μl) and was the opposite effect to that observed following acute treatment (figure 4.2 A). However the effect was similar in magnitude to that observed following the higher acute dose of 30mg/kg (figure 4.2 A). The infusion of NMDA into the RN was observed to further decrease 5-HT in the FC and levels were significantly reduced compared to treatment basal ($p<0.05, 5.5 \pm 1.5$ fmol/10μl). Sub-chronic amantadine treatment significantly decreased FC 5-HT and the subsequent infusion of NMDA was
Figure 4.10 The effect of sub-chronic dosing of amantadine and reboxetine on the extracellular levels of A) 5-HT, B) DA, C) NA and D) GLU in the frontal cortex. The bar denotes NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates extremely significantly different (p<0.0001) from controls. + indicates significantly different (p<0.05), +++ indicates extremely significantly different (p<0.0001) from reboxetine alone and # indicates significantly different (p<0.05) and ## indicates very significantly different (p<0.01) from treatment basal.
observed to briefly, but significantly, increase release. However the levels were still significantly less than those of control (figure 4.10 A). The effect of NMDA seemed to be delayed in comparison to controls and levels had returned to amantadine-basal by the end of the experiment. Sub-chronic dosing of amantadine and reboxetine resulted in an extremely significant increase in FC 5-HT (figure 4.10 A). Levels of 53 ± 5fmol/10μl were recorded - twice those of controls (p<0.0001) and four times larger (p<0.0001) than the levels observed after sub-chronic reboxetine treatment. Infusion of 100μM NMDA was seen to increase FC 5-HT further, in comparison to treatment basal (p<0.05), which was the opposite effect to that seen in controls. The effect of NMDA observed here is more like that of 25μM NMDA, as opposed to 100μM (see figure 3.5 A).

When dosed sub-chronically, reboxetine and the subsequent infusion of NMDA via the RN probe, was observed to have no significant effect on FC DA release (figure 4.10 B). The reboxetine treatment therefore appeared to have abolished the effects of NMDA in this region. Sub-chronic dosing of amantadine significantly decreased extracellular DA (26.8 ± 4fmol/10μl) and was also unaffected by the subsequent infusion of NMDA via the RN probe (figure 4.10 B). Sub-chronic dosing of both reboxetine and amantadine was observed to significantly increase FC DA to 137 ± 4fmol/10μl and this was significantly higher than both control and reboxetine alone (p<0.0001 and p<0.05 respectively). The subsequent infusion of NMDA via the RN probe had no significant effect on the release of DA in the FC (figure 4.10 B).

Extracellular FC levels of NA were seen to be reduced by about 50% to 7.4 ± 0.6fmol/10μl by sub-chronic reboxetine treatment and this decrease was unaffected by the subsequent infusion of NMDA into the FC (figure 4.10 C). The increase in NA that is normally the result of NMDA infusion therefore appeared to have been abolished by the reboxetine treatment. Sub-chronic dosing of amantadine was also observed to decrease NA in this region, although not by the same extent (12.5 ± 0.6fmol/10μl) and the subsequent infusion of NMDA resulted in a brief, but significant, increase (p<0.05 compared to treatment basal). When both amantadine and reboxetine were given sub-chronically NA levels were significantly reduced and NMDA infusion did not alter FC
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NA levels – an effect similar in magnitude to that seen following the sub-chronic administration of reboxetine (figure 4.10 C).

4 day treatment with reboxetine was observed to have no significant effect on FC GLU release, with levels remaining close to those of controls (figure 4.10 D). The subsequent infusion of NMDA, via the RN probe, resulted in a brief but significant decrease (p<0.05) in extracellular GLU in the FC. Sub-chronic dosing of amantadine had no significant effect on FC GLU release, although the subsequent infusion of NMDA via the RN probe did significantly increase levels of GLU to 30.4 ± 8pmol/10μl of basal. This was the opposite effect to that observed when NMDA was infused into controls (figure 4.10 D). Sub-chronic treatment with reboxetine and amantadine also had no significant effect on FC GLU release when compared to controls, 4day reboxetine or 4day amantadine GLU levels (figure 4.10 D). NMDA infusion via the RN probe was subsequently shown to significantly decrease FC GLU to 9 ± 1pmol/10μl and levels remained at this level at the end of the experiment.

4.2.12 The effect of sub-chronic dosing of amantadine and reboxetine on 5-HT, DA and GLU levels in the raphe nuclei

Reboxetine (10mg/kg i.p.) administered twice daily for 4 days was observed to decrease 5-HT and increase DA and GLU levels in the RN. The subsequent infusion of NMDA, via the RN probe, for 30mins was shown to potentiate the effect of reboxetine on extracellular DA but had no effect on 5-HT or GLU release in the RN (see figure 4.11 A, B, C).

When amantadine (40mg/kg/day i.p.) was administered sub-chronically, extracellular levels of both 5-HT and GLU were increased, whilst that of DA was seen to fall (figure 4.11 A, B, C). The subsequent infusion of NMDA via the RN probe, increased RN GLU, but had no effect on the release of 5-HT or DA.

Sub-chronic dosing of reboxetine was observed to decrease RN 5-HT by about 50% to 17 ± 5fmol/10μl, but the subsequent infusion of NMDA had no effect and levels of RN 5-HT remained at the same level for the duration of the experiment (figure 4.11 A).
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Figure 4.11 The effect of sub-chronic dosing of amantadine and reboxetine on the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.

The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than the symbol) from 6 experiments. * indicates significantly different (p<0.05) and *** indicates extremely significantly different (p<0.0001) from control. # indicates significantly different (p<0.05) and ## indicates very significantly different (p<0.01) from treatment basal.
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4 day dosing of amantadine significantly increased extracellular 5-HT to 66 ± 12fmol/10μl. The subsequent infusion of NMDA via the RN probe had no significant effect on RN 5-HT levels, which remained high for the duration of the experiment. Thus the effect of sub-chronic treatment with reboxetine or amantadine abolished the NMDA-induced increase in RN 5-HT. Sub-chronic treatment with amantadine and reboxetine resulted in significantly reduced extracellular 5-HT in the RN and the effect was of similar magnitude to that seen following sub-chronic reboxetine (figure 3.11 A). The subsequent infusion of NMDA was shown to further decrease RN 5-HT levels to 8 ± 2fmol/10μl, which is significantly lower than treatment basal (p<0.01).

RN DA levels were significantly increased in response to sub-chronic reboxetine treatment (figure 4.11 B). Extracellular release was increased to 81 ± 12fmol/10μl and this was further potentiated by the subsequent infusion of NMDA into the RN. Levels continued to rise for the duration of the experiment, peaking at 199 ± 13fmol/10μl, which is significantly higher than treatment basal (p<0.01). The effect of NMDA infusion on DA release in the RN has therefore been reversed by sub-chronic treatment with reboxetine. After 4 days of amantadine treatment extracellular DA levels were significantly reduced to less than 50% of controls (15 ± 2fmol10μl) and the effect of NMDA infusion was abolished (figure 3.11 B). Sub-chronic dosing of amantadine and reboxetine significantly increased RN DA (p<0.05). The magnitude of the effect was similar to that seen following sub-chronic reboxetine treatment. However the subsequent infusion of NMDA resulted in a significant decrease (p<0.05 compared to treatment basal) in DA, returning to control basal levels (figure 4.11 B).

Sub-chronic reboxetine treatment significantly increases RN GLU release (p<0.0001) to 17.2 ± 2pmol/10μl, but was unaffected by the subsequent infusion of NMDA into the RN (figure 4.11 C). 4 days of amantadine treatment was also observed to increase extracellular GLU to a similar level (20.8 ± 0.92pmol/10μl) to that seen following reboxetine treatment. The subsequent infusion of NMDA increased GLU levels further, peaking at 35.5 ± 4pmol/10μl at the end of the experiment. This effect of NMDA is more like that observed following the infusion of 25μM NMDA (see figure 3.6 C). Following sub-chronic dosing of both reboxetine and amantadine no significant change in RN GLU levels was observed (figure 4.11 C). However the levels were significantly
lower than those recorded following sub-chronic reboxetine or amantadine treatment (p<0.0001). NMDA infusion via the RN probe resulted in a significant increase (p<0.01) in RN GLU release (13 ± 2.5pmol/10μl) and this was the opposite effect to that observed when NMDA was infused into controls (figure 4.11 C).

4.2.13 The effect of 100μM NMDA infusion on the chronic reboxetine-induced changes in 5-HT, DA, NA and GLU levels in the frontal cortex

Chronic dosing of reboxetine (10mg/kg/day i.p.) for 14 days had no significant effect on FC NA levels. The subsequent infusion of NMDA into the FC had no significant effect (figure 4.12 C). However reboxetine administered twice daily (10mg/kg i.p.) for the same period did decrease FC NA and increase levels of 5-HT, DA and GLU. The subsequent infusion of NMDA had no effect on reboxetine-induced changes in DA, NA and GLU, but levels of 5-HT were reduced (figure 4.12 A, B, C, D).

Following the chronic administration of reboxetine, levels of FC 5-HT were increased almost three-fold to 56 ± 5fmol/10μl. The subsequent infusion of NMDA via the RN probe resulted in a decrease that was rapid in onset and continued for the duration of the experiment (figure 4.12 A). Extracellular 5-HT was reduced to 31 ± 7fmol/10μl (p<0.01 compared to treatment basal), but did not decrease any further towards control basal levels. The effect of NMDA infused via the RN probe on FC 5-HT release was therefore unaffected.

Chronic reboxetine treatment also increased FC DA levels (figure 4.12 B). Levels of DA were increased three-fold by reboxetine treatment (236 ± 37fmol/10μl) and the subsequent infusion of NMDA via the RN probe did not have any significant effect. The increase in FC DA release observed following NMDA infusion into the RN appears to have been abolished.

