

**The role of α_{2A} -adrenergic receptor antagonism in
therapeutic efficacy and onset of action of antidepressant
drugs in a rat model of depression**

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This study is dedicated to my dearest and beloved father and friend. I am blessed to have known such a remarkable and loving person for 22 years.

NICO LIEBENBERG

(19/08/1956 – 06/02/2004)

I will miss you every day of this life until I see you again.

Abstract

While delayed onset of antidepressant action remains a shortcoming of current antidepressants, preliminary clinical data with newer antidepressants, including mirtazapine, show promise for a hastened onset. Several potential mechanisms have been postulated and investigated. Mirtazapine displays serotonin 5-HT₂ and 5-HT₃ receptor blocking properties, while its putative earlier onset of action is thought to be related to its α_2 -adrenoceptor lytic action (at α_2 -adrenergic autoreceptors and heteroreceptors), thereby modulating central serotonergic and noradrenergic neurotransmission.

The current study investigated the role of α_2 -adrenoceptor antagonism for earlier onset of action in a rat model of depression. First of all, the treatment period necessary to produce antidepressant-like responses with fluoxetine (an antidepressant with a delayed onset of action) was established. Rats were treated for 3, 7, and 11 days with fluoxetine, whereafter the forced swim test (FST) was employed and cortical β -adrenoceptor density was measured. The results showed that fluoxetine elicits antidepressant-like behavioural and neuroreceptor effects after 7 and 11, but not 3 days of treatment. Therefore, antidepressant-like effects in this study would be recognised as early effects if they were visible after 3 days of treatment.

To investigate the role of α_2 -adrenoceptor antagonism for earlier onset of action, rats were treated for 3 and 7 days with fluoxetine, mirtazapine (α_2 -lytic mode unknown), yohimbine (α_2 -adrenoceptor inverse agonist), idazoxan (α_2 -adrenoceptor neutral antagonist), or combinations with fluoxetine, where after the FST was employed and cortical β -adrenoceptor and hippocampal 5-HT_{1A} receptor densities were measured. Results with the FST support an earlier onset of action by mirtazapine, but do not support an important role for α_2 -lytic action in this regard. β -Adrenoceptor density generally decreased with antidepressant action, but is not a good marker of hastened onset. Furthermore, 5-HT_{1A} receptor density is not a good marker for antidepressant action. It was concluded that a property different from its α_2 -adrenoceptor-lytic action may be important for the earlier onset of action by mirtazapine.

Abstrak

Terwyl 'n vertraagde aanvang van werking steeds 'n tekortkoming van antidepressante is, is daar voorlopige, maar belowende data vanaf kliniese studies met nuwere antidepressante, insluitend mirtasepien, wat 'n versnelde aanvang van werking toon. Verskeie potensiële meganismes is gepostuleer en ondersoek. Mirtasepien vertoon serotonien 5-HT₂ en 5-HT₃ reseptor antagonisme, terwyl die middel se versnelde aanvang van werking toegeskryf word aan sy α_2 -adrenoseptor litiese aksie (by α_2 -autoreseptore en heteroreseptore), en hierdeur sentrale serotonergiese, asook noradrenergiese neurotransmissie moduleer.

In die huidige studie is die rol van α_2 -adrenoseptor antagonisme in versnelde aanvang van werking in 'n diere model van depressie ondersoek. Eerstens is die behandelingstydperk wat nodig is om antidepressiewe effekte te inisieer met fluoksetien ('n antidepressant met 'n vertraagde aanvang van werking) bepaal. Rotte was behandel met fluoksetien vir 3, 7 en 11 dae, waarna die geforseerde swem toets uitgevoer en kortikale β -adrenoseptor digtheid bepaal is. Die resultate het getoon dat fluoksetien antidepressiewe effekte vertoon na 7 en 11, maar nie na 3 dae nie. Gevolglik is effekte wat na 3 dae waargeneem word in hierdie studie as vroeë effekte beskou.

Rotte was behandel vir 3 en 7 dae met fluoksetien, mirtasepien (wyse van α_2 -litiese werking onbekend), yohimbien (α_2 -adrenoseptor inverse agonis), idazoxan (α_2 -adrenoseptor neutrale antagonis), of kombinasies van hierdie middels met fluoksetien. Na behandeling is die geforseerde swem toets en meting van β -adrenoseptor en 5-HT_{1A} reseptor digtheid gebruik om antidepressante werking te identifiseer. Resultate vanaf die geforseerde swemtoets ondersteun 'n versnelde aanvang van werking van mirtasepien, maar ondersteun nie die hipotese dat α_2 -litiese werking 'n belangrike rol hier in speel nie. β -Adrenoseptor digtheid het oor die algemeen afgeneem met antidepressante werking, maar het nie gekorreleer met 'n versnelde aanvang nie. Verder is gevind dat veranderings in 5-HT_{1A} reseptor digtheid nie 'n goeie merker is van antidepressante werking nie. Daar is afgelei dat 'n ander eienskap van, wat nie verband hou met sy α_2 -litiese eienskappe nie, 'n belangrike rol speel in die versnelde aanvang van antidepressante werking van mirtasepien.

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Introduction

Chapter

1.1 PROBLEM STATEMENT

Depression is a serious and burdensome anxiety-related mood disorder. Effective treatments have been available for many years and the introduction of the selective serotonin reuptake inhibitors (SSRIs) has considerably improved the safety and tolerability of antidepressant therapy. However, despite these advances, some elements still plague the effective drug treatment of depression. Of the most troublesome factors includes the delay in the onset of the therapeutic action of antidepressants, which is typically a minimum of two weeks (Bymaster *et al.*, 2003; Leonard, 2003). Furthermore, treatment of at least 12 weeks is usually necessary to prevent relapse (Koran *et al.*, 2001). Not only is the suffering of patients prolonged after commencement of treatment, but they also remain at great risk of suicide. In addition to this, compliance is often hampered by the occurrence of adverse effects, which are frequently at their worst during the initiation of drug therapy.

The lag in onset of therapeutic activity of antidepressants is a feature of all classes of antidepressants. However, it is believed that delayed onset of antidepressant action is not a characteristic of the disease, since some treatments, such as a one night sleep deprivation and electroconvulsive shock therapy (ECT) appear to produce antidepressant effects almost immediately (Gillin, 1983; Daly *et al.*, 2001). Moreover, some recently introduced antidepressants (e.g. mirtazapine) show promise for a more rapid onset of action (Blair, 2003). This has encouraged interest in the mechanism of action of these drugs, as well as in the development of new therapeutic approaches exploiting these putative mechanisms.

Although longer-term adaptive changes in receptor sensitivity may better explain the delayed onset of action of antidepressants, the mechanism based on acutely elevated norepinephrine (*l*-NE) and serotonin (5-HT) synaptic levels remains the basis for new drug design. The dual action concept, which postulates that effects on both *l*-NE and 5-HT are more advantageous than a selective action on serotonin reuptake, has been used to design new antidepressants such as venlafaxine and mirtazapine. Mirtazapine, the prototype noradrenergic and specific serotonergic antidepressant (NaSSA), is an antagonist at 5-HT₂ and 5-HT₃ receptors, while its α_2 -adrenoceptor (α_2 -AR) blocking property is also believed to be a major mechanism by which it elicits an antidepressant response (de Boer, 1996). In addition, the α_2 -lytic action of mirtazapine

is suggested to play an important role in the putative earlier onset of antidepressant action of this drug, since this property leads to simultaneous enhancement of both *L*-NE and 5-HT neurotransmission (Blier, 2003). However, further studies are needed to either confirm or deny this hypothesis, which was also one of the objectives of the current study. Insight into the mechanisms of action of antidepressants, especially those involved in a more rapid onset of action, is of immense value to the development of novel and more effective antidepressants.

1.2 STUDY AIMS

The main aims of this project were to:

- Explore the putative ability of mirtazapine to present with an early onset of antidepressant-like action in an animal model of depression;
- Investigate the role of the α_2 -lytic properties of mirtazapine in its putative earlier onset of antidepressant-like action; and
- Explore whether an inverse agonist at α_2 -ARs is superior to a neutral antagonist in inducing a more rapid onset of antidepressant-like action.

1.3 STUDY LAYOUT

In order to recognise the onset of an antidepressant-like response as an early response, we firstly needed to establish a basal time for onset of a conventional antidepressant with a delayed onset of antidepressant action (e.g. fluoxetine). To achieve this, male Sprague Dawley rats were treated with fluoxetine for 3, 7, or 11 days whereafter behavioural and biochemical antidepressant-like responses were evaluated. Antidepressant-like responses were defined, firstly, by behavioural changes in the rat forced swim test (FST), and secondly by alterations in β -adrenoceptor (β -AR) and 5-HT_{1A} receptor densities in specific brain regions of rats.

In the core component of the current study, the importance of the role of α_2 -AR antagonism (including inverse agonism and neutral antagonism at these receptors) in the putative ability of mirtazapine to produce an earlier onset of antidepressant-like action was investigated. Animals were treated with a series of α_2 -lytic drugs (alone as well as in combination with fluoxetine) for the shortest treatment period after which fluoxetine elicited antidepressant-like responses (as observed in the initial phase of the study, described above), as well as for a shorter period after which fluoxetine was unable to produce such effects. Therefore, an antidepressant-like response was regarded as an early response if significant effects were noted after the shorter treatment period (i.e. the period after which the conventional antidepressant fluoxetine produced

no response). The series of drugs for treatment of rats included fluoxetine (20 mg/kg/day), mirtazapine (15 mg/kg/day), the α_2 -AR inverse agonist yohimbine (3 mg/kg/day), and the α_2 -AR neutral antagonist idazoxan (3 mg/kg/day), as well as fluoxetine + mirtazapine, fluoxetine + yohimbine and fluoxetine + idazoxan. This enabled us to investigate the role of α_2 -AR antagonism in eliciting an earlier onset of antidepressant-like action.

Literature overview

Chapter

This chapter presents a brief overview of some features of depression concerning prevalence, common symptoms, and the most recent classification of the disease. It discusses the various pathophysiological theories of depression and the proposed anatomical brain regions involved, as these are believed to be important for investigating and understanding the neurobiological basis of antidepressant action. This chapter also contains discussions on the drug treatments of depression, as well as the tribulations of, and proposed mechanisms and solutions for, the delayed onset of antidepressant responses. Since the current study focuses on the role of α_2 -AR antagonism in the rapidity of onset of action of antidepressants, inverse agonism is also discussed. Furthermore, this chapter confers a contextualisation of the preclinical evaluation of antidepressant action, including animal models of depression and their recent modifications, as well as the effects of antidepressant treatments on the density of β -adrenoceptors (β -ARs).

2.1 DEPRESSION

Mood disorders are amongst the most prevalent forms of mental illnesses. Severe forms of depression affect 2 to 5% of the population of the United States of America, while up to 20% of the population suffers from milder forms of the illness. Depression is almost twice as common in females as in males. Another 1 to 2% of the population suffers from bipolar disorder (also known as manic-depressive illness), which affects males and females equally. Mood disorders can be recurrent and life threatening (due to the risk of suicide), and remain a major cause of morbidity worldwide (Akiskal, 2000).

Relevant to the circumstances in South Africa, it has been reported that patients living with HIV are especially prone to develop depression (Janssen *et al.*, 1989; Ostrow *et al.*, 1989; Hays *et al.*, 1992; Perdices *et al.*, 1992; Judd & Mijch, 1996). The reasons for the high rates of symptoms found in these patients are not clear, but possible risk factors include a past history of depression, psychosocial stressors such as limited social support, stigma, bereavement and medical risk factors such as the development of AIDS- and HIV-related opportunistic conditions (Ostrow *et al.*, 1989; Gorman *et al.*, 1991; Hays *et al.*, 1992; Lyketsos *et al.*, 1996a; Lyketsos *et al.*, 1996b). The association between HIV-infection and depression, as well as the high incidence of violence and trauma in this country and the association thereof with depression (Van Winkle, 2000; Nixon *et al.*, 2004; Rayburn *et al.*, 2005; Bandelow *et al.*, 2005), further

emphasises the importance of research into depression and the effective treatment thereof in South Africa.

2.1.1 Diagnosis of depression

Depression is diagnosed as "major depression" based on symptomatic criteria described in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994), and is depicted in Table 2-1.

Table 2-1: Diagnostic criteria for major depression

Depressed mood
Irritability
Low self-esteem
Feelings of hopelessness, worthlessness, and guilt
Decreased ability to concentrate and think
Decreased or increased appetite
Weight loss or weight gain
Insomnia or hypersomnia
Low energy levels, fatigue, or increased agitation
Decreased interest in pleasurable stimuli (e.g., sex, food, social interactions)
Recurrent thoughts of death and suicide

A diagnosis of major depression is made when five of these symptoms (Table 2-1) are reported for longer than a 2-week period, and when the symptoms disrupt normal social and/or occupational functioning (American Psychiatric Association, 1994).

Milder cases are classified as "dysthymia" although there is no clear distinction between the two. It is obvious from the criteria in Table 2-1 that the diagnosis of depression, as opposed to most diseases of other organ systems (e.g., diabetes or cancer) is not based on objective diagnostic tests, but rather on a highly variable set of symptoms. Therefore, depression should not be viewed as a single disease, but rather as a heterogeneous syndrome, putatively consisting of numerous diseases of distinct causes and pathophysiologies. Attempts have been made to establish subtypes of depression defined by certain sets of symptoms, and are described in Table 2-2 (Akiskal, 2000; Blazer, 2000).