Reboxetine had no effect on FC NA release when administered chronically once a day, however the NMDA-induced increase appeared to have been blocked (figure 4.12 C). Twice-daily chronic treatment however, was observed to significantly decrease extracellular levels in the FC to 4.6 ± 2fmol/10μl. The infusion of NMDA into the FC
Figure 4.12 The effect of infusing NMDA, via the dialysis probe, on chronic reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA, C) NA and D) GLU in the frontal cortex.

NMDA was infused via the RN probe for A) 5-HT, B) DA and D) GLU and via the FC probe for C) NA measurements. The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates data points significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates extremely significantly different (p<0.0001) from control. ## indicates data points very significantly different from treatment basal (p<0.01).
again had no significant effect on NA release, levels remained low throughout the experiment (figure 3.12 C).

Chronic reboxetine treatment increased FC GLU levels to 22.2 ± 3.7pmol/10μl, which was significantly higher than control basal (p<0.0001) and 4day amantadine + reboxetine (p<0.05). The subsequent infusion of NMDA via the RN had no significant effect on the reboxetine-induced increase in FC GLU levels, therefore the effect of NMDA was abolished (figure 4.12 D).

4.2.14 The effect of 100μM NMDA infusion on the chronic reboxetine-induced changes in 5-HT, DA and GLU levels in the raphe nuclei

Chronic dosing of reboxetine (10mg/kg i.p., twice daily) for 14 days resulted in decreased RN levels of 5-HT and DA (see figure 4.13 A, B). The subsequent infusion of NMDA via the RN probe had no significant effect on transmitter release, thus NMDA-induced changes were abolished.

Following the chronic administration of reboxetine a significant decrease in RN 5-HT release was observed (figure 4.13 A). Levels fell to 17.5 ± 5fmol/10μl and were unaffected by the subsequent 30min infusion of NMDA. In control animals, 100μM NMDA infusion results in a significant increase in 5-HT release in the RN, but the chronic reboxetine treatment appears to have abolished this effect in the treated animals.

Chronic reboxetine treatment also significantly reduced extracellular levels of RN DA to 43 ± 5fmol/10μl (figure 4.13 B). As with 5-HT, the subsequent infusion of NMDA had no significant effect on DA levels, which remained low for the duration of the experiment. The chronic reboxetine treatment therefore appears to have abolished the NMDA-induced decrease in RN DA release.

Reboxetine, administered chronically, significantly increased RN GLU three-fold (compared to control) to 27 ± 7pmol/10μl (p<0.0001). This increase was also significantly higher than that observed following 4 day amantadine + reboxetine treatment (p<0.0001). The subsequent infusion of NMDA via the RN probe had no
Figure 4.13 The effect of infusing NMDA into the raphe nuclei, via the dialysis probe, on chronic reboxetine-induced changes in the extracellular levels of A) 5-HT, B) DA and C) GLU in the raphe nuclei.

NMDA was infused via the RN probe for A) 5-HT, B) DA and D) GLU and via the FC probe for C) NA measurements. The bar denotes the duration of NMDA infusion. All values are expressed as mean ± s.e.mean (shown when larger than symbol) from 6 experiments. * indicates data points significantly different (p<0.05), ** indicates very significantly different (p<0.01) and *** indicates extremely significantly different (p<0.0001) from control.
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significant effect on the reboxetine-induced increase in RN GLU and levels remained high for the duration of the experiment. The effect of NMDA in this region was therefore abolished.

4.2.15 The effect of chronic reboxetine dosing on body weight

Over a 14 day period control rats were observed to increase in body weight at a rate of approximately 4g/day (see figure 4.14). Animals of a similar starting weight were chronically dosed with reboxetine (10mg/kg i.p. twice daily for 15 days) and although an increase in weight was observed, it was at a much slower rate than controls. During the dosing period reboxetine-treated animals gained weight at a rate of approximately 1.1g/day (figure 3.14), and the amount of weight gained decreased over the course of the study. By the end of the experiment the reboxetine-treated animals had gained only 16g, which was significantly lower (p<0.0001) than the 53g gain observed in control animals.
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Figure 4.14 The effect of chronic reboxetine dosing on body weight

All values are expressed as mean ± s.e.mean (shown when larger than the symbol. * indicates significantly different (p<0.05) and *** indicates data points extremely significantly different (p<0.0001) from control.
4.3 Discussion

4.3.1 Summary

The data presented in this chapter (see table 4.2 for summary) suggests that the novel NRI antidepressant reboxetine alters NMDA receptor function. Manipulation of NMDA receptor activity by amantadine, a non-competitive NMDA receptor antagonist, appears to reduce the ‘lag’ period associated with many current antidepressant treatments, as indicated by increased levels of cortical 5-HT and DA. The effect of this treatment on NA levels appears to conflict with these results, but an explanation is offered, in terms of receptor regulation and interactions between transmitter systems. Changes in GLU levels within the FC and RN suggest an involvement for this transmitter in regulating NMDA-induced monoamine release, although the involvement of other glutamatergic receptors, AMPA/Kainate for example, should not be discounted. Collectively the data supports the hypothesis that chronic reboxetine treatment induces a series of adaptive changes in the regulation of transmitter release in the FC and RN and that the timing of these changes may be positively improved by manipulation of the NMDA receptor. It is also worth noting that the combined reboxetine/amantadine treatment appears to be more successful than the CIM/amantadine treatment, in terms of increased cortical monoamine release, with levels at 4days being equivalent to those observed after 14days of reboxetine treatment. Differences in the effect of reboxetine in the two regions and also in comparison to CIM treatment may be explained by differences in NMDA receptor subunit expression (and therefore sensitivity to modulating factors) or by interactions between transmitter systems resulting in regulated release.

4.3.2 Effect of reboxetine on neurotransmitter release in the frontal cortex and raphe nuclei

Acute administration of reboxetine was shown to increase RN 5-HT release in a dose-dependent manner. However the effect on FC 5-HT did not follow the expected pattern of decreased release. At the lower dose of 10mg/kg, FC 5-HT was unexpectedly increased, whilst levels fell following the 30mg/kg treatment. Studies of other antidepressants, given acutely, have shown different effects on FC 5-HT release. For
Table 4.2 Summary of results compared to basal or treatment-basal

Effects of drug treatments are expressed in comparison to basal or treatment-basal as appropriate. nd indicates transmitter not measured in study.

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example, acute CIM administration results in increased RN and decreased FC 5-HT (Adell & Artigas 1991, Pallotta et al. 2001), but the SNRI and SSRI venlafaxine (5-20mg/kg i.p.) and the SSRI fluvoxamine (1-10mg/kg i.p.) both increase 5-HT in the FC (Bel & Artigas 1992, Gur et al. 1999a). A review of the literature revealed few studies of the effect of reboxetine on 5-HT. Page & Lucki (2000) used in vivo microdialysis to measure 5-HT release in the FC of freely moving rats and found that acute administration of reboxetine had no significant effect at any dose tested (1-20mg/kg). However reboxetine has also been shown to attenuate forced swim test-induced behavioural and neurochemical changes (Connor et al. 1999). The forced swim test induces increased 5-HT turnover in the FC and amygdala of the rat (Connor et al. 1999) and acute reboxetine treatment (10 and 30mg/kg) was shown to attenuate this (Connor et al. 1999).

A comprehensive study of the pharmacological properties of reboxetine revealed that reboxetine was highly selective for the NA transporter in synaptosomes: $K_i$ 8nmol/L compared to 1070nmol/L for 5-HT reuptake (Wong et al. 2000). Receptor binding and biochemical assays of a number of CNS receptors (including adrenergic, dopaminergic, serotonergic, histaminergic and muscarinic) showed the poor affinity of reboxetine at these receptors. Of the receptors tested, $H_1$ histamine and 5-HT$_{2C}$ receptors have the highest affinity ($K_i = 1.4 \pm 0.2$ and $1.5 \pm 0.5$ µmol/L respectively) but its affinity was still 1000-fold less than its affinity at the NA transporter (Wong et al. 2000). Therefore this may suggest that the effects of reboxetine on 5-HT may be indirect and a result of an interaction between transmitter systems e.g. NA from the LC tonically regulates output from the RN via inhibitory $\alpha_2$ heteroreceptors located on 5-HT neurones, thereby inhibiting 5-HT release (Haddjeri et al. 1996). As the available data on the effects of reboxetine on 5-HT release is conflicting, further studies are required.

The effect of 30mg/kg reboxetine on 5-HT release (increased RN and decreased FC 5-HT) is similar to that observed following the acute administration of CIM in this study and may be explained in the same way. Pazos & Palacios (1985) and Pedigo et al. (1981) both observed that the RN is enriched with 5-HT$_{1A}$ receptors displaying nanomolar affinity for 5-HT. Therefore increased RN 5-HT would activate these receptors in cell bodies and dendrites, inhibiting cell firing and reducing the release of 5-HT in terminal regions, including the FC. Hutson et al. (1989) supported this theory.
and showed that the local application of the specific 5-HT$_{1A}$ agonist 8-OH-DPAT to the RN resulted in decreased hippocampal 5-HT. Why a similar effect is not observed with the lower dose of reboxetine is unknown, but it could be a result of neurotransmitter interactions or that 10mg/kg reboxetine was not a high enough dose to sufficiently activate the RN receptors and modulate terminal release.

Sub-chronic (4day) reboxetine was administered twice daily following preliminary studies in this lab and also because of the relatively short half-life of reboxetine (terminal half-life of elimination ($t_{1/2}$) = 1-2h) in rodents (Dostert et al. 1997). Levels of 5-HT were decreased in both the RN and FC, therefore the increase in RN 5-HT following acute treatment was reversed. By comparison, levels of FC 5-HT following sub-chronic dosing appeared to be lower than those observed after acute treatment. Finally, chronic (14day) reboxetine treatment was shown to decrease extracellular RN and increase FC 5-HT release. The magnitude of the increase in FC 5-HT was less than that observed after 14day CIM treatment and the decrease in RN 5-HT was the opposite to that seen in this region after chronic CIM treatment. The effect of chronic reboxetine treatment (15mg/kg /day i.p.) on striatal 5-HT release was studied by Sacchetti et al. (1999), they observed that extracellular concentrations of 5-HT were not significantly different to those observed after saline treatment. A study of the effects of reboxetine (10mg/kg/day i.p.) on the olfactory bulbectomized (OB) rat model of depression looked at changes in amygdaloid cortex 5HIAA and also showed no significant effects in either OB or sham-operated rats over a 14day period (Harkin et al. 1999). The lack of effects in both these studies may be explained by the daily dosing protocol. Studies have shown that the rate of metabolism of reboxetine in rats is such that twice-daily dosing or continual release by osmotic minipump is required in order to maintain plasma concentrations (Dostert et al. 1997).