Table 2-2: Examples of proposed subtypes of depression

Depression subtype	Main features
Melancholic depression ¹	Severe symptoms; prominent neurovegetative abnormalities
Reactive depression ²	Moderate symptoms apparently in response to external factors
Psychotic depression	Severe symptoms; associated with psychosis
Atypical depression	Associated with labile mood, hypersomnia, increased appetite, and weight gain
Dysthymia	Milder symptoms, but with a more protracted course
¹ Melancholic depression is similar to a syndrome classified as "endogenous depression".	
² Reactive depression is similar to a syndrome classified as "exogenous depression".	

Note: These subtypes are, however, based only on symptomatic differences and there is no evidence to date that they reflect different underlying disease states (Nestler *et al.*, 2002).

2.1.2 Aetiology of depression

Epidemiologic studies suggest that 40 to 50% of the risk for depression is genetic (Sanders *et al.*, 1999; Fava & Kendler, 2000). This would imply that depression is a highly heritable disorder, although there is still only speculation as to the specific genes that could be involved in the aetiology of depression. There are many challenges facing researchers when attempting to identify these genes, which are reviewed elsewhere (Burmeister, 1999), and include the fact that depression is a complex phenomenon with many genes potentially involved in its aetiology. In addition, vulnerability to depression is only partly genetic, with non-genetic factors also playing an important role. These are as diverse as stress and emotional trauma and viral infections (e.g., Borna virus). Even some random processes during brain development have been implicated in the aetiology of depression (Akiskal, 2000; Fava & Kendler, 2000).

The role of stress seems to be of particular importance. Depression is often described as a stress-related disorder, and there is good evidence that episodes of depression often occur in the presence of some form of stress (Kendler *et al.*, 1999). However, stress *per se* is not sufficient to cause depression, since most people do not become depressed after serious stressful experiences, and those who do become depressed sometimes do so following mild stress. Also, severe stress, such as that experienced during war or rape, does not typically induce depression, but instead causes post-traumatic stress disorder (PTSD) (Nestler *et al.*, 2002), which is symptomatologically distinct from depression. There are, however, significant interactions between the two states (Shalev *et al.*, 1998). The observations mentioned above suggest that depression may be caused by interactions between a genetic predisposition and

some environmental factors, rendering the mechanisms of such interactions an important focus of investigation (Van Praag, 2004).

2.1.3 Neuroanatomy implicated in depression

Although many brain regions have been implicated in depression, there is non consensus on the particular neural circuitry(ies) underlying normal mood and mood abnormalities (Nestler *et al.*, 2002). This is in sharp contrast to neuropsychiatric disorders such as Parkinson's disease, Huntington's disease, and Alzheimer's disease for which pathological lesions in specific brain regions, involving known well-defined neural circuitries have been identified.

It is likely that many brain regions mediate the diverse symptoms of depression. This is supported by human brain imaging studies, which have demonstrated changes in blood flow or other measures in several brain regions, including regions of the prefrontal and cingulate cortex, hippocampus, striatum, amygdala, and thalamus, to name a few (Drevets, 2001; Liotti & Mayberg, 2001). Similarly, anatomical studies of the brains of depressed patients, as obtained with autopsy have reported abnormalities in many of these same brain regions (Rajkowska, 2000; Drevets, 2001; Manji *et al.*, 2001). However, since some of the imaging and autopsy studies have yielded contradictory findings, there is still no consensus on the role of these regions in depression.

Knowledge of the normal neuropsychological and other mental functions of the respective brain regions implicated to be defective in depression, may also suggest the symptoms of depression to which they may contribute. For example, the neocortex and hippocampus may mediate cognitive aspects of depression, such as the impairment of memory and feelings of worthlessness, hopelessness, guilt and suicidal thoughts. The striatum (particularly the ventral striatum or nucleus accumbens (NAc)) and amygdala are involved in emotional memory, and could therefore mediate anhedonia (decreased drive and reward for pleasurable activities), anxiety, and reduced motivation that is present in many patients (Nestler *et al.*, 2002). It has been speculated that dysfunction of the hypothalamus may be involved in the neurovegetative symptoms of depression, including excessive or impaired sleep, appetite and energy, as well as a loss of interest in sex and other pleasurable activities (Nestler *et al.*, 2002). These various brain regions operate in a highly interactive manner, which could represent the neural circuitry involved in depression.

Figure 2-1 shows a simplified illustration of a series of neural circuits in the brain that may contribute to depressive symptoms. While most research in the field of depression has focused on the hippocampus and prefrontal cortex (PFC), there is increasing realisation that several subcortical structures are implicated in reward, fear, and motivation (Yadid *et al.*, 2001). These

include the nucleus accumbens (NAc), amygdala, and hypothalamus. The figure shows only a few of the many known interconnections among these brain regions, as well as the innervation of several brain regions by monoaminergic neurons. The ventral tegmental area (VTA) provides dopaminergic input to the NAc, amygdala, PFC, and other limbic structures. Noradrenergic (from the locus coeruleus, LC) and serotonergic (from the dorsal raphe, DR) neurons innervate all of the regions shown in the figure. In addition, there are strong connections between the hypothalamus and the VTA-NAc pathway.

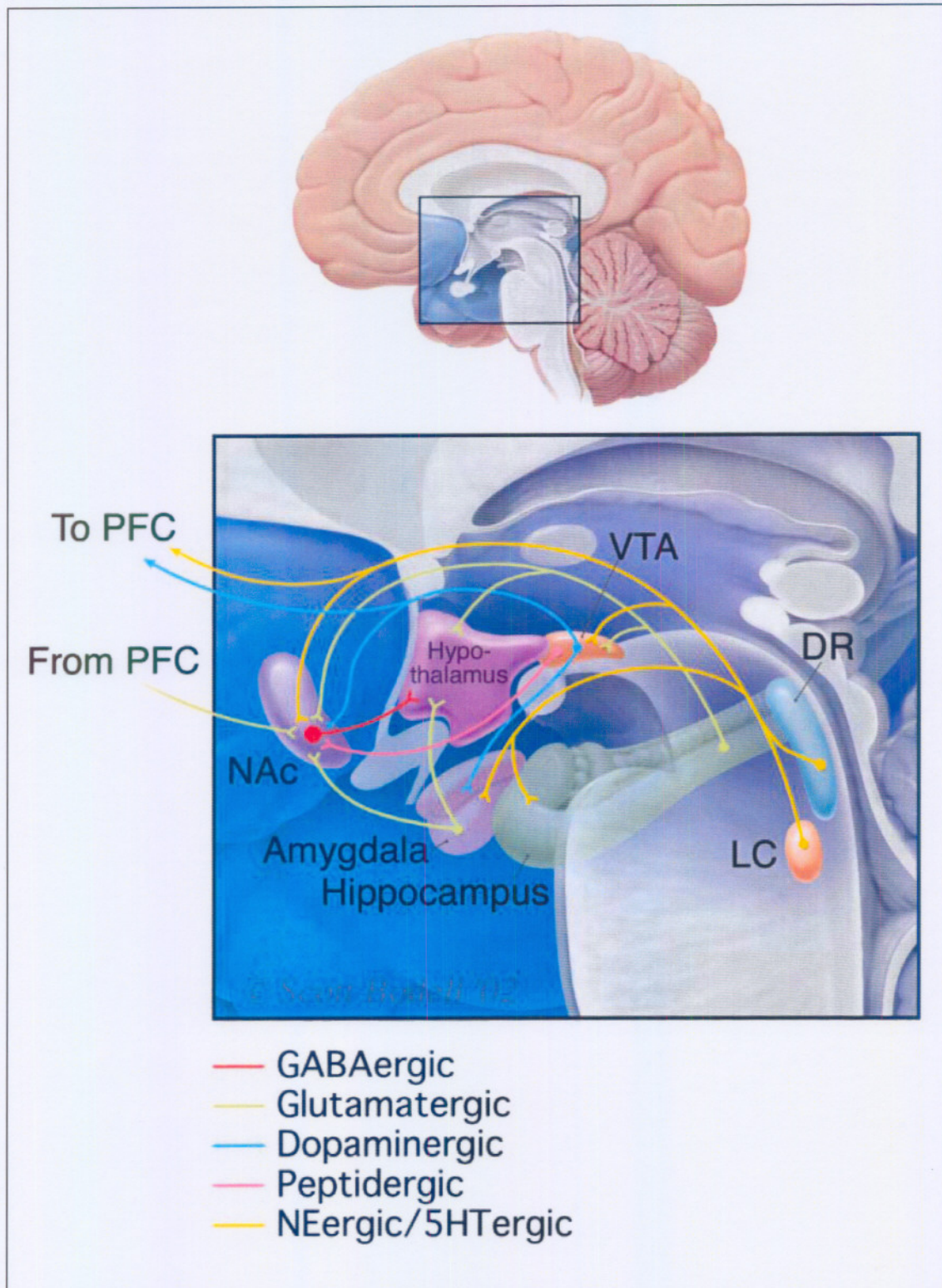


Figure 2-1: Neural circuitry of depression. Abbreviations: PFC = prefrontal cortex; VTA = ventral tegmental area; NAc = nucleus accumbens; DR = dorsal raphe; LC = locus coeruleus. (Nestler *et al.*, 2002).

2.1.4 Neuropathological hypotheses of depression

The four hypotheses presented below are not comprehensive of the field of depression, but provide examples of traditional, as well as recent approaches toward understanding depression and antidepressant action. These discussions (with the exception of the monoamine hypothesis, which contain facts presented in Elhwuegi (2003)) mostly reflect particulars presented in Nestler *et al.* (2002).

2.1.4.1 Monoamine hypothesis

2.1.4.1.1 The old monoamine theory of depression

The modern history of drug therapy of depression started in the early 1950s when iproniazid, which was developed for the treatment of tuberculosis, was found to have mood elevating effects in patients with tuberculosis and depression. One year later it was found that iproniazid was capable of inhibiting monoamine oxidase (MAO) (Delay *et al.*, 1952). A few years later, the antidepressant efficacy of imipramine, a tricyclic compound with some structural resemblance to the antipsychotic drug chlorpromazine, was discovered accidentally (Kuhn, 1958). The search for compounds related chemically to imipramine has yielded several successful tricyclic drugs that are still currently in clinical use. To varying degrees, these compounds share the capability of inhibiting neuronal uptake of monoamines. A number of the tricyclic antidepressants (TCAs) block the reuptake of both 5-HT and *L*-NE (e.g. imipramine and amitriptyline), while others are more selective in blocking the reuptake of 5-HT (e.g. clomipramine) or *L*-NE (e.g. desipramine). Mianserin was the first atypical antidepressant discovered that lacked an inhibitory effect on the reuptake of monoamines and did not inhibit MOA (Leonard, 1978). Mianserin was found to act by blocking the presynaptic α_2 -ARs. However, the adverse effects of this antidepressant, namely postural hypotension and sedation, have been attributed to its antagonistic action on α_1 -ARs and histaminergic H_1 receptors, respectively (Asakura & Tsukamoto, 1985). In a search for better antidepressants with fewer side-effects and a larger therapeutic index, the selective serotonin reuptake inhibitors (SSRIs), e.g. fluoxetine and paroxetine were introduced in the early 1990s (Fuller, 1995). During the same period, the highly potent α_2 -AR antagonist mirtazapine (Smith *et al.*, 1990) was discovered. The last antidepressant introduced by the end of the last century was the highly selective *L*-NE reuptake inhibitor (NRI), reboxetine (Dencker, 2000).

Although these drugs belong to different chemical groups and act at different sites of the monoaminergic neuron, they all share the property of acutely modifying monoamine levels at the synapse. Some antidepressants (e.g. TCAs, SSRIs and NRIs) block the reuptake (the physiological inactivation process) of certain monoamines into the monoaminergic nerve

terminal, increasing their availability at the synapse. Others (e.g. tranylcypromine and phenelzine, not mentioned above) inhibit the intraneural metabolism of monoamines by inhibiting MAO, thus increasing the amount of the monoamines stored and released. The more recent antidepressants introduced acts by blocking presynaptic inhibitory α_2 auto- and heteroreceptors, thus increasing the amount of the monoamines released at the synapse. On the other hand, it was found that drugs that cause depletion of monoamines (e.g. the antihypertensive reserpine may induce depression (Goodwin & Bunney Jr, 1971). There are also clinical data which indicate that acute depletion of *L*-tryptophan (a precursor metabolite of 5-HT) may induce depressive symptoms in susceptible persons (Schmeck *et al.*, 2002).

Some of these facts lead to the suggestion of the monoamine theory of depression in the 1960s (Schildkraut, 1965). This theory simply states that depression is due to the deficiency of monoaminergic activity in the brain and that depression is treated by drugs that increase this activity.

However, this theory suffered from several drawbacks and failed to explain several facts. Firstly, there are drugs that can increase brain monoaminergic activity (e.g. cocaine and amphetamine) but are not clinically effective as antidepressants. Secondly, and most importantly, these changes in the monoamine levels at the synapse take place within hours after the administration of the antidepressants, but the therapeutic response requires the continuous administration of these drugs for weeks (Baldessarini, 1989).

2.1.4.1.2 The modern monoamine theory of depression

Four decades of research on the mechanisms involved in depression has led to the accumulation of a large amount of evidence supporting the idea of an important role for the monoamines in depression. This evidence was obtained by studying the long-term effects of antidepressant treatments on the monoamines and their receptor density and function both in animals and in depressed patients.

The modified monoamine theory suggests that the acute increase in the levels of monoamines at the synapse may only be an early step in a potentially complex cascade of events that ultimately results in antidepressant activity (Pineyro & Blier, 1999). This acute increase in monoamine concentration at the synapse has been found to induce desensitisation of the inhibitory auto- and heteroreceptors located in certain brain regions. The desensitisation of these receptors would result in higher central monoaminergic activity that coincides with the appearance of the therapeutic response. These adaptive changes responsible for the therapeutic effect depend on the availability of the specific monoamine at the synapse. Furthermore, blocking the somatodendritic as well as the nerve terminal autoreceptors, increased the response rate in the treatment of major and treatment-resistant depression,

providing further support for the assumption that the antidepressant effect results from the long-term adaptive changes in the monoamine auto- and heteroreceptors. In the following sections, evidence from the literature for the abovementioned assumptions will be presented.

2.1.4.1.2.1 Changes in noradrenergic receptor sensitivity

It is a well documented fact that the continuous exposure of the receptor to an agonist for a certain period may result in adaptive changes in the receptor sensitivity and/or density. It can therefore be predicted that the acute increase of the monoamine level at the synapse will result, after a certain period, in a decrease in the number and/or sensitivity of the noradrenergic receptors. This prediction was found to be true both in depressed patients and animals. It was reported that β -AR concentration and function are consistently decreased in the rat cortex by the chronic administration (14 days) of desipramine, electroconvulsive therapy (ECT) (Heal *et al.*, 1987; Heal *et al.*, 1989) or reboxetine (Harkin *et al.*, 2000) (see § 2.5.2 for more detailed evidence for alterations in β -AR density following antidepressant treatment). Furthermore, it was reported that upregulation of β -ARs has been consistently observed in patients with depression, and downregulation of β -ARs is regarded as a marker for antidepressant activity (Leonard, 1997) (see § 2.5.2).

In contrast, the number and function of postsynaptic α_1 -ARs in the rat cortex were reported to be increased by the chronic administration a large number of antidepressants (Maj *et al.*, 1985). Similarly, changes in α_2 -AR sensitivity after chronic therapy with antidepressants have also been reported. The inhibitory α_2 -heteroreceptors are believed to control 5-HT release from the serotonergic nerve terminal (Limberger *et al.*, 1986), while α_2 -autoreceptors control the release of *I*-NE from noradrenergic nerve terminals (Dennis *et al.*, 1987) (see § 2.2.1). It would be expected, therefore, that desensitisation of these receptors will increase the availability of *I*-NE and 5-HT at the synapse.

In summary, the chronic administration of antidepressants desensitises α_2 -ARs and increases the sensitivity of stimulatory α_1 -ARs, thus increasing the release of both 5-HT and *I*-NE at certain synapses that would result in antidepressant activity (see § 2.2.1). On the other hand, the downregulation of β -ARs by chronic antidepressant therapy might be a useful tool for the assessment of antidepressant action, which is also employed for this purpose in the current study.

2.1.4.1.2.2 Changes in serotonergic receptor sensitivity

Reports regarding the sensitivity and density of 5-HT_{2A} receptors are not very consistent. Several studies in rodents have shown a decrease in the number or function of 5-HT_{2A} receptors in the frontal cortex after short-term treatment with antidepressant drugs that increase the synaptic availability of 5-HT, such as imipramine, desipramine, citalopram and paroxetine

(Goodnough & Baker, 1994; Klimek *et al.*, 1994; Maj *et al.*, 1996). However, another study found no changes in the density of 5-HT_{2A} receptors in the cerebral cortex of the guinea pig after the chronic daily treatment with paroxetine, fluoxetine or amitriptyline (Cadogan *et al.*, 1993), but did report an increase in the sensitivity 5-HT_{2A} receptors. On the other hand, an increase in the density of 5-HT_{2A} receptors in the rat frontal cortex after chronic treatment with fluoxetine or levoprotiline was reported (Hrdina & Vu, 1993; Klimek *et al.*, 1994).

Serotonergic 5-HT_{1A} receptor regulation is thought to be essential to the antidepressant response (Blier & Ward, 2003). These receptors are located presynaptically in the dorsal raphe nuclei, where they act as somatodendritic autoreceptors to inhibit the firing rate of 5-HT neurons (see § 2.2.1), and are located postsynaptically in limbic and cortical regions where they also attenuate firing activity. It was reported that the sustained treatment of rats with citalopram for 2 weeks produced desensitisation of 5-HT_{1A} autoreceptors (Invernizzi *et al.*, 1994), and similar results were reported in the dorsal raphe (Hervas *et al.*, 2001). In contrast, chronic treatment with fluoxetine or imipramine was reported to produce hypersensitivity of postsynaptic 5-HT_{1A} receptors in the dorsal hippocampus (Shen *et al.*, 2002; Elena Castro *et al.*, 2003). These differential adaptive changes of pre- and postsynaptic 5-HT_{1A} receptors could underlie the mechanism of action of antidepressants, and also contribute to their clinical effects (Elena Castro *et al.*, 2003).

Serotonergic 5-HT_{1B} receptors are located in the axon terminals of both 5-HT and non-serotonergic neurons, where they act as inhibitory autoreceptors or heteroreceptors, respectively. It would be expected, therefore, that decreasing the activity of these receptors will increase central monoaminergic activity. Several studies have demonstrated that antidepressants may facilitate 5-HT neurotransmission through the desensitisation of 5-HT_{1B} receptors (Pineyro & Blier, 1996; Pineyro & Blier, 1999). This may account for, at least in part, the antidepressant activity of these drugs.

In summary, results regarding 5-HT receptors are not very consistent, where the majority of evidence supports regional differences in the regulation of central 5-HT receptor function following repeated antidepressant treatments (Hensler, 2003). There is also convincing evidence that antidepressant therapy may relieve depression through an increased efficacy of the 5-HT system as a result of the desensitisation of somatodendritic 5-HT_{1A} and terminal 5-HT_{1B} autoreceptors, thereby restoring the normal function of this system.

2.1.4.2 Dysregulation of the hippocampus and hypothalamic-pituitary-adrenal axis

A prominent response of the brain to acute and chronic stress includes the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 2-2) (Ehlert *et al.*, 2001). Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing hormone

(CRH), which stimulates the synthesis and release of adrenocorticotropin (ACTH) from the anterior pituitary. ACTH then stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. Glucocorticoids exert extensive effects on general metabolism and also dramatically affect behaviour via direct actions on numerous brain regions (Schimmer & Parker, 2001).

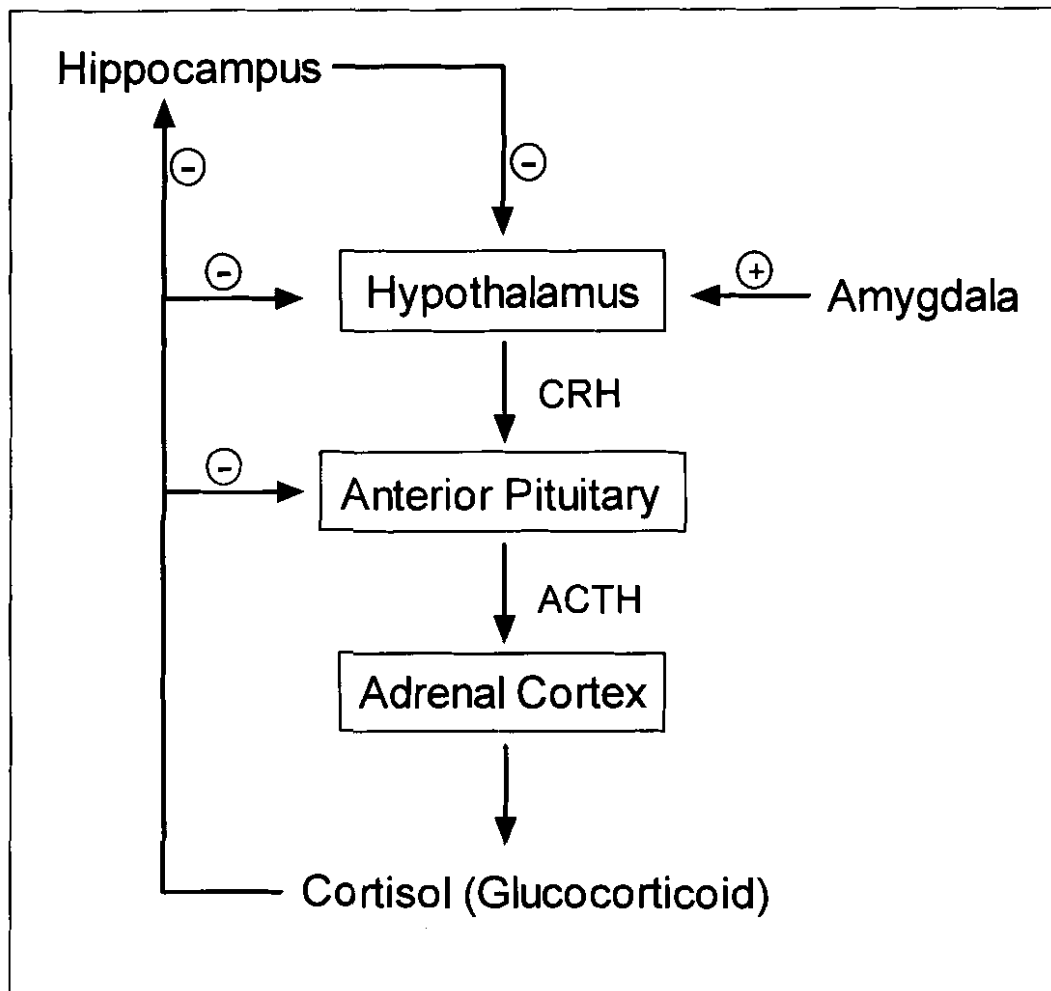


Figure 2-2: The hypothalamic-pituitary-adrenal (HPA) axis. Abbreviations: CRH = corticotrophin-releasing hormone; ACTH = adrenocorticotropin. Produced according to particulars discussed in Schimmer & Parker (2001).

The activity of the HPA axis is controlled by several brain pathways, including the hippocampus (which exerts an inhibitory influence on hypothalamic CRH-containing neurons) and the amygdala (which exerts an excitatory influence). Glucocorticoids, by potently regulating hippocampal and PVN neurons, exert powerful feedback effects on the HPA axis (Schimmer & Parker, 2001). Levels of glucocorticoids that are seen under normal physiological circumstances enhance hippocampal inhibition of HPA activity. They may also enhance hippocampal function in general and thereby promote certain cognitive abilities (Nestler *et al.*, 2002). However, sustained elevation of glucocorticoids, seen under conditions of severe stress, may damage hippocampal neurons, particularly CA₃ pyramidal neurons (McEwen, 2000b;

Sapolsky, 2000). Such damage would be expected to reduce the inhibitory control that the hippocampus exerts on the HPA axis, which would further increase the levels of circulating glucocorticoids and subsequent hippocampal damage. Abnormal, excessive activation of the HPA axis is observed in approximately half of individuals with depression, and these abnormalities are corrected by antidepressant treatment (Sachar & Baron, 1979; De Kloet *et al.*, 1988; Arborelius *et al.*, 1999; Holsboer, 2001).

In addition to the effects of cortisol, there are also striking parallels between some aspects of the stress response, severe depression, and the effects of centrally administered CRH. These include increased arousal and vigilance, decreased appetite, decreased sexual behaviour, and increased heart rate and blood pressure (Arborelius *et al.*, 1999; Holsboer, 2001). In addition, it has been demonstrated that antagonism of CRF₁ receptors produces antidepressant-like effects in an animal model of depression (Chaki *et al.*, 2004). Following these observations, it can be suggested that a hyperactive HPA axis may contribute to depression not only via hypercortisolemism, but also via enhanced CRH transmission in the hypothalamus and other brain regions that are innervated by these neurons.

Despite the compelling evidence supporting this model, it is still unknown whether HPA axis abnormalities are a primary cause of depression or, instead, secondary to some other initiating cause. Nevertheless, a strong case can be made for its role in the generation of some symptoms of depression.

2.1.4.3 Impairment of neurotrophic mechanisms

The pathologic effects of stress on the hippocampus described above, have contributed to another recent hypothesis, which proposes a role for neurotrophic factors in the aetiology of depression and its treatment (Altar, 1999). Neurotrophic factors were first characterised for regulating neuronal growth and differentiation during development, but are now known to be potent regulators of plasticity and survival of adult neurons (Ghosh *et al.*, 1994; Sklairavron & Nestler, 1995; Mamounas *et al.*, 2000). The neurotrophic hypothesis of depression states that a deficiency of neurotrophic support may contribute to depressive symptoms.

Work on this hypothesis has focused on brain-derived neurotrophic factor (BDNF), one of the most prevalent neurotrophic factors in adult brain. Acute and chronic stress decreases levels of BDNF expression in the dentate gyrus and pyramidal cells in the hippocampus in rodents (Smith *et al.*, 1995). This reduction appears to be mediated partly via stress-induced glucocorticoids and partly via other mechanisms, such as stress induced increases in serotonergic transmission (Smith *et al.*, 1995; Vaidya *et al.*, 1997). In support of this theory, chronic (but not acute) administration of virtually all classes of antidepressants increase BDNF expression in the abovementioned regions (Nibuya *et al.*, 1995) and can prevent the stress-induced decreases in

BDNF levels. There is also evidence that antidepressants increase hippocampal BDNF levels in humans (Chen *et al.*, 2001). Antidepressant-mediated induction of BDNF is at least partly mediated via the transcription factor CREB (cAMP response element binding protein), and is described below. These findings suggest that antidepressant-induced upregulation of BDNF could help repair some stress-induced damage to hippocampal neurons and protect vulnerable neurons from further damage. This theory could also explain why an antidepressant response is delayed: it would require sufficient time for levels of BDNF to gradually rise and exert their neurotrophic effects. Some compelling evidence for this hypothesis comes from a recent study where administration of BDNF or a related neurotrophin (neurotrophin-3) into the dentate gyrus or CA₃ region of the rat hippocampus causes antidepressant-like effects as measured by the forced swim and learned helplessness tests (Shirayama *et al.*, 2002).