Due to the lack of further studies of the effects of chronic reboxetine treatment, comparisons between the known effects of other antidepressants and reboxetine will be made. It is interesting to note that changes in 5-HT release are occurring after as little as 4days of treatment. Chronic treatment therefore seems to reverse the increase in RN 5-HT and this change occurs after as little as 4days, whilst the change in FC 5-HT release takes longer (4-14days). Taken together with the CIM data this would seem to confirm the theory that adaptive changes must occur to allow the potential clinical benefit of the
antidepressant (Artigas et al. 1996, Blier & de Montigny 1994). The increases in terminal levels of 5-HT initiated by 5-HT uptake blocking drugs can be potentiated by the addition of a 5-HTIA autoreceptor antagonist (Artigas et al. 1994, Blier & Bergeron 1995). Pindolol and WAY100635 have both been tested in this way and shown to potentiate the effect of the antidepressant (Artigas et al. 1994, Blier & Bergeron 1995, Blier et al. 1997, Cryan et al. 1999, Gartside et al. 1995, Millan et al. 1998, Romero & Artigas 1997, Romero et al. 1996). This would indicate that the effects of serotonergic drugs can be potentiated by 5-HTIA autoreceptor blockade and therefore emphasises the role of this receptor in the mechanism of action of antidepressants. However reboxetine is not classed as a serotonergic drug (Wong et al. 2000) and it may therefore be an interaction between the noradrenergic and serotonergic systems which fully explains the effects of reboxetine on 5-HT. The study by Harkin et al. (1999) using the OB rat model of depression showed the attenuation of hypothermia induced by 8-OH-DPAT (0.05mg/kg s.c.) and clonidine (0.1mg/kg s.c.) following 7 or 14 days treatment with a combination of reboxetine (10mg/kg/day) and the SSRI sertraline (5mg/kg/day). This suggests changes to 5-HTIA and α2-adrenoceptor sensitivity are occurring during this time period. Changes in response to clonidine are indicative of a reduction in the sensitivity of α2 receptors and it was shown that a faster onset of this response was observed when reboxetine was combined with sertraline than when either drug was given alone (Harkin et al. 1999). Similarly the 8-OH-DPAT-induced changes suggest that reboxetine can alter 5-HTIA receptor sensitivity as previously demonstrated with other antidepressants including the SNRI desipramine and ECT (Goodwin et al. 1987, Kelly & Leonard 1994, Wozniak et al. 1988). Long-term adaptive changes in β-adrenoceptors are also known to occur following the administration of both NA and 5-HT reuptake inhibitors (Koe et al. 1983, Riva et al. 1989). The rapid down-regulation of β-adrenoceptors following the co-administration of the TCA and SNRI desipramine and the SSRI fluoxetine to rats, for example, is believed to require an interaction with a functional serotonergic system (Baron et al. 1988). Such studies suggest that both NA and 5-HT are involved in the response to antidepressants and lend some support to the hypothesis that a dual action on both biogenic amine uptake sites may have a broader spectrum of therapeutic action (Harkin et al. 1999).

It is demonstrated here that acute reboxetine treatment results in a dose-dependent increase in RN DA levels. In the FC the effect is similar to that seen in 5-HT, with the
lower dose increasing FC DA and the higher dose having the reverse effect. The literature shows one study of the effect of reboxetine on DA output in the FC (Linner et al. 2000). They investigated the effects of reboxetine on terminal DA output in the medial pre-FC and the nucleus accumbens following systemic administration and also local infusion into the medial pre-FC, nucleus accumbens and ventral tegmental area (VTA). Reboxetine (0.65-10mg/kg i.v.) was shown to dose-dependently increase DA output in the medial pre-FC but not the nucleus accumbens. Local application increased DA output preferentially in the medial pre-FC but also in the nucleus accumbens, whereas administration of reboxetine in the VTA did not effect terminal DA release (Linner et al. 2000). The effect observed by Linner et al. (2000) was therefore similar to that observed in this study following 10mg/kg reboxetine, but due to the different routes of administration it is difficult to compare them in more detail. A number of studies have reported increased FC DA in response to acute antidepressant treatment (Carlson et al. 1996, Tanda et al. 1994, 1995, 1996, Wedzony & Golembiowska 1993), the drugs studied included fluoxetine, CIM, imipramine, desipramine, mianserin, nortryptiline and paroxetine. However Pallotta et al. (1999b) did observe a decrease in FC DA following the acute administration of CIM, suggesting that the effect of antidepressants on DA release in this region to be complex. There are few studies of the effects of antidepressants on RN DA release, but the dose-dependent increase in RN DA was similar to that observed following acute CIM treatment (Pallotta et al. 1999a). The results of a number of acute CIM studies (Ichikawa & Meltzer 1995, Pallotta et al. 1999a, b) collectively suggest that the effects observed are due to secondary changes in the reuptake of other transmitters (e.g. 5-HT) more potently effected by CIM (Pallotta et al. 1999b). This may also be the case for reboxetine and may be related to its noradrenergic component, as reboxetine is more selective for the NA transporter than the CIM-metabolite DCIM, which has been shown to have a higher affinity for the NA transporter than CIM (Benfield et al. 1980, Thomas & Jones 1977). The results of receptor binding and biochemical assays also seem to confirm this theory as the K_i for D_2, D_3 and D_4 receptors ranges from 9 to >49μmol/L, showing the poor affinity of reboxetine for these receptors (Wong et al. 2000).

Sub-chronic (4day) reboxetine was observed to increase RN DA and have no significant effect on FC release. The acute effect of reboxetine in the FC was therefore abolished, whilst that in the RN was unaffected. Following chronic (14day) treatment, these effects
were further changed: FC DA levels increased and RN DA decreased. These effects are similar in magnitude to those seen following chronic CIM treatment (Pallotta et al. 1999a,b) and represent a complete reversal of the acute effects of reboxetine. These results show that changes in release of DA are occurring after as little as 4 days but that they are complete by day 14 of dosing and therefore follow the pattern observed for 5-HT above and also for CIM. Dopaminergic pathways are known to project to the RN from the VTA (Ferre & Artigas 1993) and D2 receptors have been shown to mediate increased release of 5-HT (Kalén et al. 1988, Stern et al. 1981) in this region. Thus changes in the RN concentration of DA may influence RN 5-HT and therefore FC 5-HT and DA release. An alternative interaction, which would seem more applicable due to the noradrenergic nature of reboxetine, is that suggested by Pozzi et al. (1994) who provided evidence that extracellular FC concentrations of DA are regulated by noradrenergic neurones in rats. The same group also went onto suggest that as the selective NA uptake blocker desipramine significantly increased output selectively in the FC (Pozzi et al. 1994), a different organisation and regulation of DA and NA terminals must occur in this region (Pozzi et al. 1994). Therefore it may this interaction, resulting from the noradrenergic property of both reboxetine and CIM that explains the effect of antidepressants on DA release, rather than a direct effect on the dopaminergic system itself.

The effect of acute reboxetine on NA release in the FC was surprising considering the classification of the drug as a NRI (Wong et al. 2000). A dose-dependent decrease in extracellular FC NA was recorded when the drug was administered by injection (10 and 30mg/kg). A similar effect was also observed when reboxetine (10 and 100μM) was infused directly into the FC, via the dialysis probe, in an attempt to bypass the controlling influence of the LC. These results initially seem to conflict with those of Sacchetti et al. (1999) and Page & Lucki (2000) that both showed an increase in FC NA release in response to acute reboxetine treatment (1-20mg/kg i.p.). Sacchetti et al. (1999) also recorded a similar increase in hippocampal NA after 15mg/kg reboxetine. However Sacchetti et al. (1999) also observed that blockade of α2-adrenoceptors by idazoxan (1mg/kg s.c.) further potentiated the reboxetine-induced increase in FC NA release.
Yohimbine was infused into the FC because the infusion of reboxetine via the FC probe resulted in a decrease in FC NA, suggesting that a mechanism within the FC was causing the effect. Studies of idazoxan have suggested that presynaptic α₂ adrenoceptors play a major role in modulating idazoxan-induced cortical NA release in the rat (Dennis et al. 1987). The infusion of yohimbine was shown to reverse the reboxetine-induced decrease in FC NA release and this effect was significantly greater than that observed following the infusion of yohimbine alone. It can be suggested, therefore, that the discrepancy between the studies of Sacchetti et al. (1999) and Page & Lucki (2000) and the data presented here is due to different α₂ tone in the experimental settings (e.g. rat strain, holding conditions, anxiety) or even α₂-adrenoceptor subtype (Bylund 1985, Cheung et al. 1982, Summers et al. 1983), since the dose-dependent decrease in NA levels observed after both systemic and locally administered reboxetine can be reversed by infusion of yohimbine via the dialysis probe. Therefore reboxetine may require the desensitisation of α₂ adrenoceptors (or some other similar process) in the FC before increased NA levels and therefore the antidepressant benefits are observed. Yohimbine was thought to be a selective α₂-adrenoceptor antagonist (Goldberg & Robertson 1983) but evidence indicating an interaction between α₂-adrenoceptors and 5-HT systems in the CNS led to the suggestion of the involvement of the serotonergic system in yohimbine-induced effects (Cheng et al. 1993, Fuxe 1965, Maura et al. 1982, Raiteri et al. 1990). Kawai et al. (1992) investigated the properties of yohimbine and a number of its stereoisomers. They showed that yohimbine potently inhibited the binding of [³H] 8-OH-DPAT, a selective 5-HT₁A agonist, to rat hippocampal membranes as well as the binding of [³H] idazoxan, a selective α₂-adrenoceptor antagonist, to rat cortical membranes (Kawai et al. 1992). To further determine whether yohimbine acts through 5-HT₁A receptors, the effect of yohimbine on adenylate cyclase activity were studied, as adenylate cyclase is negatively coupled to 5-HT₁A receptors (Kawai et al. 1992). Yohimbine was shown to reduce forskolin-induced adenylate cyclase activity to the same extent as 5-HT and 8-OH-DPAT and therefore it was concluded that yohimbine is a potent full agonist of 5-HT₁A receptors as well as an antagonist of α₂-adrenoceptors.