The BDNF hypothesis predicts that agents that promote BDNF function might be clinically effective antidepressants. However, no such drugs are available. Another approach would be to intervene earlier in the process, that is, in the mechanisms by which antidepressants induce BDNF expression. There is now considerable evidence that CREB is involved and that the BDNF gene is induced *in vitro* and *in vivo* by CREB (Tao *et al.*, 1998; Conti *et al.*, 2002). Also, virtually all major classes of antidepressants increase levels of CREB expression and function in several brain regions, including the hippocampus (Nibuya *et al.*, 1996; Thome *et al.*, 2000). Increased CREB activity in the rat hippocampal dentate gyrus, achieved by injection of a viral encoding CREB directly into this brain region, exerts an antidepressant-like effect in the forced swim and learned helplessness tests (Chen *et al.*, 2001).

While these effects of CREB could be mediated via numerous target genes in addition to BDNF, it does create opportunity for novel strategies to influence hippocampal function in depression.

The role of stress, glucocorticoids and glutamate in synaptic remodelling has recently been reviewed (Harvey *et al.*, 2003). Conditions of excessive glutamatergic activity, associated with elevated levels of glucocorticoids, have been implicated in structural remodelling in the hippocampus, involving decreased branching of dendrites and loss of dentate granule cells (McEwen, 1999; McEwen, 2000a; McEwen, 2000b). It is now becoming increasingly clear that modifications induced by stress or antidepressants in the strength of synaptic transmission in the hippocampus and the molecular modifications induced by antidepressants have their origins in effects at the N-methyl-D-aspartate (NMDA) receptor (Popoli *et al.*, 2002), which is stimulated by glutamate. This synaptic remodelling and eventual damage may underlie the neurodegenerative pathology documented in patients suffering from severe depression (Sapolsky, 2000).

In animal models of stress, acute stress significantly suppresses neurogenesis in the dentate gyrus (Gould *et al.*, 1998), while a similar effect is obtained with the administration of adrenal glucocorticoids (McEwen, 1999) and repeated stress (Pham *et al.*, 2003). Up to 3 weeks of repeated stress allow for virtually complete recovery of neuronal modelling after termination of stress (Conrad *et al.*, 1999; Sousa *et al.*, 2000), but 6 weeks of repeated stress leads to changes that suggest that reversal may not occur so readily (Pham *et al.*, 2003). Thus, the resilience of the brain to repeated stress may give way to permanent damage. Stress-mediated glucocorticoid release has been proposed to promote neuronal death or necrosis, by an increased release of glutamate, disturbed Ca^{2+} homeostasis via excessive activation of NMDA receptors, inhibition of glucose transport and an increase in oxygen radical production (Sapolsky, 2000).

In addition to the possible mechanisms outlined above, dysfunction of the immune system during depression may also have neurodegenerative effects (Wichers *et al.*, 2006). Major depression has been associated with increased, cell-mediated immune activation (Maes, 1995; Sluzewska *et al.*, 1996; Mikova *et al.*, 2001; Zorrilla *et al.*, 2001). Increased concentrations of the proinflammatory cytokines tumour necrosis factor alpha (TNF- α) (Tuglu *et al.*, 2003), interleukin-1 beta (IL-1 β) (Thomas *et al.*, 2005), and interleukin-6 (IL-6) (Maes *et al.*, 1997) are present during depression. The question remains whether altered immune activation in depressed patients is a causal risk factor for the development of depression or an epiphenomenon. Studies have shown that interferon-alpha (IFN- α) treatment in hepatitis C patients results in major depressive disorder (MDD) in about 30% of the patients (Bonaccorso *et al.*, 2002; Hauser *et al.*, 2002; Dieperink *et al.*, 2003). In a previous study, it was observed that immune-induced neurotoxic substances were associated with IFN- α -induced depressive symptoms over the course of treatment (Wichers *et al.*, 2005), suggesting that the association between immune activation and depressive symptoms may be mediated by neurotoxicity. Although this finding suggests that immune activation be a causative factor in depression, it is unclear whether natural occurring inflammation also has the potential of putting subjects at risk of depression (Wichers *et al.*, 2006).

2.1.4.4 Impairment of brain reward pathways

It is evident from the above discussion that most preclinical studies have focused on the hippocampus as the site involved in the generation and treatment of depression. However, it is unlikely that the hippocampus on its own accounts completely for the symptoms of depression. As mentioned earlier, brain imaging and autopsy studies suggest abnormalities in several brain areas of depressed individuals. There has been increasing recognition of the role played by some subcortical structures (e.g., the NAc, hypothalamus, and amygdala) in the regulation of

motivation, sleep, appetite, energy level, circadian rhythms, and responses to pleasurable and aversive stimuli, factors which strongly relate to depression (see Table 2-1).

2.1.4.4.1 Nucleus accumbens

The NAc is a target of the mesolimbic dopamine system, which arises in dopaminergic neurons in the ventral tegmental (VTA) area in the midbrain. These VTA neurons also innervate several other limbic structures, including the amygdala and limbic regions of the neocortex (see Figure 2-1). The NAc, and its dopaminergic inputs, play critical roles in reward (Ikemoto & Panksepp, 1999). It is reported that virtually all drugs of abuse increase dopaminergic transmission in the NAc, which partly mediates their rewarding effects (Koob *et al.*, 1998; Wise, 1998).

The possible involvement of the VTA-NAc pathway in mood regulation and depression is not well understood. There have been some publications over the past several years reporting an association between the two (Brown & Gershon, 1993; Willner, 1995; Di Chiara *et al.*, 1999; Pallis *et al.*, 2001; Yadid *et al.*, 2001), but the majority of research has focused largely on *I*-NE and 5-HT mechanisms in other brain circuits (e.g., the hippocampus and neocortex).

An interesting finding relevant to the current study, is a reported relationship between serotonin-induced dopamine (*I*-DA) release in the NAc and onset of antidepressant action (Dremencov *et al.*, 2004; Dremencov *et al.*, 2005). The study proposes that 5-HT_{2C} receptors might be hyperfunctional in depressed individuals, where these receptors play an inhibitory role in serotonin-induced *I*-DA release in the NAc. It is suggested that the onset of an antidepressant-like response is dependent on the reversal of 5-HT_{2C} receptor hyperfunctionality, which would allow 5-HT to effectively increase *I*-DA levels in the NAc.

2.1.4.4.2 Hypothalamus

The hypothalamus has long been known to mediate many neuroendocrine and neurovegetative functions. The hypothalamus has been studied in the context of depression, although most of this work has focused on the HPA axis (as described in § 2.1.4.1). Other hypothalamic functions and nuclei have remained largely unexplored in depression research, despite the fact that these nuclei and their peptide transmitters are crucial for appetite, sleep, circadian rhythms, and interest in sex, which are abnormal in depressed patients (Nestler *et al.*, 2002).

2.1.4.4.3 Amygdala

The amygdala is well studied for its role in conditioned fear (Davis, 1998; Cahill *et al.*, 1999; Ledoux, 2000). It mediates the ability of previous non-threatening stimuli to elicit a wide range of stress responses when associated with naturally frightening stimuli (eg. exposure to a predator or other severe stress).

The amygdala is equally important for conditioned responses to rewarding stimuli, including drugs of abuse and natural rewards (Everitt *et al.*, 1999). In fact, some view the amygdala as part of a larger circuit, termed the extended amygdala, which also includes the NAc and other brain regions (de Olmos & Heimer, 1999). It is proposed that the circuits formed by these structures are critical for emotional memory, as well as in mental strength and persistence.

The amygdala and its related structures have been the focus of a great deal of work in the anxiety, PTSD, and drug addiction fields, but have received relatively little attention on depression. This is despite the fact that symptoms of anxiety and fear, and abnormal responses to pleasurable stimuli, are prominent in many depressed individuals.

2.2 DRUG TREATMENT OF DEPRESSION

2.2.1 Alteration of monoaminergic neurotransmission

Most drug treatments of depression target presynaptic processes to increase the concentrations of 5-HT, *l*-NE, and in some cases also *l*-DA, at postsynaptic monoamine receptors. Principally, two different strategies are used. Once released from presynaptic vesicles, the monoamines are cleared from the synaptic cleft via a reuptake transporter located on the presynaptic nerve terminal. Blockade of this transporter is the principal action underlying most antidepressants, particularly the TCAs, SSRIs and related drugs. The alternative mechanism is the inhibition of monoamine degradation by specific oxidases within the presynaptic terminal. Only a few drugs are marketed that target the monoamine oxidases, mostly because of severe side effects following dietary intake of tyramine. Of the two major molecular species of MAO, type A is selectively inhibited by clorgyline, while type B is selectively inhibited by selegiline (also known as *[-]*-deprenyl). 5-HT and *l*-NE terminals contain mainly MAO-A, while MAO-B is predominant in blood platelets (Baldessarini, 2001). Except for selegiline, clinically employed MAO inhibitors (phenelzine and tranylcypromine) inhibit both MAO-A and MAO-B.

More recently introduced antidepressants (e.g. mianserin and mirtazapine) presumably produce antidepressant effects by blocking α_2 auto- and heteroreceptors, thereby increasing postsynaptic 5-HT and *l*-NE availability (see § 2.2.1.3.3). Lastly, recent evidence suggest that 5-HT_{1A} receptor agonists may have antidepressant activity (Blier & Ward, 2003), most probably by stimulating postsynaptic 5-HT_{1A} receptors on pyramidal neurons in the hippocampus (see Figure 2-3).

The most serious problem with antidepressants is their delayed onset of action. Although drug-induced inhibition of the reuptake transporter occurs within minutes or hours, successful therapy

takes a minimum of two weeks before the first signs of symptom alleviation are observed (Bymaster *et al.*, 2003; Leonard, 2003), and a few additional weeks until full remission. This observed delay in therapeutic action, despite immediate changes in monoamine concentrations, raises the idea that enhancement of monoaminergic neurotransmission may be distinct from the mechanism by which these drugs alleviate depression.

Figure 2-3 illustrates the interaction between 5-HT and *l*-NE neurons and their projections to pyramidal neurons of the hippocampus. In order to understand the proposed mechanism of action of antidepressants (conventional and putative early onset antidepressants), it is necessary to be familiar with the neurotransmitter systems involved and their interrelationships.

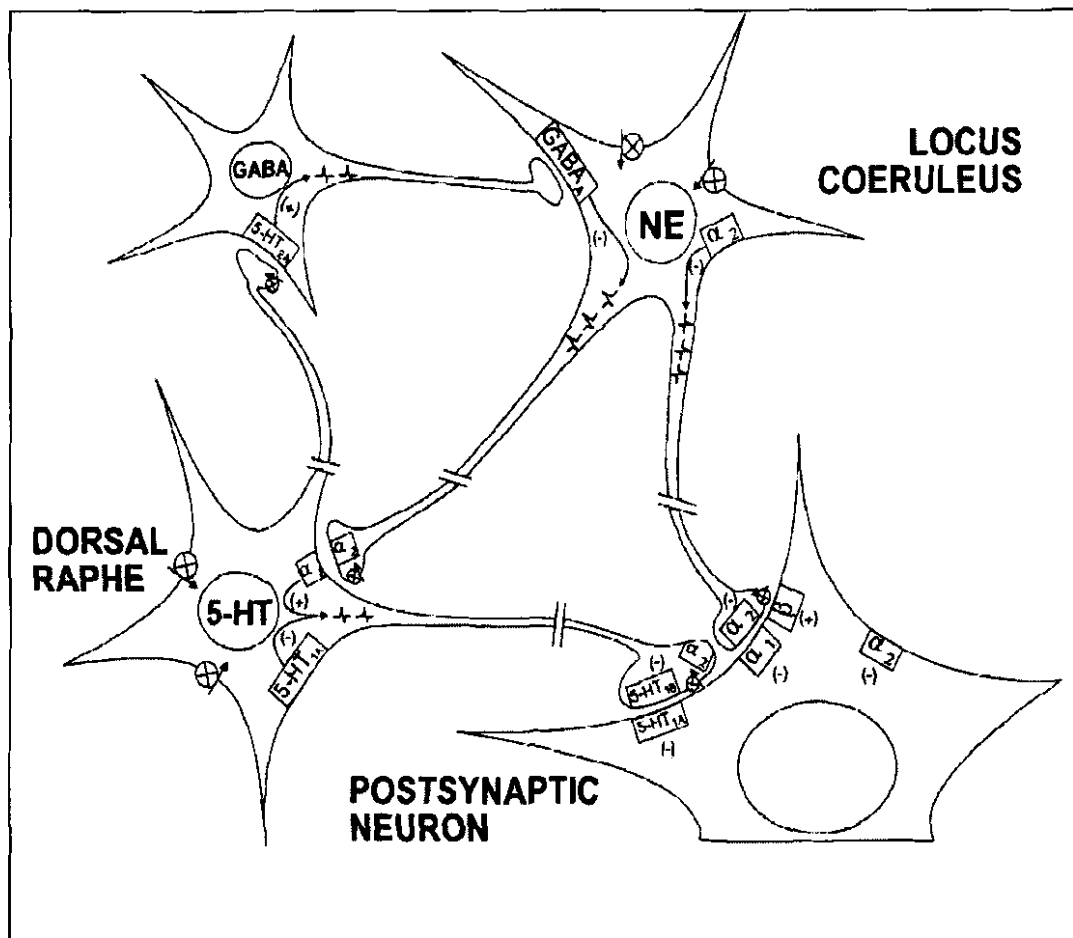


Figure 2-3: Interaction between 5-HT and *l*-NE neurons and their projections to pyramidal neurons of the hippocampus. The cog wheels represent the reuptake transporters responsible for the inactivation of the various neurotransmitters. The pharmacological subtypes of the pre- and postsynaptic receptors identified within the boxes of cell bodies and axon terminals are indicated using the international classification. The '+' and '-' signs in parentheses depict the influence of these receptors on neuronal firing. (Blier, 2003).

The cell bodies of 5-HT neurons are located in the raphe nuclei in the brainstem and project to all areas of the brain, including the limbic system which is involved in mood (Blier *et al.*, 1990). The cell bodies of *l*-NE neurons are located in the locus coeruleus and project to all parts of the

brain, including the hippocampus and the raphe nuclei (Takagi *et al.*, 1981). Not only do *I*-NE and 5-HT neurons interact at the level of their cell bodies in the raphe nuclei and locus coeruleus, but they both project to the same neurons in the forebrain (the pyramidal cells of the hippocampus) where interactions (pre and postsynaptic) occur (Mongeau *et al.*, 1997). The observation that all types of antidepressants have been shown to enhance 5-HT and/or *I*-NE neurotransmission in the hippocampus, suggests that this system could be directly relevant to the clinical situation (Blier & de Montigny, 1999). The following description is a proposed hypothesis of the interactions of 5-HT and *I*-NE neurons in the raphe nuclei, locus coeruleus, and hippocampus (Chaput *et al.*, 1991; Haddjeri *et al.*, 1996; Mongeau *et al.*, 1997; Szabo & Blier, 2001; Blier, 2003) and relates to Figure 2-3.

The *I*-NE and 5-HT neuronal cell bodies possess autoreceptors (of the α_2 -AR and 5-HT_{1A} receptor subtypes, respectively) which, when stimulated, inhibit the firing activity of their respective neurons. The firing of 5-HT neurons is also under the tonic excitatory control of an *I*-NE projection to the raphe nucleus, and is mediated by α_1 -heteroreceptors on the raphe cell bodies. These synapses are also regulated by α_2 -AR autoreceptors which, when stimulated, reduce the release of *I*-NE, and hence the firing of 5-HT neurons in the raphe nuclei. The raphe and locus coeruleus projections to the hippocampus are mainly inhibitory via postsynaptic 5-HT_{1A} and α_1 -ARs. Therefore, increasing *I*-NE and 5-HT tone would inhibit pyramidal cells in the hippocampus. The 5-HT neurons of the dorsal raphe also project to the locus coeruleus where they inhibit *I*-NE neuronal activity via an excitatory postsynaptic 5-HT_{2A} receptor located on a GABA neuron.

These interactions are complex, and they form a mutual feedback control system that resists alterations under normal physiological conditions. Antidepressant drugs, however, have the ability to modify the sensitivity of these neuronal systems through their immediate biochemical effects, provided they are administered for a sufficient period (Blier, 2003).

Table 2-3 contains the affinities of some antidepressants for the 5-HT, *I*-NE, and DAergic transporters, and will be referred to in the discussion on the mechanisms of action of some of these drugs.

Table 2-3: Potencies of antidepressants at human transporters for monoamines

Drug	<i>I</i> -NE transporter	5-HT transporter	<i>I</i> -DA transporter
<u><i>I</i>-NE-selective drugs</u>			
Desipramine	0.83	17.5	3200
Protriptyline	1.4	19.6	2130
Nortriptyline	4.35	18.5	1140
Oxaprotiline	5	4000	4350
Lofepramine	5.3	71.4	18,500
Reboxetine	7.14	58.8	11,500
Maprotiline	11.1	5900	1000
Nomifensine	15.6	1000	55.6
Amoxapine	16.1	58.5	4350
Doxepin	29.4	66.7	12,200
Mianserin	71.4	4000	9100
Viloxazine	156	17,000	100,000
Mirtazapine	4760	100,000	100,000
<u><i>5</i>-HT-selective drugs</u>			
Paroxetine	40	0.125	500
Clomipramine	37	0.28	2200
Sertraline	417	0.293	25
Fluoxetine	244	0.81	3600
Citalopram	4000	1.16	28,000
Imipramine	37	1.41	8300
Fluvoxamine	1300	2.22	9100
Amitriptyline	34.5	4.33	3200
Dothiepin	45.5	8.33	5300
Venlafaxine	1060	9.1	9100
Milnacipran	83.3	9.1	71,400
Trimipramine	2400	1500	10,000
Trazodone	8300	160	7140
Nefazodone	60	200	360
Bupropion	52,600	9100	526

Note: Potency is expressed as inhibition constant (K_i) in nM, based on radiotransporter competition binding assays with membranes from cell lines transfected with human genes for specific transporter proteins. (Frazer, 1997a; Owens *et al.*, 1997; Baldessarini, 2001)

2.2.1.1 Tricyclic antidepressants

Figure 2-4 illustrates the chemical structure of some tricyclic antidepressants.

Tricyclic antidepressants with secondary amine side chains are relatively selective inhibitors of *I*-NE transport, while most tertiary-amine tricyclic compounds inhibit 5-HT reuptake in addition to *I*-NE reuptake (see Table 2-3 and Figure 2-4). The monoamine-transport inhibiting effects of antidepressants occur immediately and are sustained for the duration of the treatment, and lead to complex secondary responses. These mechanisms are thought to be the major mode of action of the tricyclic antidepressants in alleviating depression (Baldessarini, 2001).

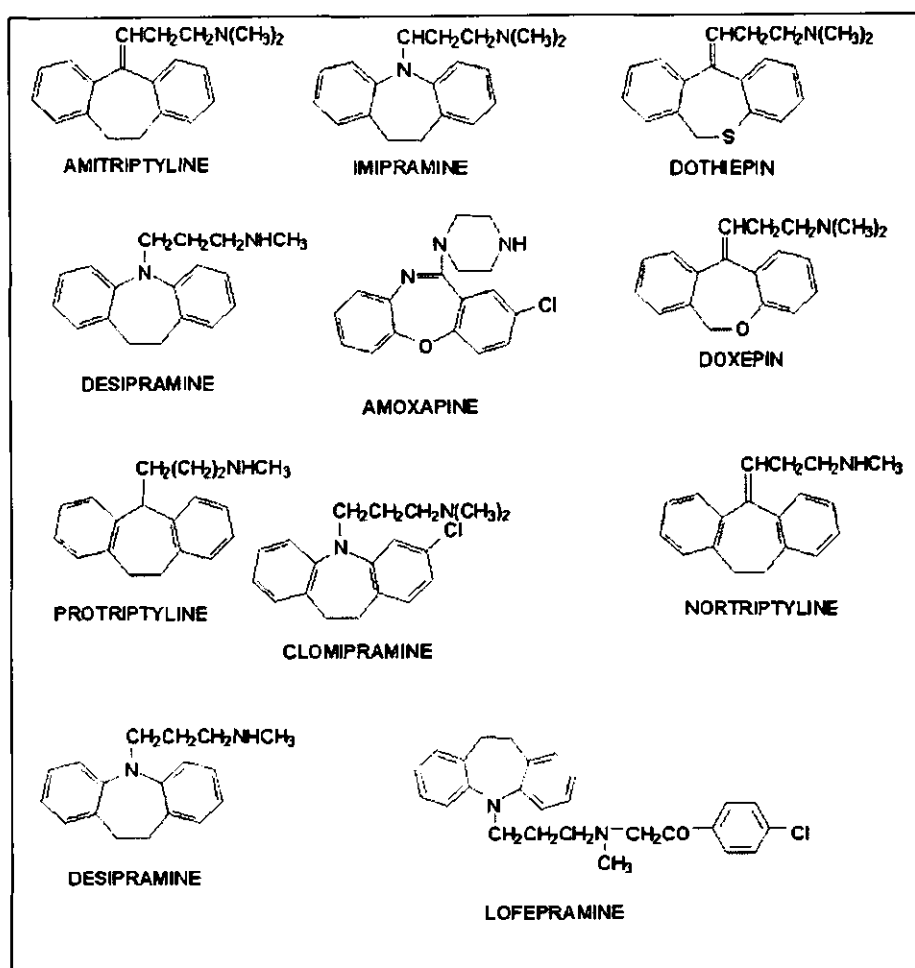


Figure 2-4: The chemical structure of some tricyclic antidepressants

The tricyclic antidepressants do not block *I*-DA transport (see Table 2-3) and in that way differ from central nervous system stimulants (e.g., cocaine, methylphenidate, and the amphetamines) (Baldessarini, 2001). However, it is reported that tricyclic antidepressants can desensitise dopaminergic D₂-receptors (autoreceptors), which could lead to enhanced forebrain *I*-DA neurotransmission, and this, together with the inhibition of transport of *I*-NE and/or 5-HT, could contribute to elevation of mood and behavioural activity (Potter *et al.*, 1998).

In addition to their transport-inhibiting effects, tricyclic antidepressants have notable interactions at *I*-NE receptors. Most tricyclic antidepressants have at least moderate affinity for α_1 -ARs, much less for α_2 -ARs, and virtually none for β -ARs (Baldessarini, 2001). However, the mechanism of antidepressant action of these drugs is thought to be mainly the result of the secondary effects caused by increased *I*-NE and/or 5-HT levels (Frazer, 1997b). These presumably include indirect activation of postsynaptic α_1 -ARs and 5-HT_{1A} receptors located on pyramidal neurons of the hippocampus (Figure 2-3).

An acute increase in postsynaptic *I*-NE availability would lead to enhanced stimulation of α_2 -ARs on *I*-NE cell bodies and nerve terminals. These α_2 -ARs would include presynaptic autoreceptors which inhibit *I*-NE neurons projecting from the locus coeruleus and supplying

other brain regions such as the hippocampus (see Figure 2-1 and Figure 2-3). Activation of these receptors inhibits transmitter release by molecular and cellular actions that are not yet fully understood, but most likely include suppression of voltage-gated Ca^{2+} currents and activation of G protein-coupled K^{+} currents (Foote & Aston-Jones, 1995). Stimulation of these autoreceptors also reduces the synthesis of *I*-NE through the rate-limiting step at tyrosine hydroxylase, presumably through α_2 -AR attenuation of cAMP-mediated phosphorylation (Baldessarini, 2001).

The α_2 -AR negative feedback response is rapidly activated upon administration of tricyclic antidepressants, however, with repeated drug exposure, α_2 -AR responses are eventually reduced, which may be a result of desensitisation secondary to increased exposure to *I*-NE (Baldessarini, 2001). This adaptation allows the presynaptic production and release of *I*-NE to return to normal, or even exceed baseline levels (Baldessarini, 1989), which is thought to be the restrictive factor before the onset of an antidepressant response can occur (Blier, 2003).

In summary, tricyclic antidepressants most probably exert their mood elevating effects by increasing *I*-NE and 5-HT levels in areas such as the hippocampus and prefrontal cortex which leads to secondary effects by these neurotransmitters. However, the major drawback of these drugs, as with all the other classes of antidepressants, is their delayed onset of antidepressant action.

2.2.1.2 Selective serotonin reuptake inhibitors

The SSRIs are the most commonly prescribed antidepressants today. Unlike tricyclic antidepressants, which block the reuptake of *I*-NE or *I*-NE and 5-HT, the SSRIs selectively block the reuptake of 5-HT (Table 2-3), which also leads to complex secondary responses. Increased synaptic availability of 5-HT stimulates a large number of postsynaptic 5-HT receptor subtypes, where activation of postsynaptic 5-HT_{1A} receptors are thought to be critical for antidepressant action (Blier, 2003). Stimulation of 5-HT₃ receptors is suspected to contribute to common adverse effects which are characteristic of this class of drugs, including gastrointestinal and sexual effects, whereas activation of 5-HT_{2C} receptors may contribute to the risk of agitation or restlessness which occasionally occurs with SSRIs (Baldessarini, 2001).