A study by Haddjeri et al. (1997) showed that the firing activity of NA neurones in the rat LC could be modulated by the 5-HT system. The 5-HT₁A antagonist WAY100635
and 5-HT both failed to modify the spontaneous firing of LC NA neurones when applied by microiontophoresis, but this was rapidly suppressed by the i.v. injection of WAY100635 (Haddjeri et al. 1997). Therefore their data supports the notion that the 5-HT system tonically modulates NA neurotransmission, however the location of the 5-HT1A receptors involved in this complex circuitry remains to be elucidated (Haddjeri et al. 1997). They also showed that the suppressant effect of WAY100635 on the firing activity of LC NA neurones was a result of the enhancement of 5-HT function via presynaptic 5-HT1A receptors and that the postsynaptic 5-HT receptor mediating this effect of WAY100635 on NA neurones appears to be of the 5-HT2A subtype (Haddjeri et al. 1997). This may be important in the interpretation of the results presented above and may suggest that even antidepressants with a predominantly noradrenergic component require some serotonergic activity in order to function fully as an antidepressant.

Pre-clinical studies have shown that the concurrent administration of an α2-adrenoceptor antagonist, such as yohimbine, with a TCA yields a greater and more rapid decline in β-adrenergic receptor density (Crews et al. 1981, Johnson et al. 1980) and it has been reported that a patient with chronic depression exhibited marked improvement with the addition of yohimbine to ongoing antidepressant treatment (Pollack & Hammerness 1993). Another explanation for the decreased release of NA in response to acute reboxetine may be the anxiety levels of the animals. Yohimbine has been shown to possess anxiogenic-like effects in animal models of anxiety (Pellow et al. 1985, 1987) and this is believed to be due to its ability to increase the activity of the central 5-HT system (Cheng et al. 1993). The interaction between 5-HT1A and the α2 adrenoceptor activities of yohimbine described above could then combine to effect the reversal of the reboxetine-induced decrease in NA, as observed in this study. This would therefore suggest that changes in anxiety levels of the rats resulting from subtle variants, in holding conditions for example, may explain the different results obtained in this study, in comparison to those of Sacchetti et al. (1999).

Following 4day sub-chronic reboxetine treatment, levels of NA were still observed to be decreased and this effect was unchanged after chronic treatment. Invernizzi et al. (2000, 2001) also dosed reboxetine sub-chronically (2days) and chronically (14days) but used osmotic minipumps to ensure delivery of a constant dose of reboxetine (3, 10 and


30mg/kg/day). They observed levels of NA to be dose-dependently increased in the FC after 2 days of treatment and that this effect was significantly larger after 14 days (Invernizzi et al. 2000). The same group assessed the sensitivity of the α2-adrenoceptors by comparing the inhibitory effects of clonidine (0.01 and 0.03mg/kg i.p.) on NA release in rats given vehicle or 10mg/kg/day reboxetine for 14 days. Extracellular NA was decreased in response to clonidine in control animals, but had no significant effects on the reboxetine-induced increase in the FC. Invernizzi et al. (2000, 2001) therefore suggested that the effect of reboxetine on extracellular NA in the FC is enhanced by chronic treatment and that desensitisation of α2-adrenoceptors may be involved in this effect. The results of these chronic reboxetine studies also seem to conflict with those demonstrated in this study, but the same explanation of differing receptor tone (i.e. α2 or 5-HT1A) and delivery method may be applied.

The observation that a dysfunction of glutamatergic-NMDA receptors may be involved in the aetiology of depression (Capello et al. 1997, Layer et al. 1998) has led to more interest in the role of GLU. It is shown here that acute reboxetine treatment decreases GLU outflow in both the RN and FC, though no dose-effect was recorded. There are no published studies of the effect of reboxetine on GLU release. However Bouron & Chatton (1999) did look at the effect of acute desipramine on GLU release in the hippocampus. Desipramine has been shown to interfere with serotonergic and/or noradrenergic cells (Blier & de Montigny 1994, Potter et al. 1995) and was shown to rapidly enhance the release of GLU from cultured hippocampal neurones at therapeutic concentrations (Bouron & Chatton 1999). By acting presynaptically, desipramine is believed to activate a protein kinase and led to the speculation that TCA's may act to modify glutamatergic neurone activity, as well as acting on serotonergic and noradrenergic cells (Bouron & Chatton 1999). This was the opposite effect to that observed in the study of reboxetine presented here, but other studies have shown similar effects. A number of antidepressants, including desipramine, imipramine and citalopram, were infused directly into the FC at a concentration of 0.1mM by Golembiowska & Zylewska (1999), this resulted in a significant inhibition of veratridine-evoked GLU release in this region. A more recent study involving phenelzine and imipramine also showed that these antidepressants result in a rapid, significant decrease in stimulated GLU outflow in brain slices, but this was only observed in the pre-FC (Michael-Titus et al. 2000).
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Following sub-chronic dosing (4 day) of reboxetine, GLU levels in the RN were increased whilst those in the FC remained similar to control basal. After 14 days of reboxetine levels of GLU in both the RN and FC were significantly increased. Few studies have been carried out to investigate the chronic effect of antidepressant treatment on amino acid release. Michael-Titus et al. (2000) demonstrated that pre-FC GLU release was decreased following 21 days of imipramine or phenelzine treatment, whilst Nowak et al. (1996) recorded an increase in FC and hippocampal aspartate after 21 days of citalopram administration to mice. From this it can be deduced that chronic treatment with reboxetine allows adaptive changes to occur that affect the release of GLU. It is known that GLU release in the FC may be directly affected by antidepressants acting on Na⁺ channels or indirectly by the involvement of D₂/D₃, α₂ or 5-HT₁B heteroreceptors activated by the increased levels of monoamines in response to the blockade of their respective transporters (Golembiowska & Zylewska 1999). Although both DA and 5-HT have been shown to regulate GLU release (Maura et al. 1998, Otani et al. 1998, Vandermaelen et al. 1986), it is unlikely that this would be the mechanism following reboxetine treatment, as studies by Wong et al. (2000) have shown this compound to be highly selective for the noradrenergic system. It may well be that the effects on DA and 5-HT are secondary and that these in turn may affect GLU. Alternatively NA may modulate GLU release more directly, Crowder & Bradford (1987) observed NA inhibiting veratrine-induced GLU release from cortical slices. Further studies are required to confirm whether the effects of antidepressants are a result of their intrinsic effects on cellular release and uptake mechanisms or by their affects on monoamine levels (Bouron & Chatton 1999, Michael-Titus et al. 2000, Popoli et al. 2000).

In summary it has been shown that chronic treatment with reboxetine induces changes in the release of the neurotransmitters 5-HT, DA, NA and GLU and that these changes require adaptation within the systems involved in order for the full benefits of the antidepressants to be appreciated. Early effects of these changes can be seen after 4 days but the presumed therapeutic effects (increased cortical release of 5-HT, DA and NA) are not realised until later.
4.3.3 Effect of NMDA on basal and reboxetine-induced neurotransmitter release in the frontal cortex and raphe nuclei

Pallotta et al. (1998) showed that NMDA has a biphasic effect on 5-HT release in the FC and RN and this was confirmed by work carried out for this study. Pallotta et al. (1998) hypothesised that the degree of NMDA receptor activation regulates the outcome with regard to serotonergic transmission to the FC. Dramatically different outcomes are seen and this may be a result of a changing role of the 5-HT$_{1A}$ receptor in regulating these effects. Increased RN 5-HT release accompanied by a concomitant decrease in the FC was observed following the infusion of 100μM NMDA into the RN of control animals. Infusion of 100μM NMDA following acute administration of reboxetine (10mg/kg i.p.) reversed the reboxetine-induced increase in the RN and abolished changes in the FC. Therefore the reboxetine treatment appears to block the effects of NMDA and even reverse them completely in the RN. A similar experiment was carried out after sub-chronic (4days) of reboxetine treatment (10mg/kg i.p., twice daily). The decreased RN release induced by the reboxetine treatment was unaffected by the subsequent infusion of NMDA. However the decrease observed in the FC was further potentiated by NMDA infusion and this was a similar effect to that observed after NMDA was administered to controls.

After chronic (14day) reboxetine treatment (10mg/kg i.p., twice daily) 5-HT levels are reduced in the RN and this is accompanied by an increased release in the FC. An infusion of NMDA had similar effects to those observed after 4days of reboxetine treatment. This suggests that the effects of NMDA have been abolished in the RN following treatment with reboxetine, similar to the effect observed with CIM. However CIM treatment also resulted in the abolition of NMDA effects in the FC and this is not observed after reboxetine treatment, in fact with the exception of the acute effect, reboxetine has no effect on NMDA-induced changes in the FC. It is clear that adaptive changes are occurring over the 14day period and that the NMDA receptor may be involved, but the degree of this involvement is unknown at the present time.