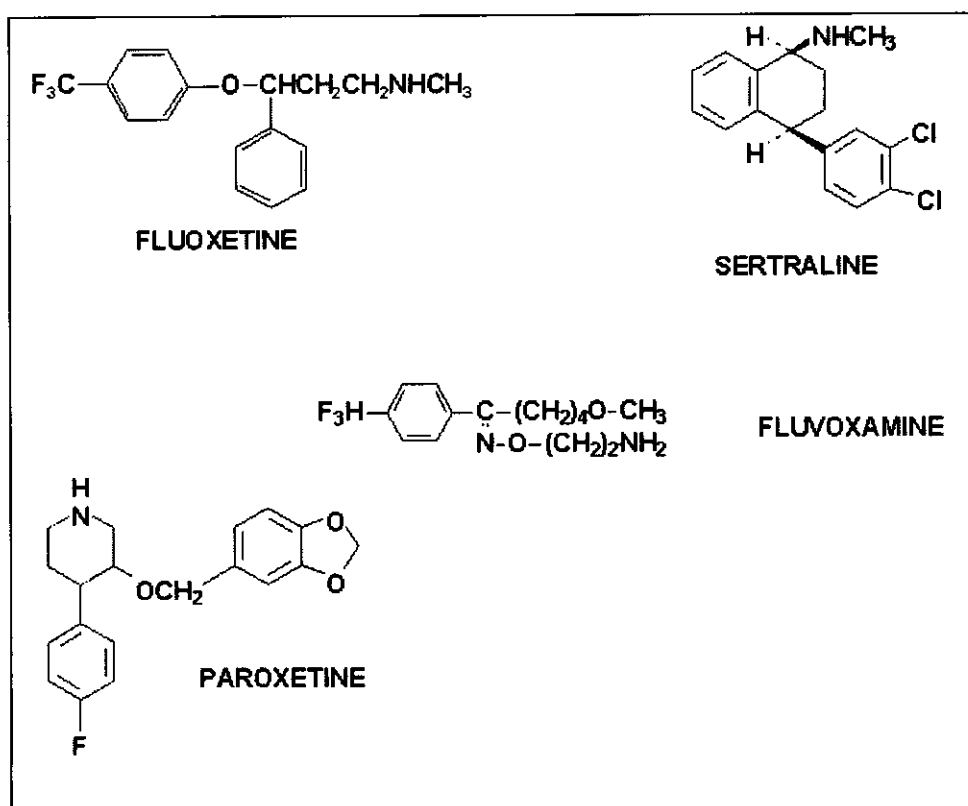


Figure 2-5: The chemical structure of some selective serotonin inhibitors

A feature of both 5-HT and *I*-NE neurons is that negative feedback mechanisms rapidly emerge to restore homeostasis (Blier, 2003). In the 5-HT system, 5-HT_{1A} and 5-HT_{1B} autoreceptors suppress 5-HT synthesis, as well as neuronal 5-HT release (Figure 2-3). Repeated treatment leads to gradual downregulation and desensitisation of these autoreceptors over several weeks (particularly 5-HT_{1B} receptors at nerve terminals) with a resulting return, or increase in production and release, of 5-HT (Blier *et al.*, 1990; Chaput *et al.*, 1991). Additional long-term changes include gradual downregulation of postsynaptic 5-HT_{2A} receptors that may contribute to antidepressant effects, and indirect enhancement of *I*-NE transmission by a reduction of tonic inhibitory effects of 5-HT_{2A} heteroreceptors located on GABA neurons which project to the locus coeruleus (see Figure 2-3) (Baldessarini, 2001; Blier, 2003). As with the tricyclic antidepressants, the delay in downregulation and/or desensitisation of receptors implicated in negative feedback mechanisms is thought to be the major restrictive factor for the onset of an antidepressant response (Blier, 2003).

In summary, it seems that the principal mechanism of antidepressant action of the SSRIs is an elevation in postsynaptic availability of 5-HT, and also the secondary mechanisms that begin after sustained treatment. The SSRIs significantly improved the treatment of depression, pertaining to compliance to treatment, with their favourable side-effect profile. However, they do not address the problematic aspect of delayed onset of antidepressant action.

2.2.1.3 Atypical antidepressants

Recent research on antidepressant development has focused on discovering new drugs that have greater specificity (and therefore fewer side-effects), and a more rapid onset of antidepressant action than preceding drug classes. As a result, four antidepressants with significantly distinct pharmacological characteristics have been recently introduced: venlafaxine, nefazodone, mirtazapine, and reboxetine. These drugs offer equivalent or better efficacy while improving on many of the undesirable side-effects of the earlier classes (Kent, 2000), and have been dubbed atypical due to their distinctive pharmacology compared to previous antidepressants. Mirtazapine is of particular interest in the current study and will be discussed in more detail relative to the other atypical antidepressants.

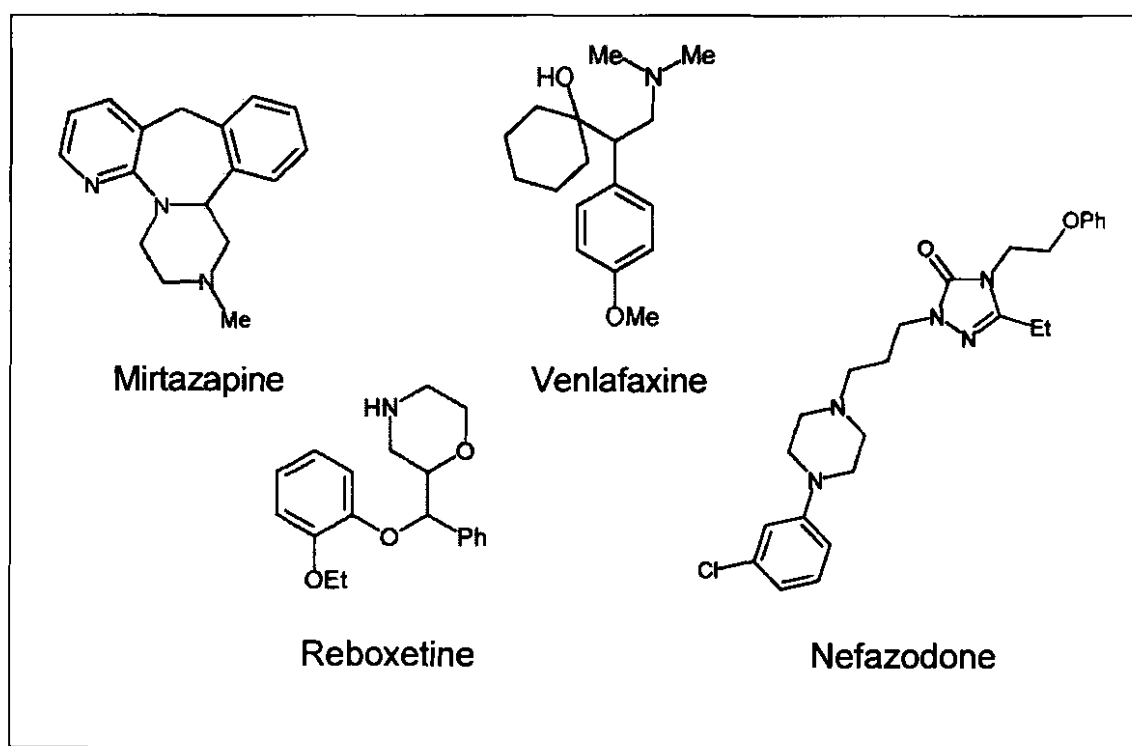


Figure 2-6: The chemical structure of some atypical antidepressants

Figure 2-6 illustrates the chemical structure of some atypical antidepressants. The structure of venlafaxine and reboxetine differ from earlier marketed antidepressants, while nefazodone and mirtazapine are structurally related to the earlier antidepressants trazodone and mianserin (not shown) respectively.

Table 2-4 contains the receptor binding affinities of atypical antidepressants for a selection of receptors (α_1 - and α_2 -ARs, histaminergic 1 (H_1) receptors, and muscarinic acetylcholine (mACh) receptors), compared to those of some SSRIs. The affinities for *I*-NE and 5-HT transporters of these drugs are displayed in Table 2-3.

Table 2-4: Receptor binding profiles of atypical antidepressants compared to serotonin reuptake inhibitors

Drug	Receptor affinity Ki (nM)			
	α_1	α_2	H ₁	mACh
Venlafaxine	>10,000	>10,000	>10,000	>5000
Nefazodone	42	1200	370	>10,000
Mirtazapine	372	58	0.5	794
Reboxetine	>10,000	>10,000	1400	3900
Fluoxetine	5900	>10,000	1000	1300
Sertraline	300	5000	>10,000	500
Paroxetine	>10,000	>10,000	1000	89
Citalopram	4500	>10,000	470	2900

(Hall & Ogren, 1981; de Boer *et al.*, 1988; Eison *et al.*, 1990; Hyttel, 1994; Taylor *et al.*, 1995; Kent, 2000; Wong *et al.*, 2000; Wikstrom *et al.*, 2002)

2.2.1.3.1 Venlafaxine

Venlafaxine is the first antidepressant in a new class referred to as the serotonin noradrenergic reuptake inhibitors (SNARIs). Although venlafaxine shares the dual 5-HT and *I*-NE reuptake properties of many tricyclic antidepressants (Table 2-3), it lacks anticholinergic, antihistaminic, and α_1 -lytic properties (Table 2-4). The putative earlier onset of action of venlafaxine is discussed in § 2.3. Another drug in this class is the newly introduced SNARI duloxetine, but the superiority of this drug to other antidepressants remains to be proven.

2.2.1.3.2 Nefazodone

Nefazodone is a weaker inhibitor of 5-HT and *I*-NE reuptake (Table 2-3), but is a potent antagonist at 5-HT₂ receptors (Eison *et al.*, 1990). This drug was developed to improve the unwanted side-effects caused by the earlier antidepressant, trazodone, which is associated with sedation and postural hypotension. Structural alteration led to a compound with significantly less affinity for α_1 -ARs (Table 2-4), which is most likely responsible for these effects (Eison *et al.*, 1990). An important mechanism of antidepressant action is believed to be indirect postsynaptic 5-HT_{1A} activation, secondary to increased levels of 5-HT (Eison *et al.*, 1990). The putative earlier onset of action of nefazodone is discussed in § 2.3. However, severe hepatotoxic side-effects were observed with nefazodone, and the drug was subsequently withdrawn from clinical use.

2.2.1.3.3 Mirtazapine

Mirtazapine has a unique pharmacology among antidepressants (receptor binding profile described in Table 2-5). Although it does not inhibit the reuptake of *I*-NE or 5-HT, or inhibit monoamine oxidase, mirtazapine potentiates both *I*-NE and 5-HT neurotransmission at selected receptor subtypes. Its principal mechanism of antidepressant action is thought to be the

antagonism of α_2 -ARs (de Boer, 1996), however, it also blocks 5-HT₂ and 5-HT₃ receptors (Table 2-5) which may play a role in its antidepressant mechanism, since antagonism of these receptors produced antidepressant-like effects in animal models of depression (Redrobe & Bourin, 1997; Dremencov *et al.*, 2005). The putative earlier onset of action of mirtazapine is discussed in § 2.3.

2.2.1.3.4 Reboxetine

Reboxetine is the first selective *L*-NE reuptake inhibitor (NaRI) to be introduced since the tricyclic antidepressants. This drug has no direct 5-HT effects and also has little affinity for α -AR, histaminergic and muscarinic receptors (Table 2-4). It has been suggested that reboxetine is not superior to sertraline regarding an earlier onset of action in an animal model of depression. However, a combination of the drugs produced earlier effects than either of the drugs alone (Harkin *et al.*, 1999). In another study, it was reported that reboxetine could be an effective alternative in the treatment of "retarded" post-stroke depression in the elderly, in which SSRIs are often ineffective (Rampello *et al.*, 2005). Furthermore, it has been demonstrated that reboxetine could be a safe and effective add-on therapy to SSRIs in the treatment of major depression (Rapaport *et al.*, 2002; Tavormina *et al.*, 2002).

Table 2-5: Receptor binding profile mirtazapine

Receptor	Tissue	Affinity (pA ₂ or pK _i)
<u>I-NE interactions</u>		
α ₂ -autoreceptor	Synaptosomes	7.7
α ₂ -heteroreceptor	Synaptosomes	8
α ₂ -postsynaptic	Rat cortex	7.3
α ₂ -presynaptic	Rat vas deferens	6.8
α ₁ -adrenoceptor	Rat cortex	6.3
α ₁ -adrenoceptor	Rat vas deferens	6.5
β ₁ -adrenoceptor	Rat atrium	< 4.5
β ₂ -adrenoceptor	Rat trachea	< 4.5
I-NE -transporter	Synaptosomes	5.8
<u>5-HT interactions</u>		
5-HT _{1A} receptor	Rat hippocampus	5.3
5-HT _{1B} receptor	Rat striatum	4.9
5-HT _{1D} receptor	Pig striatum	5.3
5-HT _{2A} receptor	Rat cortex	8.2
5-HT _{2B} receptor	Rat fundus	6.7
5-HT _{2C} receptor	Pig choroid plexus	7.9
5-HT ₃ receptor	Mouse neuroblastoma	8.1
5-HT ₃ receptor	Mouse neuroblastoma	7.1
5-HT ₄ receptor	Rat esophagus	< 4.5
5-HT transporter	Synaptosomes	< 4.5
<u>I-DAergic, histaminergic, and cholinergic interactions</u>		
D ₁ -dopaminergic	Rat striatum	5.8
D ₂ -dopaminergic	Rat striatum	5.6
M ₁ -muscarinic	Rat forebrain	6.2
H ₁ -histaminergic	Guinea pig ileum	9.9
H ₂ -histaminergic	Rat atrium	4.8

(de Boer *et al.*, 1988; de Boer *et al.*, 1994; Kooyman *et al.*, 1994; de Boer, 1996).

2.2.1.4 Other α₂-AR antagonists

The α₂-AR antagonists, idazoxan and yohimbine, are not generally classified as antidepressant drugs. However, the pharmacology of these drugs are important in the current study since these drugs are employed in order to investigate the role of α₂-autoreceptor antagonism in the efficacy and onset of action of antidepressants that block these receptors (i.e., mirtazapine).

2.2.1.4.1 Idazoxan

Although idazoxan is not a recognised antidepressant drug, there is some clinical evidence that suggests antidepressant efficacy for this drug (Osman *et al.*, 1989; Grossman *et al.*, 1999). Idazoxan is a potent and selective antagonist at α₂-ARs (Doxey *et al.*, 1984) and is reported to

act as a neutral antagonist at these receptors (Wade *et al.*, 2001) (see § 2.4.4.2). The drug also has high affinity for I_2 -imidazoline receptors (Miralles *et al.*, 1993), and acts agonistically at 5-HT_{1A} receptors (Kawai *et al.*, 1994).

It has been demonstrated that idazoxan increases the synthesis of *I*-NE in the cerebral cortex and hippocampus of rats following the powerful blockade of α_2 -autoreceptors but, in contrast, decreases 5-HT synthesis in these brain regions via stimulation of presynaptic 5-HT_{1A} autoreceptors (Llado *et al.*, 1996). These contrasting effects of idazoxan have to be considered when speculating on the putative antidepressant activity of this drug, since an elevation of *I*-NE and 5-HT are both implicated in the alleviation of depressive symptoms.