Antidepressant treatment has been hypothesised to play a smoothing role in the regulation of NMDA receptor-mediated fluctuations in serotonergic transmission to the FC (Pallotta et al. 2001). Trullas & Skolnick (1990) suggested that the NMDA receptor
may be important in the aetiology of depression and since then a large number of studies have indicated a role for the NMDA receptor (see 1.8.6). Antidepressant treatment, when chronically administered, has been shown to reduce the potency of GLY to inhibit 5,7-DCKA binding to the NMDA receptor-associated GLY sites in neocortical membrane (Nowak et al. 1993, Paul et al. 1993, 1994). Harkin et al. (2000a) have carried out a similar study with reboxetine and looked at changes in binding properties of cortical NMDA receptors and cortical β₁-adrenoceptors after chronic (14day) treatment in the OB rat model of depression. Reboxetine produced a small decrease in the potency of GLY at the NMDA receptor complex, when compared to vehicle treated controls (Harkin et al. 2000a), similar to that observed with other antidepressants (Nowak et al. 1993, Paul et al. 1993, 1994). This effect was further potentiated by the co-administration of the SSRI sertraline. Reboxetine alone, and in combination with sertraline down-regulated binding of the selective β-adrenoceptor antagonist [³H]-CGP 12177 in both OB and sham operated-animals. From these results, Harkin et al. (2000a) suggest that due to the lack of effect in the [³H]-CGP 12177 binding assay, and the fact that olfactory bulbectomy and antidepressants produce similar changes in the potency of GLY at the NMDA receptor, these tests do not provide a neurochemical marker for either the behavioural hyperactivity deficit or antidepressant response in the model. Porter & Greenamyre (1995) showed that there were regional variations in the pharmacology of NMDA receptor channel blockers (e.g. MK801, amantadine) and suggested that the pharmacological properties of NMDA receptors are region-specific. Boyer et al. (1998) are the only group to date to have published work on the effects of chronic antidepressant treatment on the levels of mRNA encoding for NMDA receptor subunits. They carried out their study in mouse brain and looked at the effects of imipramine and citalopram, observing distinct, region-specific effects (Boyer et al. 1998, see 3.4.3 for more detail). An unpublished study (F. Murray, Personal Communication) has looked at the effect of chronic reboxetine treatment (10mg/kg i.p., twice daily for 14days) on NR1 protein levels in different brain regions. The results show that reboxetine significantly reduces levels of the transcript in the FC, whilst levels in the hippocampus also appear to be reduced, although this is not statistically significant. Both of these brain regions have been shown to be important in depression, as they are especially important in the processing of emotions (Marano 1999). The same group also studied the effect of chronic CIM (10mg/kg/day i.p. for 14days) on the NR1 transcript and showed significantly reduced levels in the FC,
hippocampus and amygdala (F. Murray, Personal Communication). These observations therefore confirm and extend those of Boyer et al. (1998) and it can be suggested that chronic antidepressant treatment produces region-specific changes in NMDA receptor subunit expression. It may be, therefore, that adaptive changes in the NMDA subunit composition are required before the full therapeutic benefit of an antidepressant may be observed and it is these changes that are behind the 'lag' period associated with antidepressant therapy. Although the effects of reboxetine treatment on 5-HT release are different to those observed after similar CIM treatment, it can still be suggested that reboxetine treatment results in adaptive changes in the NMDA receptor and that such changes are beginning as early as day 4 of treatment. The differing effects of the two antidepressants used in this study may be a result of their different functions – CIM is predominately an SSRI/TCA, whilst reboxetine is a NRI.

The infusion of 100μM NMDA into the RN was observed to dose-dependently decrease RN DA release in this study. The effect in the FC was observed to be biphasic, 100μM NMDA increases FC DA, whilst 25μM NMDA decreases release. The literature shows a large number of studies that support the modulation of DA release by NMDA (Feenstra et al. 1995, Kretschmer 2000, Kretschmer et al. 2000, Pallotta et al. 1999a, b, Smith & Whitton 2001, Whitton 1997, Whitton et al. 1992b). This suggests that modulation of NMDA-evoked DA release in the FC, at least, is affected by an external factor and lends support to the involvement of other transmitter systems in regulating NMDA-evoked DA release in the FC. Del Arco & Mora (1999) suggested that endogenous GLU preferentially acts thorough NMDA receptors to decrease DA metabolism in rat FC, whilst the theory of Feenstra et al. (1995) suggests that NMDA-induced decreases in DA release are a result of an indirect action via an inhibitory interneurone or polysynaptic circuit. An alternative theory resulted from the observation that infusion of the NMDA antagonist CGS-19755 into the FC resulted in a concentration-dependent decrease in DA and this could be inhibited by the sodium channel blocker tetrodotoxin (Nishijima et al. 1994). Therefore DA neurones, projecting to the FC may be tonically inhibited by excitatory amino acid neurotransmission via the NMDA receptor at the level of DA terminal fields (Nishijima et al. 1994).

The infusion of 100μM NMDA into the RN of animals that have been acutely dosed with reboxetine had no further effect on the magnitude of DA release in the RN but the
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effect was extended for the duration of the experiment. In the FC, NMDA infusion was observed to decrease DA release, therefore reversing the effects of acute reboxetine treatment (10mg/kg), although levels were similar to those recorded following acute treatment with 30mg/kg reboxetine. The effects of 100μM NMDA have therefore been reversed by the acute reboxetine treatment and this may suggest that it is somehow interfering with NMDA receptor function. These effects were further changed by sub-chronic (4day) reboxetine treatment. The reboxetine-induced increase in RN DA was potentiated by NMDA infusion but FC levels were unaffected by both the reboxetine treatment and the subsequent NMDA infusion. As observed following acute reboxetine treatment, the effect of NMDA infusion is altered by sub-chronic reboxetine treatment and because the effect changes with time, suggests that adaptation is occurring within the systems involved. Chronic (14days) reboxetine treatment resulted in increased FC DA and a decrease in RN release, the subsequent infusion of NMDA into the RN was observed to have no significant effect on DA levels in either brain region, suggesting that long term reboxetine treatment inhibits the NMDA-induced changes in DA release. As with 5-HT, the changes observed following reboxetine treatment are not the same as those observed after similar treatment with CIM. However it is clear that during chronic reboxetine treatment, marked and qualitative adaptive changes are occurring in the manner in which NMDA receptors regulate the release of DA and this may explain the effect of reboxetine on chronic basal DA release in the RN and FC. Although little work has been carried out with reboxetine, studies of other antidepressants may be important in understanding its effects. Chronic imipramine treatment has been shown to induce behavioural supersensitivity to DA agonists and this can be reversed by chronic, but not acute, treatment with the NMDA receptor antagonist MK801 (D'Aquila et al. 1992, De Montis et al. 1993). Chronic MK801 administration also blocks changes in D1 receptor number and adenylyl cyclase response to DA stimulation induced by chronic imipramine treatment (De Montitis et al. 1993). However, in contrast, a number of studies of NMDA receptor antagonists have shown that they display antidepressant-like properties in a number of animal depression models (Maj et al. 1992a, Papp & Moryl 1994, Trullas & Skolnick 1990). The study by Wedzony & Golembiowska (1993) confirmed these results when they observed that acute administration of MK801 and desipramine increased extracellular DA levels in the pre-FC.
In this study it has been shown that infusion of NMDA into the FC has a biphasic effect on the release of NA in this region. 25μM NMDA was observed to decrease NA release, whilst 100μM significantly increased extracellular levels of FC NA. Lehman et al. (1992) observed an increase in FC NA when 1mM NMDA was contained in the perfusion medium. This increase was shown to be largely reversible and could be prevented by the infusion of the NMDA receptor antagonist MK801 at a concentration of 300nM (Lehmann et al 1992). Therefore the effect observed by Lehmann et al. (1992) is similar to that observed in this study, although the concentration of NMDA used by Lehmann et al. (1992) was much higher than the one employed here. However the effect of 25μM NMDA was observed to have the opposite effect on NA release, a significant decrease in FC NA levels. A study by Yoshida et al. (1997) also supports this result, as they observed a similar reduction in NA levels following NMDA administration. They locally injected 10-20nmol NMDA into the pre-frontal cortex and noted a dose-related and NMDA antagonist-reversible facilitation of NA uptake in the FC during a period of tyrosine hydroxylase inhibition, therefore resulting in decreased extracellular levels in this region. Bilateral application of 6-hydroxydopamine into the superior cerebellar peduncle failed to affect the binding of [3H]N-(1-[2-thienyl]cyclohexyl)piperidine to the NMDA receptor associated ion channel in the pre-FC. Yoshida et al. (1997), on the basis of these results, hypothesised that in the freely moving rat, pre-FC NA neurones may be under glutamatergic facilitatory control mediated by the NMDA receptors located on the non-NA systems in the FC. From the studies of Lehmann et al. (1992) and Yoshida et al. (1997) and the data presented in this study it may be suggested that NMDA has a biphasic effect on FC NA release. Such a biphasic effect has been observed in NMDA-induced 5-HT release, although the nature of the changes is not the same (Pallotta et al. 1998). There have also been a number of studies of isolated tissues that have suggested a role for NMDA in regulating NA release. Ransom & Deschenes (1988) observed increased [3H]NA release from rat hippocampal slices following the application of NMDA. They went on to suggest that as kynurenic acid non-competitively inhibited NMDA-induced NA release and that this was in turn blocked by GLY, NMDA-induced hippocampal [3H] NA release is modulated by GLY (Ransom & Deschenes 1988). The ability of GLY to prevent kynurenic acid induced inhibition of NA release was shared by other amino acids, including isomers of serine and leucine, but GLY was shown to be the most potent (Ransom & Deschenes 1988). Fink et al. (1989) saw a similar increase in FC NA
release when cortical brain slices and synaptosomes were exposed to NMDA. They observed that NMDA concentration-dependently increased stimulated outflow from slices preloaded with \[^{3}H\]NA. This increase could be blocked by tetrodotoxin, the presence of Mg\(^{2+}\) or absence of Ca\(^{2+}\) from the perfusion fluid (Fink et al. 1989). A similar experiment carried out on human cerebral cortex brain slices was observed to have the same effects as those noted above and was further extended to conclude that NMDA receptors as well as kainate- and AMPA-recognising non-NMDA receptors stimulate NA release in the human cerebral cortex (Fink et al. 1992).