2.2.1.4.2 Yohimbine

Yohimbine is well recognised as an effective treatment for impotence (Sala *et al.*, 1990; Montorsi *et al.*, 1994), but is not generally acknowledged as an effective antidepressant drug. However, there is some clinical evidence suggesting that yohimbine may be effective as an additive to fluvoxamine in the treatment of depression (Cappiello *et al.*, 1995). The principal pharmacological action of yohimbine is thought to be its antagonism of α_2 -ARs (Doxey *et al.*, 1984), while more data suggest that it is an inverse agonist at these receptors (Wade *et al.*, 2001). If one assumes the hypothesis that α -lytic action may contribute to antidepressant action, and in particular to earlier onset of action, inverse agonism could render yohimbine superior to neutral antagonists, regarding antidepressant-like properties (see § 2.4.4.2).

2.2.2 Alteration of neurogenesis

Another hypothesis to explain the mechanism of action of antidepressants is one that states that antidepressants activate several signalling cascades, including those that lead to activation of transcription factors such as cAMP response element binding protein (CREB), which activates brain derived neurotrophic factor (BDNF) which, in turn, binds to tyrosine kinase B (trkB) receptors (Altar, 1999; Chen *et al.*, 2001; Nestler *et al.*, 2002) (see § 2.1.4.3). The BDNF-trkB signalling pathway increases formation and stabilisation of synaptic connectivity and promotes neurogenesis, which is stimulated by chronic antidepressant treatment in adult rats (Malberg *et al.*, 2000), and is thought to be mediated via stimulation of 5-HT_{1A} receptors (Gould, 1999). Although it is not clear whether neurogenesis is also important for antidepressant activity in humans, the hypothesis has received some attention because hippocampal size is decreased in patients with longstanding/recurrent major depression (Frodl *et al.*, 2002). However, it is not yet clear whether this change is due to hippocampal cell loss and/or decreased axonal sprouting. This is a relatively novel hypothesis, and antidepressants that specifically target neurogenesis are still to be introduced.

An alternative mechanism by which antidepressants may alter neurogenesis is via their actions on NMDA receptor functioning (see § 2.1.4.3). Antidepressants reduce glutamatergic transmission by reducing the proportion of high affinity glycine sites on the NMDA receptor (Skolnick *et al.*, 1996). *In situ* hybridisation studies suggest that antidepressants reduce NMDA subunit messenger RNA (mRNA) in various limbic and subcortical structures (Boyer *et al.*, 1998). Since excessive stimulation of NMDA receptors is implicated in the neuronal damage associated with severe depression (McEwen, 1999; McEwen, 2000a; Sapolsky, 2000; McEwen, 2000b; Popoli *et al.*, 2002), inhibition of the functioning of these receptors may play a part in the mechanism of action of antidepressants. Another mechanism by which decreased NMDA receptor functioning may contribute to antidepressant effects of antidepressants is via a resulting decrease in NO synthesis (Wegener *et al.*, 2003), since structurally distinct NOS inhibitors have been shown to have antidepressant-like behavioural effects in rats and mice (Harkin *et al.*, 1999; Yildiz *et al.*, 2000), and inhibition of the NO-sensitive guanylyl cyclase (sGC) produces antidepressant-like effects in the rat forced swim test (Heiberg *et al.*, 2002).

2.3 ONSET OF ACTION OF ANTIDEPRESSANTS

2.3.1 Delayed onset of antidepressant action

As noted in the introductory section of this chapter, all conventional antidepressants have a lag in onset of antidepressant response, which is usually a minimum of two weeks (Bymaster *et al.*, 2003; Leonard, 2003). This delay in onset of action is a particularly troublesome factor in the treatment of depression, since it not only prolongs the suffering of depressed patients, but also causes them to remain under great risk of suicide. Also, compliance is also often hampered by a delay in symptom relief, since adverse effects are frequently at their worst during the early stages of treatment. In addition, drug resistance is seen all too frequently. Some recently introduced antidepressants appear to have a faster onset of action than earlier generation drugs. This has encouraged the development of new therapeutic approaches that preserve the therapeutic action of existing treatments, but accelerate their onset of action.

2.3.2 Pharmacological strategies for earlier onset of action

A putative mechanism of action of antidepressants proposed by Blier (2003), suggests that conventional antidepressants acutely increase the availability of 5-HT and/or *L*-NE, preferentially at their cell body level, which triggers negative feedback mechanisms. After continued stimulation, these feedback mechanisms become desensitized and only then are the levels of 5-HT and/or *L*-NE allowed to be effectively increased to alleviate depressive symptoms. Putative

fast onset antidepressants, on the other hand, may uncouple these feedback control systems and enhance 5-HT and/or *I*-NE neurotransmission more rapidly.

2.3.2.1 Mirtazapine

Mirtazapine enhances 5-HT and *I*-NE neurotransmission by mechanisms that exploit the presynaptic regulation of these systems. In addition, the mechanisms by which mirtazapine accomplishes this are synergistic and could possibly explain its more rapid onset of therapeutic action (Blier, 2003).

2.3.2.1.1 Effects on *I*-NE neurotransmission

As described in § 2.2.1 and as depicted in Figure 2-3, *I*-NE transmission is under negative feedback control via α_2 -autoreceptors, which, when stimulated inhibit the release of *I*-NE. Mirtazapine blocks the cell body autoreceptors and thereby uncouples the feedback inhibition on *I*-NE neuronal firing, therefore increasing electrical impulse flow in terminal projection areas such as the hippocampus (de Boer *et al.*, 1995; Haddjeri *et al.*, 1996). In the normal course of events this increase in firing would be resisted by terminal α_2 -autoreceptors which reduce the amount of *I*-NE released. However, these receptors are also blocked by mirtazapine, and this permits the increase in *I*-NE neurotransmission (Figure 2-3). This increase in *I*-NE neurotransmission is believed to be at least part of the mechanism of antidepressant action of mirtazapine (Blier, 2003).

2.3.2.1.2 Effects on 5-HT neurotransmission

As described above, mirtazapine increases *I*-NE neurotransmission in terminal projection areas. These include projections to the dorsal raphe where it stimulates 5-HT neurotransmission via indirect activation of α_1 -heteroreceptors on 5-HT cell bodies secondary to an increased release of *I*-NE (Figure 2-3). This enhancing effect would normally be dampened by the activation of α_2 -heteroreceptors on 5-HT nerve terminals (Figure 2-3) resulting from the enhanced release of *I*-NE by mirtazapine. However, these receptors are also blocked by mirtazapine (de Boer, 1996).

This modulation of 5-HT and *I*-NE neurotransmission is thought to be crucial in defining the mechanism of action of mirtazapine, and in particular, its early onset (Blier, 2003). The experimental evidence for this mechanism is therefore of significant importance. Convincing evidence for the indirect stimulation of 5-HT neurotransmission comes from electrophysiological studies of the firing of 5-HT cell bodies in the raphe nucleus (Haddjeri *et al.*, 1996), and also direct microdialysis measurement of 5-HT and *I*-NE release in the hippocampus (de Boer *et al.*, 1996).

These suggested effects of mirtazapine, in addition to the possibility that mirtazapine could act as an inverse agonist at these receptors (see § 2.4.4.2 for the implications of this) could

possibly explain its putative rapid onset of antidepressant action. However, these hypotheses need to be rigorously tested and verified, and is an objective of the current study.

2.3.2.2 Venlafaxine

It has been suggested that venlafaxine has an early onset of action compared to earlier antidepressants (Guelfi *et al.*, 1995; Montgomery, 1995). The unique pharmacological properties of venlafaxine (see § 2.2.1.3.1) may explain its better tolerability, but not its reported rapid onset of action. There is speculation that the earlier onset of antidepressant response of the drug could most likely be related to the fact that it is possible to administer higher doses of the drug early during treatment due to the minimal incidence of adverse effects (Blier, 2003).

2.3.2.3 Nefazodone

It has been shown in an animal model of depression that nefazodone may have a faster onset of antidepressant action than desipramine (Dremencov *et al.*, 2005). This is suggested to be a result of the ability of nefazodone to antagonise 5-HT₂ receptors, since these receptors inhibit the serotonin-induced release of *L*-DA in the NAc (see § 2.1.4.4.1).

2.3.2.4 SSRI augmentation with pindolol

The suggestion that pindolol might be effective in accelerating the onset of action of SSRI antidepressants arose from a rational analysis of the physiology and pharmacology of 5-HT neurons. This seems contradictory, since pindolol, as a β -AR antagonist, belongs to a class better known for causing depression. However, its properties as a β -AR antagonist are irrelevant in the current context (Zanardi *et al.*, 1997). Uniquely among β -AR antagonists, pindolol antagonises 5-HT_{1A} and 5-HT_{1B} receptors (Barnes & Sharp, 1999). One study has suggested that pindolol may block, specifically, the inhibitory 5-HT_{1A} autoreceptors on the cell bodies of 5-HT neurons, and less effectively, the postsynaptic 5-HT_{1A} receptors in the hippocampus (Romero & Artigas, 1997). This is an important property since postsynaptic 5-HT_{1A} receptors are thought to play an important role in alleviating depressive symptoms.

Simultaneous administration of pindolol and an SSRI would prevent the initial decrease in firing activity of 5-HT neurons (mediated by presynaptic 5-HT_{1A} autoreceptors) normally occurring with SSRIs (see § 2.2.1.2). Therefore, by giving pindolol, desensitisation of 5-HT_{1A} autoreceptors (which could require approximately 2 weeks of treatment) is not necessary, since this drug simply blocks these receptors and allows an immediate increase in 5-HT transmission.

An interesting observation with SSRI augmentation with pindolol is that patients do not relapse when pindolol is ceased during therapy (Blier, 2003). The persistence of the beneficial action of SSRIs in the absence of pindolol could be explained by its partial 5-HT_{1A} receptor agonistic

property. It was indeed shown that a 21 day treatment can desensitise the rat 5-HT_{1A} autoreceptor in the dorsal raphe (Haddjeri & Blier, 2000). Therefore, while preventing an excessive activation of 5-HT_{1A} autoreceptors resulting from the SSRI, pindolol triggers a low level of stimulation producing a concurrent desensitisation.

2.3.3 Clinical evidence for early onset of antidepressants

Although many claims for a more rapid onset of action have been made for antidepressants, few have been confirmed clinically. Also, there is considerable debate about how an early onset should be measured and identified. Specifically designed, double-blind randomised controlled trials seem to be a viable option (Blier, 2003). However, such trials need to be large enough to allow them to resolve differences between active treatments.

To date, a complete and convincing proof of early onset of action has not been demonstrated for a single antidepressant drug. However, recent studies with two antidepressants and an antidepressant adjunctive therapy have provided some strong evidence that suggests an early onset of action. A number of clinical studies have shown a possible earlier onset of antidepressant action for mirtazapine (Wheatley *et al.*, 1998; Leinonen *et al.*, 1999; Benkert *et al.*, 2000; Vester-Blokland & Van Oers, 2002), venlafaxine (Guelfi *et al.*, 1995; Benkert *et al.*, 1996; Entsuah *et al.*, 1998), and also for SSRI augmentation with pindolol (Perez *et al.*, 1997; Tome *et al.*, 1997; Zanardi *et al.*, 2001). There are, however, studies that did not report statistically significant differences between the onset of action of some of these newer drugs or augmentation strategies compared to those of conventional antidepressants (e.g. TCAs and SSRIs). These include a study comparing mirtazapine to amitriptyline (Kasper *et al.*, 1997), and a trial comparing the onset of SSRI augmentation with pindolol to that of SSRI treatment alone (Magyaros & Haraszti, 2002). The majority of these data are from double-blind, randomised, placebo-controlled clinical trials. In addition to the clinical data, there is also some preclinical evidence for early antidepressant-like effects with nefazodone (Dremencov *et al.*, 2005).

These data are important, not only because of the possibility of improving therapy, but also because the pharmacology of these treatments might shed light on the aetiology of depression and the mechanism of action of antidepressants.

2.4 α_2 -ARs: Ligand actions and signalling mechanisms

2.4.1 Background information on signal transduction systems

Research of affective disorders classically focused on the primary messengers (neurotransmitters and/or drugs) and the associated receptor subtypes, distribution and

expression levels. Especially in the past few decades research extended to the subcellular level of signal transduction mechanisms, including G-protein-mediated mechanisms. G-proteins (also GTP-binding regulatory proteins) are a family of proteins that play a crucial role in transducing extracellular signals to cellular targets such as adenylyl cyclase, phospholipase C, and ion channels. G-proteins are heterotrimeric molecules composed of three subunits termed α , β and γ . The α -subunits can be classified into families depending on whether they are targets for cholera toxin (G_s proteins, which activate adenylyl cyclase), pertussis toxin (G_i and G_o proteins, which inhibit adenylyl cyclase and Ca^{2+} channels, as well as promote K^+ channel function) or neither (G_q proteins, which stimulate phospholipase C activity) (Simon *et al.*, 1991). $G_{12/13}$ proteins primarily regulate small GTP binding proteins and other G protein effectors (Brink *et al.*, 2004).

The family of seven transmembrane (7TM) spanning receptors that interact with G-proteins is referred to as G protein-coupled receptors (GPCRs), and include receptor types such as muscarinic acetylcholine (mACh) receptors, 5-HT receptors, α - and β -ARs, and *I*-DA receptors. Various drugs interact with these types of receptors including antidepressant drugs.

GPCRs convey extracellular instructions from ligands to the heterotrimeric G-protein which, in turn, stimulate cellular proteins that form part of cellular signalling systems (e.g., adenylyl cyclase) referred to as G protein effectors. Activation of these receptors leads to the release of active second messengers, alterations in ion-channel functioning and/or other signalling cascades, resulting in diverse cellular responses.