Infusion of 100\(\mu\)M NMDA into the FC following the acute administration of reboxetine (10mg/kg i.p.) had no significant effect on the reboxetine-induced decrease in FC NA release. The effect of NMDA had therefore been abolished by the reboxetine pre-treatment. A similar infusion following sub-chronic (4day) and chronic (14day) reboxetine treatment also had no significant effect on the reboxetine-induced decrease in FC NA levels. These results suggest that reboxetine is rapidly blocking the effects of NMDA and that this effect is maintained throughout the treatment period. A review of the literature failed to locate any studies of the direct effect of reboxetine on the NMDA receptor. Chronic antidepressant treatment, including desipramine, has been observed to decrease the potency of GLY to displace \[^{3}H\]5,7-DCKA in neocortical membranes (Nowak et al. 1993, Paul et al. 1993, 1994). Harkin et al. (2000b) then extended this by suggesting that noradrenergic function is critical to the action of SNRI such as desipramine at the NMDA receptor. Mice were treated with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) preceded by inhibition of the 5-HT transporter to destroy NA neurones and the ligand binding properties of the GLY recognition site of the NMDA receptor complex were assessed in vehicle and DSP-4 pre-treated animals following chronic (28day) desipramine treatment. They reported that noradrenergic lesion inhibited the increase in the IC\(_{50}\) of GLY to displace \[^{3}H\]5,7-DCKA binding to cortical membranes normally observed after chronic desipramine treatment (Harkin et al. 2000b). In contrast, lesion of noradrenergic neurones in the absence of desipramine treatment did not affect \[^{3}H\]5,7-DCKA binding or its displacement by GLY (Harkin et al. 2000b). It was therefore concluded that these data demonstrate a functional link between antidepressant actions at NA neurones and the adaptation of the NMDA receptors. The mechanism by which desipramine affects the potency of GLY at NMDA receptors is unknown, though it has been shown that some antidepressants directly
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interact with the NMDA receptor complex itself (Kitamura et al. 1991, Reynolds & Miller 1988). However their affinity for the receptor complex is considered to be too low to account for the effects reported (Harkin et al. 2000b). An alternative explanation is that antidepressant-induced adaptations to the NMDA receptor complex may be secondary to antidepressant action at monoaminergic terminals (Harkin et al. 2000b). A number of studies implicate NA neurones in the modulation of GLU release. Mori-Okamoto et al. (1991) have shown that NA can modulate GLU neurotransmission, whilst Kamisaki et al. (1991) observed NA reducing GLU release from rat cortical and spinal cord synaptosomes and reducing glutamatergic transmission in the entorhinal cortex via presynaptic \( \alpha_2 \)-adrenoceptors. A number of studies have also reported converse effects, activation of \( \beta \)-adrenoceptors, or direct activation of adenylate cyclase by forskolin, increases GLU release in the hippocampus (Chavez-Noriega & Stevens 1994, Gereau & Conn 1994). Therefore the alterations to the GLY site on the NMDA receptor complex induced by chronic antidepressant treatment may be accounted for by action at NA nerve terminals. Harkin et al. (2000b) therefore hypothesises that antidepressant interactions with monoaminergic neurotransmitter systems are required in order for these treatments to effect adaptation of the NMDA receptor complex.

The degree of NMDA receptor involvement in antidepressant action can be studied by observing the effects of NMDA and reboxetine on GLU release in the RN and FC. However it is worth remembering that glutamatergic transmission involves AMPA and Kainate receptors as well as NMDA receptors and these too may play a role in depression aetiology. It has been shown here that NMDA dose-dependently decreases FC GLU levels, but that the effects of NMDA in the RN are more complex. 25\( \mu \)M NMDA increases RN GLU, but 100\( \mu \)M NMDA is only observed to briefly increase GLU release in this region before an overall decrease is observed. This would suggest that the sensitivity of the NMDA receptors in the two regions studied differ. Individual subunits making up the receptor may determine the properties of the receptor and it is possible that the composition of NMDA receptors in the RN differs from those found in the FC, explaining the different responses to NMDA. The literature contains few examples of the effects of NMDA infusion on excitatory amino acid release. Palmer et al. (1989) suggested that a complex system of modulation exists within the FC as NMDA infusion into the FC substantially increased aspartate release in the neostriatum (Palmer et al. 1989). GABA antagonists also increased aspartate release when applied to
the neocortex and this appeared to be selective, as GLU levels were unaffected (Palmer et al. 1989). However when NMDA was co-administered with GABA antagonists, the levels of both aspartate and GLU were observed to rise (Palmer et al. 1989). Dijk et al. (1995) also reported dose-dependent increases in striatal amino acid release following the administration of NMDA. The topical application of NMDA directly to the frontal cortex meant that the concentrations of 2 and 20mM employed by Dijk et al. (1995) were significantly higher than those used in the current study. Tetrodotoxin was used to suggest that the amino acid release was a result of a non-specific, exocytotic mechanism within the striatum (Dijk et al. 1995). By applying the 5-HT1A antagonist WAY100135 or the 5-HT1A agonist 8-OH-DPAT it was concluded that a selective 5-HT1A antagonist can increase the activity of the corticostriatal pyramidal neurones and this may explain the increased levels of GLU in response to the infusion of NMDA (Dijk et al. 1995).

The reboxetine-induced decreases in FC and RN GLU release were reversed by the subsequent administration of 100μM NMDA via the RN probe. The effects of NMDA alone were also reversed by the reboxetine treatment and this suggests that reboxetine somehow affects the NMDA receptor, be it directly or via a secondary mechanism. After 4 days of reboxetine treatment levels of GLU in the RN have increased, whilst those in the FC are not significantly different from those of control basal. The subsequent infusion of NMDA to these animals has no significant effect on the increased RN GLU levels but does decrease FC release, as seen when 100μM NMDA was administered to controls. After chronic (14 day) reboxetine treatment GLU levels have increased significantly in both brain regions and are unaffected by the subsequent infusion of NMDA via the RN probe. By observing the effects of NMDA on reboxetine-induced GLU release it is clear that the sensitivity of the NMDA receptor is being affected by the reboxetine treatment and that adaptive changes are taking place during this period. It is worth noting that sub-chronic CIM treatment also results in the abolition of the NMDA effect and this may be important in understanding the role of NMDA in the mechanism of action of antidepressants. It is possible that the effects observed here are not a result of a direct action of reboxetine on NMDA receptors but of an interactive involvement between GLU and the monoamines. A number of studies have shown DA release to be modulated by GLU (Hu et al. 1999, Kretschmer 2000, Wu et al. 2000) and the interaction between these two transmitters is well recognised (Biggs & Starr 1997, Hu et al. 1999, Wu et al. 2000). Alternatively a dysfunctional interaction
between glutamatergic and serotonergic transmission, possibly at the level of the RN, may be significant (Pallotta et al. 2001) and this theory is supported by the observation that GLU release is regulated via 5-HT$_{1A}$ receptors in the guinea pig dentate gyrus (Matsuyama et al. 1996).

In summary these results have shown that the release of 5-HT, DA, NA and GLU can be modulated by NMDA receptors and that these effects may be a result of interactions between these systems. As with CIM, the effects of NMDA are altered over a period of chronic reboxetine administration, suggesting that the antidepressant is inducing adaptive changes within the system. The data presented here also supports the hypothesis for the involvement of the NMDA receptor in the aetiology of depression.

4.3.4 Effect of the NMDA antagonist amantadine on basal and reboxetine-induced neurotransmitter release in the raphe nuclei and frontal cortex

Based upon the results of the CIM studies and due to time constraints it was decided to restrict the study of the effects of NMDA antagonists on reboxetine-induced transmitter release. Amantadine was chosen because of its positive effects on CIM-induced transmitter release, which indicated that this was the most likely compound of those tested to reduce the 'lag' phase observed before the therapeutic effects of an antidepressant are observed. A review of the literature has also shown that amantadine has been shown to alleviate depressive symptoms in patients suffering from Parkinson’s disease (Vale et al. 1971) and its potential antidepressant properties have recently been put to the test (Huber et al. 1999, Moryl et al. 1993).

Trullas & Skolnick (1990) have suggested that the behavioural deficits induced by inescapable stress may be due to an involvement of NMDA receptors. They suggested that pathways subserved by the NMDA receptor may be involved in the pathophysiology of depression and that NMDA receptor antagonists may have antidepressant potential (Trullas & Skolnick 1990). The effects of various NMDA receptor antagonists (including MK801, ketamine, (+)-HA-966, memantine) on transmitter release have been studied and are discussed in more detail in 3.4.4 (Callado et al. 2000, Lindefors et al. 1997, Goldstein et al. 1994, Quack et al. 1995, Whitton et al, 1992 a, b). Tanaka et al. (1973) found that amantadine had no significant effect on 5-
HT levels or 5HTP decarboxylase activity, but, that one-hour after 100mg/kg amantadine 5HIAA levels were reduced and MAO activity was inhibited. It was concluded that as MAO inhibition induced 5-HT accumulation in the brain was enhanced by the amantadine treatment, amantadine must affect the serotonergic system (Tanaka et al. 1973). DA levels have been shown to be increased by amantadine in both the FC (Spanagel et al. 1994) and the striatum (Quack et al. 1995, Spanagel et al. 1994) and these studies suggest that the effects of NMDA antagonists may be region-specific. In support of this theory is the study by Porter & Greenamyre (1995) that used quantitative autoradiography to study the regional binding characteristics of a diverse group of NMDA receptor antagonists, including amantadine. Their results provide further support for the theory that cerebellar and forebrain NMDA receptors exhibit different pharmacological properties (Porter & Greenamyre 1995).

Amantadine is believed to function by weakly interacting with the MK801 binding site in the NMDA receptor channel, blocking the flow of ions through the receptor. When administered alone it was observed to have no significant effect on 5-HT release in either the FC or RN, or on RN levels of DA and GLU. NA levels are reduced in the FC and although no definite effects on DA and GLU are observed, there are trends of increase and decrease respectively. Amantadine was therefore shown to modulate the release of transmitters when administered alone, but it seems that the effects on 5-HT and NA are the most potent. The effect of amantadine on reboxetine-induced changes in transmitter release proved to be more interesting. Amantadine was observed to reverse the reboxetine-induced increase in RN 5-HT and this was accompanied by a potentiation of the reboxetine-induced increase in the FC. Reboxetine-induced DA release was decreased in both regions by amantadine – this was a reversal of the reboxetine effect in the FC and potentiated the decrease in the RN. The effect of amantadine on reboxetine-induced NA release in the FC was also very interesting, a reversal of the reboxetine-induced decrease was observed and a brief but significant increase recorded. Levels of FC 5-HT and NA were therefore seen to be increased following acute treatment with reboxetine and amantadine, without the usual ‘lag period’ associated with reboxetine treatment. Although a similar effect on DA was not observed this might be dependent on changes in other systems occurring before increased DA release can be observed. Reboxetine-induced changes in FC GLU appear to be unaffected by the subsequent administration of amantadine and levels remain
lower than those of controls. However in the RN, the reboxetine-induced decrease is reversed and a significant and persistent increase in RN GLU levels was observed. This effect was similar to that observed following the local infusion of 25\mu M NMDA and also after 100\mu M NMDA was infused into the RN of CIM-treated animals. This suggests a possible common mechanism between the two antidepressants used in this study. The change in NMDA effect may be a result of a reduced sensitivity of the NMDA receptor as 100\mu M NMDA has been shown to elicit a response similar to that initiated by 25\mu M NMDA in control animals. The principal observation of this study is that amantadine alters the effect of reboxetine on neurotransmitter release in the RN and FC of rats and that by combining these two drugs the latent period typically observed before increased cortical 5-HT and NA release is observed following treatment with reboxetine is reduced. Amantadine may decrease NMDA receptor function and prevent reboxetine-induced increases in RN 5-HT and the consequent activation of 5-HT\textsubscript{1A} receptors. Artigas et al. (1996) hypothesise that RN 5-HT\textsubscript{1A} receptor desensitisation is a requirement for antidepressant action and compounds that reduce the time taken for this to occur may be of future clinical benefit. Both pindolol and WAY100635 have already been shown to potentiate antidepressant action (Artigas et al. 1994, Blier & Bergeron 1995, Blier et al. 1997, Cryan et al. 1999, Gartside et al. 1995, Millan et al. 1998, Romero & Artigas 1997, Romero et al. 1996). Amantadine also has a number of dopaminergic effects, including inhibition of uptake, stimulation of uptake and altered receptor confirmation (Allen 1981, Gianutsos et al. 1985, Heikkila & Cohen 1972), that may explain the dopaminergic effects observed in this study. Finally Fisher & Starr (2000) studied the effects of amantadine on two enzymes important in monoamine biosynthesis, DDC and 5HTPDC, in the substantia nigra and corpus striatum of reserpine-treated rats. The activity of DDC was strongly increased, whilst no effect on 5HTPDC was noted in either region tested (Fisher & Starr 2000). They came to the conclusion that DA and 5-HT were being differentially regulated by GLU, DDC being tonically suppressed and 5HTPDC being tonically stimulated (Fisher & Starr 2000). Alternatively amantadine could be acting via an unknown, indirect pathway e.g. intracellular signalling, Ca\textsuperscript{2+} level modulation.

In summary these results have shown that the addition of amantadine will effect the release of transmitters induced by acute reboxetine treatment. Although not all the changes are the same as those observed following CIM treatment, there are a number of
important similarities, including the increase in RN GLU and increased cortical levels of 5-HT. I therefore believe that a common mechanism has been revealed in these studies, as two antidepressants with different modes of action have been modulated in the same way by the NMDA ion channel antagonist amantadine.

4.3.5 Effects of sub-chronic amantadine treatment on sub-chronic reboxetine-induced changes in transmitter release in the frontal cortex and raphe nuclei

Sub-chronic dosing of amantadine alone resulted in decreased FC 5-HT, DA and NA and also RN DA. Levels of RN 5-HT and GLU were observed to increase following the treatment. These results do not correspond with those observed following the acute injection of amantadine and therefore suggest that adaptive changes within the NMDA receptor complex itself, in transmitter interactions or in intracellular signalling cascades are occurring after as little as 4 days of treatment. The combination of amantadine and reboxetine sub-chronic treatment resulted in increased cortical release of both 5-HT and DA, to similar levels observed following 14 days of reboxetine treatment. Levels of FC NA remain decreased and this may be a result of α2-adrenoceptor control via the LC or differing receptor tone (see 4.4.2 for further explanation). GLU appears to be unaffected by the 4 day treatment with reboxetine and amantadine, a reversal of acute effects and also when the two drugs are administered alone sub-chronically. It is worth noting that infusion of 100μM NMDA has differing effects on the transmitters and there does not appear to be a uniform response. For example, infusion of NMDA into animals treated sub-chronically with reboxetine and amantadine results in increased FC 5-HT release but has no effect in the RN. These effects do not resemble the effects of NMDA in controls and suggest that, in the RN at least, NMDA action is being blocked, possibly the result of adaptive changes in the receptor itself. Similar effects are observed for DA, NA and GLU and suggest that some form of adaptation is occurring at the NMDA receptor itself, but that interactions between the transmitters themselves may also be involved. The effects of amantadine may be dependent on the type of NMDA receptor subtype present, the NMDA receptors present in the RN may be of different composition to those in the FC or display different sensitivities. This theory was discussed briefly above and will be looked at in more detail in chapter five.
In comparison to the sub-chronic study of CIM and amantadine, the reboxetine and amantadine treatment appears to be successful in terms of increased cortical 5-HT and DA release. This may well be due to the nature of reboxetine and it could be that the noradrenergic component is more important than first appreciated. It is also possible that reboxetine itself is interfering with the NMDA receptor directly and a study of reboxetine on the binding properties of cortical NMDA receptors in the OB model of depression did note a decrease in the potency of GLY to displace \[^{3}H\]5,7 DCKA in cortical homogenates of OB rats when compared to sham-operated controls (Harkin et al. 2000a).

In summary the present findings support the involvement of the NMDA receptor in the mechanism of action of the antidepressant reboxetine. The combined treatment with amantadine significantly increases the cortical levels of both DA and 5-HT, and as this is believed to be a pre-requisite for antidepressant action, this may be important in understanding the aetiology of depression. It would be interesting to further explore the differences between CIM and reboxetine and may be elucidate why the reboxetine/amantadine combination is more successful, in terms of cortical monoamine release, than CIM/amantadine.

4.3.6 Effects of chronic reboxetine treatment on body weight

There appears to be no published studies of the effects of reboxetine treatment on weight gain, either in clinical or pre-clinical studies. Over 14 days of reboxetine treatment, average body weight was seen to increase at an overall rate of approximately 25% of that of control animals. This was significantly lower than the rate of weight gain observed when CIM was administered for a similar period.

Weight gain is a frequent and inconvenient side effect of TCA treatment (Berken et al. 1984) and this is believed to be related to antagonism of 5-HT\(_2\) and histamine receptors (Bernstein 1988, Fernstrom 1995, Garland et al. 1988). Subtypes of these receptors have been shown to have higher affinity for reboxetine in a CNS receptor binding study (Wong et al. 2000). This may suggest that reboxetine would be expected to induce weight gain, though its affinity is more than 1000-fold less than its affinity for the NA transporter. The effect of the TCA and SNRI desipramine on food intake and body
weight in rats has been studied (Nobrega & Coscina 1987) and this is the closest comparison that can be made to the effects of reboxetine observed in this study. Chronic desipramine treatment was administered at a range of drug doses (2.5-17mg/kg), by different routes of administration (i.p., s.c., oral, daily injections, continuous release from osmotic minipumps), in various diet compositions and palatability (regular chow pellets, powder, added high fat or carbohydrate, high or low protein diets) to animals of either sex in single and group housing (Nobrega & Coscina 1987). None of these treatments were shown to result in an increase in daily food intake or rates of body weight gain, in fact desipramine treatment was observed to cause decreased food intake and weight loss (Nobrega & Coscina 1987), similar to those observed following chronic reboxetine treatment. It has been observed that routes of metabolism of reboxetine vary between species (Dostert et al. 1997). For example, humans have been shown to absorb reboxetine rapidly ($t_{\text{max}} \approx 2$ hours), with a $t_{1/2}$ of 13 hours, which allows for twice daily dosing (Dostert et al. 1997). Animal models also absorb reboxetine rapidly ($t_{\text{max}} 0.5-2$ hours), but $t_{1/2}$ was much faster at 1-2 hours (Dostert et al. 1997). When comparing reboxetine to other currently used antidepressants, the most striking metabolic difference is the absence of inhibitory effects on the major human CYP isoforms involved in the majority of drug metabolism (Dostert et al. 1997). These differences may explain the effects of reboxetine on weight gain in rats, but make it difficult to hypothesise about the effect of reboxetine in humans. The observation that chronic reboxetine treatment reduces the rate of weight gain in rats may be important in the study of eating disorders, especially anorexia nervosa and bulimia and when compared to the effects of chronic CIM, it may be possible to understand the mechanisms involved in these disorders.

4.5 Conclusions

1) Reboxetine regulates the release of neurotransmitters in the FC and RN of the freely moving rat, as measured by in vivo microdialysis. Over a treatment period of 14 days, the effects of reboxetine are observed to change and this suggests that adaptation is occurring within the system.

2) NA levels are unexpectedly reduced in response to reboxetine treatment and this may be a result of $\alpha_2$-adrenoceptor action, as yohimbine was shown to reverse this
effect. However yohimbine is also known to possess serotonergic activity and it may be an interaction with this system that results in the increased levels of NA.

3) Activation of the NMDA receptor also modulates the release of the transmitters in the FC and RN.

4) NMDA-evoked release may be the result of interactions between the NA, 5-HT, DA and GLU systems as opposed to direct effects on the individual systems.

5) NMDA also modulates reboxetine-induced changes in transmitter release, confirming a role for NMDA receptors in antidepressant action.

6) The non-competitive NMDA receptor ion channel blocker amantadine was observed to effect the release of transmitters in both the FC and RN.

7) Addition of amantadine to a reboxetine treatment regime appears to increase cortical levels of both 5-HT and DA. The effect on NA levels may be a result of interactions between transmitter systems, masking the increased release that was observed following acute treatment.

8) The differences in GLU release in the two regions following this treatment suggests that antidepressant treatment may be dependent on the subtype of NMDA receptor present in important brain areas.

9) A highly reduced rate of weight gain is observed following the chronic administration of reboxetine. Although the effects in humans is unknown this may be important in determining the suitability of this drug for use in certain categories of patients.

10) Chronic reboxetine treatment results in a reduced rate of weight gain compared to similar CIM treatment, suggesting that the differing properties of these two antidepressants may be important in the elucidation of the mechanisms of eating disorders.
Chapter Five

Concluding Remarks
5.1 Overview

There were three main aims of this study:

1) Do antidepressants of different classes, e.g. TCA and SNRI, have a similar effect on NMDA-evoked monoamine and amino acid release in the RN and FC?
2) Do NMDA antagonists have a similar effect on monoamine and amino acid release in the RN and FC?
3) Do NMDA antagonists and currently used antidepressants show synergism that may lead to a more rapid onset of increased monoamine release in the FC and thereby antidepressant effect? Is this effect restricted to a particular class of antagonist?

The results of this study indicate that, in general, the two classes of antidepressants exert similar effects on basal monoamine and amino acid release in the FC and RN when administered acutely. However the effects of NMDA on CIM and reboxetine-induced release are different and may be a result of the different properties of the antidepressant or due to the dose given. Following sub-chronic dosing of CIM and reboxetine, changes in basal and NMDA-evoked monoamine and amino acid release were again differentially affected. Chronic dosing resulted in similar effects on terminal monoamine release and increases in 5-HT and DA were recorded following CIM or reboxetine treatment. It was disappointing that increased FC NA levels were not observed following reboxetine treatment and this was especially surprising as reboxetine is classified as a SNRI. This may be a result of $\alpha_2$ receptor tone or subtle variations in holding conditions and is more fully explored in 4.3.2.

The effect of a range of NMDA antagonists on CIM-induced release was studied in chapter three and although all effected the release of monoamines and amino acids in the regions studied, only one, amantadine, was shown to positively effect terminal transmitter release. This is interesting as amantadine is a low affinity channel blocker and its co-administration with CIM resulting in increased terminal 5-HT suggests that NMDA receptors and Ca$^{2+}$ levels may be important in the mechanism of action of antidepressants (see 1.8.6 and 1.6.3). It was therefore decided to observe the effects of amantadine on reboxetine-induced release of both monoamines and amino acids following acute and sub-chronic dosing. The treatment resulted in significantly increased terminal release of 5-HT and DA in comparison to CIM + amantadine,
although levels of NA were still decreased in this region. To my knowledge this is the first study to demonstrate that amantadine can be used to potentiate the effect of antidepressants from different classes on terminal transmitter release. NMDA also differentially affected transmitter release following the combined treatment and this may be explained in terms of individual NMDA receptor subtypes present in the FC and RN. For example, the NMDA receptor polyamine site antagonist ifenprodil has been shown to selectively bind to the NR2B subunit over the NR2A subunit (Williams 1993) and may explain its effects in the current study.

The functional distribution of NMDA receptor subtypes has been studied by a number of groups (Masu et al. 1993, Monyer et al. 1992, 1994) and radioligand and electrophysiological studies indicate that NMDA receptor properties vary throughout the CNS (Laurie & Seeburg 1994). As outlined in 1.8.3, NMDA receptors are heteromultimers composed of multiple protein subunits. The mRNA encoding for the individual subunits is differentially expressed during development. The NR1 subunit mRNA is expressed from an early stage of development and is found in almost every neuronal type in the adult CNS (Monyer et al. 1994, Moriyoshi et al. 1991, Watanabe et al. 1992), whilst the mRNA encoding for NR2 subunits is spatially more restricted and differentially regulated during development (Ishii et al. 1993, Kutsuwada et al. 1992, Monyer et al. 1992, 1994, Pollard et al. 1993, Sheng et al. 1994, Watanabe et al. 1992). Monyer et al. (1992) visualised three NR2 subunit mRNAs in rat whole rat brain using in situ hybridisation and noted differential expression patterns. NR1 subunits are prominently and ubiquitously expressed throughout the brain and this is in contrast to the restricted distribution of the NR2 subunits. NR2A mRNA is found in the forebrain and cerebellum and its distribution is the most closely related to that of the NR1 subunit mRNA (Monyer et al. 1992). NR2B is present in the forebrain but not in the cerebellum, whilst NR2C shows the highest levels in the cerebellum. The thalamic nuclei contain all three NR2 subunits, but there is less NR2A mRNA, whilst the amygdaloid nuclei express mRNA encoding NR2A and NR2B (Monyer et al. 1992). Laurie & Seeburg (1994) demonstrated that ligand affinities at recombinant NMDA receptors depend on subunit composition and as the involvement of NMDA receptors in the aetiology of depression is well founded (for review see Skolnick 1999 and 1.8.6) this may explain the regional-specific actions of drugs such as the antidepressants used in this study.
It is also worth considering a new hypothesis concerning another glutamatergic receptor, AMPA. The theory suggests that the availability of the AMPA subtype can be regulated by endocytosis (Beattie et al. 2000, Ehlers 2000, Lin et al. 2000). Although initially controversial, the theory is now more widely accepted as studies into the cellular mechanisms regulating the internalisation of AMPA receptors are carried out. Both Beattie et al. (2000) and Ehlers (2000) showed that application of NMDA triggers internalisation of the AMPA receptors and I feel that this would be of interest in the current study. However there appear to be a number of different pathways through which AMPA receptors become internalised and these appear to be dependent on the nature of the stimulus (Beattie et al. 2000, Ehlers 2000, Lin et al. 2000), suggesting a complex system of receptor regulation. If such a system of regulating AMPA subtype availability is in place it may act alongside NMDA receptors in regulating antidepressant-induced transmitter release or alternatively a similar system may govern the availability of NMDA receptor subtypes, with the antidepressant/amantadine combination acting as a stimulus to induce endocytosis and effect transmitter release in specific regions.

From the results presented in this study I would suggest that the effect of the antidepressant/amantadine combination treatment on transmitter release is the result of regional variation in NMDA receptor subtype distribution. Therefore I would suggest that a specific NMDA receptor subtype distribution is necessary before the clinical effects of the antidepressant can be fully appreciated. The mechanisms governing distribution appear to be more complex than originally thought and the antidepressant treatment itself may act as a stimulus to regulate the expression of certain NMDA receptor subtypes. It appears that the use of the NMDA antagonist amantadine accelerates the effects of the antidepressants on transmitter release and this further reinforces the involvement of the NMDA receptor in the possible aetiology of depression. Amantadine may therefore act as a stimulus in intracellular signalling pathways or modulate NMDA receptor subtype availability via its action at the NMDA receptor, in order to accelerate antidepressant action.

Recent research into depression has moved to the study of intracellular signalling pathways, especially those involving the neurotrophic factor BDNF. Both NMDA antagonists and BDNF have been shown to be protective against neuronal insults,
presumably by dampening NMDA receptor function (Choi 1988, Kornhuber & Weller 1997). Long term exposure to imipramine or BDNF has been shown to result in reduced levels of NR2A subunit mRNA (Boyer et al. 1998, Brandoli et al. 1998), showing a similar mechanism of action. If increased BDNF levels are the key to the antidepressant action of current therapies it may be possible to develop new treatments which bypass the monoaminergic synapse altogether. The results presented in this study suggest that the mechanism of the antidepressant/amantadine treatment should be further investigated in order to determine the effects of the treatment on BDNF levels.

I believe that the results presented in this study suggest a novel mechanism of decreasing the ‘lag’ period prior to the onset of antidepressant action. This may be a result of changes in NMDA receptor subunit availability in terminal regions, in BDNF levels or by some currently undetermined mechanism. The different effects observed with CIM and reboxetine are most likely a result of their individual properties, i.e. TCA/SSRI and SNRI respectively, however the results do indicate that a common pathway is followed but the exact nature remains to be determined.

5.2 Future directions

The work presented here provides the basis for a number of research directions and these can be summarised under the following aims:

1) Is reboxetine active at the NMDA receptor and if so what are its properties?
2) Do different classes of antidepressant drugs elicit time-dependent changes in the NMDA-receptor regulation of monoamine and amino acid release?
3) Are such changes in NMDA receptor regulation maintained following the cessation of antidepressant treatments?
4) Are changes in NMDA regulation of monoamine and amino acid release accompanied by changes in the brain regional expression levels of the NMDA receptor subunits, especially in terminal regions?
5) Does antidepressant treatment affect NMDA receptor subunit localisation?
6) Are similar changes observed following combined treatment with an NMDA antagonist?
7) What is the involvement of other glutamatergic receptors, e.g. AMPA/Kainate, in antidepressant action?

8) Is NMDA receptor subunit availability regulated in the same fashion as AMPA receptors i.e. endocytosis?

9) Do different classes of antidepressants modulate BDNF levels in the same fashion?

10) Do different classes of NMDA receptor antagonists have similar effects on BDNF levels?

11) Are such changes in BDNF potentiated or inhibited by the addition of an NMDA receptor antagonist to the treatment regime?
Appendices
Appendix I: Serotonergic receptor nomenclature
(Reproduced from Alexander & Peters 2000)

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>5-HT&lt;sub&gt;1A&lt;/sub&gt;</th>
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### Appendix I: Serotonergic receptor nomenclature continued

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### Appendix II: Dopaminergic receptor nomenclature

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