The α_2 -AR is a GPCR, and of particular interest in the current study. Therefore, the interaction of this receptor subtype with G proteins will be discussed in more detail, as well as the effects of antidepressant drugs on these interactions.

2.4.2 Distribution and function of α_2 -AR receptors

The α_2 -ARs couples to G_i proteins (GPCRs). The amino sequences for three distinct subtypes of α_2 -ARs (i.e. α_{2A} , α_{2B} , and α_{2C}) have been deduced (Bylund, 1992). These receptors are located in the periphery on pancreatic islet cells (β -cells) where they inhibit insulin secretion and on platelets where they promote aggregation, on nerve terminals where they decrease the release of *I*-NE, and on vascular smooth muscle where they mediate contraction (Hoffman & Taylor, 2001). Furthermore, α_2 -ARs are also found in the nucleus tractus solitarius (NTS), where they play an important role in the regulation of blood pressure (Sved *et al.*, 2003).

The α_{2A} -AR subtype has an important role in the regulation of the central sympathetic nervous system. As mentioned above, activation of α_2 -ARs inhibits the release of *I*-NE from nerve endings. Therefore, activation of α_{2A} -ARs in the central nervous system inhibits sympathetic

nervous system activity, while blockade of α_{2A} -AR receptors by antagonists such as yohimbine can increase the release of *I*-NE from sympathetic nerve terminals (Hoffman, 2001). It can be accepted that all referrals to α_2 -ARs in this dissertation in the context of presynaptically located receptors on nerve cells, is indeed of the α_{2A} -AR subtype.

2.4.3 Signal transduction of the α_2 -AR

α_2 -ARs couple to a variety of effectors (Bylund, 1992; Aantaa *et al.*, 1995). As an example, G_i protein-mediated inhibition of adenylyl cyclase activity results in decreased cAMP formation. However, the ability of these receptors to reduce the release of *I*-NE in the central nervous system is more dependent on the activation of G_i protein-mediated activation of K^+ channels, resulting in membrane hyperpolarisation and decreased cell firing (Hoffman & Taylor, 2001). α_2 -ARs are also known to inhibit voltage-gated Ca^{2+} channels, mediated via G_o proteins.

2.4.4 Inverse agonism and the α_2 -AR receptor

2.4.4.1 Constitutive activity and inverse agonism at G-protein coupled receptors

An important development in understanding of GPCR signalling mechanisms includes the observation that they can couple to, and functionally activate, G proteins in the absence of an agonist (Costa & Herz, 1989). This is also referred to as basal (constitutive) activity. This observation was supported by the identification of mutant receptors with substantial constitutive activity (Kjelsberg *et al.*, 1992). This led to the postulation of the "Extended Ternary Complex (ETC) model" (Figure 2-8) (Samama *et al.*, 1993), the cubic ternary complex model (Weiss *et al.*, 1996), and later the two- and three-state receptor models (Leff, 1995; Leff *et al.*, 1997; Strange, 1998). Today it is commonly accepted that GPCRs exist in multiple states of activation, referred to as the N-state model (Kenakin, 1995; Strange, 1998; Gether & Kobilka, 1998; Kenakin, 2001).

These models suggest that the receptor exists in equilibrium between an inactive state (R^0) and an active state (R^*) in the absence of an agonist. This equilibrium is dependent on various factors such as receptor type and expression level and determines its basal or constitutive activity. Wild-type receptors (non-mutated, naturally occurring receptors) expressed at normal concentrations, usually predominantly exist in the inactive (R^0) state and have minimal basal activity in the absence of an agonist. An agonist binds preferentially to R^* , thereby shifting the equilibrium towards R^* and in effect stabilising this conformation. This then leads to enhanced G protein coupling and hence associated cellular responses.

The concept of inverse agonism arose from the observation that certain drugs were able to reduce the activity of receptor systems that were active in the absence of agonists

(constitutively active receptors). Since agonists increase the activity of receptor systems, this opposite or “negative activity” was coined “inverse agonism”, and has been extensively described for GPCRs (Milligan & Bond, 1997; Leurs *et al.*, 1998; Kenakin, 2001).

In contrast with the traditional view that receptor ligands can only be divided into two classes (agonists and antagonists), it is now believed that drugs acting at GPCRs exhibit a spectrum of efficacy from inverse agonism through neutral antagonism to agonism. This concept is explained in Figure 2-7.

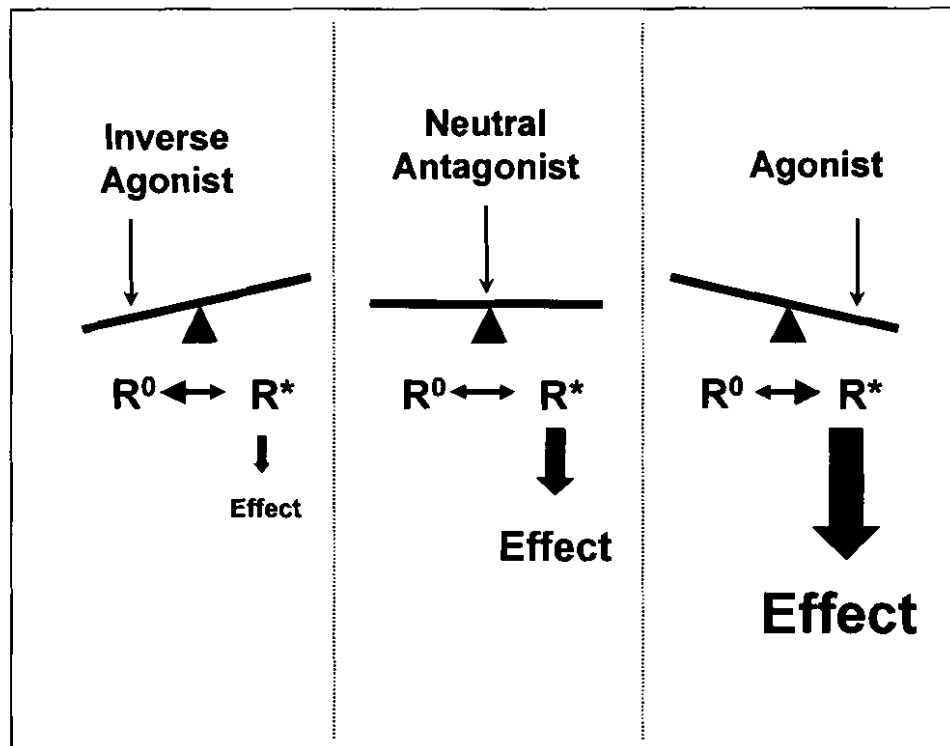


Figure 2-7: The spectrum of efficacy of drugs acting at GPCRs. (R^0) resembles the inactive conformation of the receptor while (R^*) resembles the activated state. An agonist increases basal activity by stabilising R^* . A neutral antagonist binds equally well to both conformations and does not alter the basal activity of the receptor system. On the other hand, an inverse agonist stabilises R^0 , thereby decreasing the basal activity of the receptor system.

According to the extended ternary complex model (Figure 2-8) (Samama *et al.*, 1993), inverse agonists preferentially bind to R^0 , thereby shifting the equilibrium towards R^0 , stabilising this conformation and reducing G_i protein coupling. Neutral antagonists have equal affinity for both states, and merely block the effect of the endogenous ligands. They do not alter spontaneous (basal) G protein coupling.

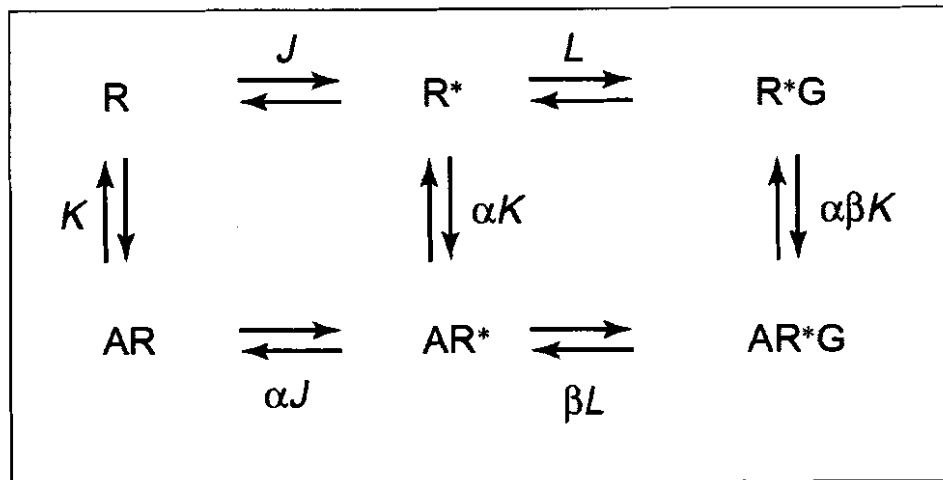


Figure 2-8: The extended ternary complex model. Abbreviations: A = agonist; G = G-protein; K = association constant for the binding of A to R; J = equilibrium constant governing the $R:R^*$ equilibrium; L = equilibrium constant governing the $R^*:R^*G$ equilibrium; R = inactive receptor; R^* = partially activated receptor; α and β = allosteric constants governing the effect of the agonist on the $R:R^*$ and $R^*:R^*G$ equilibria respectively. (Strange, 2002).

2.4.4.2 Inverse agonism at α_2 -ARs in neuronal cells

The same theory described in the previous section (§ 2.4.4.1) can be applied to α_2 -ARs located in the central nervous system. Under normal neurophysiological conditions, a large fraction of α_2 -ARs in the CNS can be expected to exist in the inactive state ($\alpha_2\text{-AR}^0$), while a small fraction will be in the active state ($\alpha_2\text{-AR}^*$). Upon stimulation of endogenous *I*-NE the equilibrium is shifted towards $\alpha_2\text{-AR}^*$, leading to increased coupling to G_i proteins and hence, either an inhibition of adenylate cyclase with reduced cAMP production or activation of K^+ -channels causing hyperpolarisation of the cell (Hoffman & Taylor, 2001). This will eventually decrease neuronal cell firing and neurotransmitter release (Foote & Aston-Jones, 1995; Baldessarini, 2001).

A neutral antagonist at α_2 -ARs, such as idazoxan (see below), will compete with *I*-NE for binding to α_2 -ARs. At relatively high concentrations of the neutral antagonist, when all of the *I*-NE is displaced, the equilibrium between $\alpha_2\text{-AR}^0$ and $\alpha_2\text{-AR}^*$ will return to basal levels (since there is no preferential binding of the neutral antagonist to any of the conformations), and the effect will return to basal (i.e. in the absence of *I*-NE). An inverse agonist, such as yohimbine (see below), will also compete with *I*-NE for binding to α_2 -ARs. However, at relatively high concentrations of the inverse agonist, when all of the *I*-NE is displaced, the equilibrium will shift towards $\alpha_2\text{-AR}^0$ thereby preventing any spontaneous coupling to G_i proteins (see Figure 2-7 and Figure 2-8). If there is any constitutive activity of α_2 -ARs, the basal effect will be reduced. Even if constitutive activity for major cellular responses is limited (e.g. inhibition of cAMP production), there may be an effect of the inverse agonist on other effects, such as alteration of membrane trafficking of

receptors, leading to alteration in expression levels, opposite to what would be achieved with, for example, an agonist. The latter has not been investigated for α_2 -ARs and remains speculative, although data for other GPCRs suggest that this may be possible.

Relevant to the current study, the α_2 -ARs are located presynaptically on *I*-NE neuronal cell bodies and terminals (α_2 -autoreceptors) and on 5-HT neuronal cell bodies and terminals (α_2 -heteroreceptors), where they inhibit the synthesis and release of the respective neurotransmitters. Inverse agonists at these receptors would therefore be able to reduce spontaneous inhibition of *I*-NE and 5-HT neurotransmission mediated by α_2 -ARs, whereas neutral antagonists will not.

Recent studies report that drugs acting at α_2 -ARs that have previously been thought to be neutral antagonists are indeed inverse agonists at this receptor subtype. Rauwolscine and yohimbine act as inverse agonists at α_2 -ARs (Wade *et al.*, 2001), and recent results from our laboratory suggest that mirtazapine could act either as an inverse agonist or as a neutral antagonist at these receptors (Khoza, 2004). It is suggested that idazoxan is a neutral antagonist at α_2 -ARs (Wade *et al.*, 2001). The differences between the modes of antagonism of these drugs at α_2 -ARs could account for *in vivo* pharmacological differences between them.

Since the α_2 -AR is implicated in the mechanism of action of some atypical antidepressants (§ 0), the question can be asked whether the mode of antagonism (i.e., neutral antagonism or inverse agonism) could have an effect on the efficacy and/or the onset of action of these antidepressants. Indeed, the suggested earlier onset of action by mirtazapine (see § 2.3.3) could be related to a possible inverse agonistic effect at α_2 -ARs (Khoza, 2004). Since α_2 -AR antagonism in general is an attractive putative mechanism for early onset of antidepressant response (see § 0), an inverse agonist could potentially have a potentiated action in this regard, with an even more accelerated onset and greater efficacy of antidepressant action.

2.5 PRECLINICAL EVALUATION OF THE ACTION OF ANTIDEPRESSANT DRUGS

2.5.1 Animal models of depression

2.5.1.1 Overview of animal models of depression

Animal models of depression have been used extensively in the development of novel therapeutic compounds, and for understanding the neural processes underlying depressive behaviour (McKinney, Jr. & Bunney, Jr., 1969; Cryan *et al.*, 2002; Holmes, 2003; Uys *et al.*,

2003). As portrayed in Table 2-1, depression is a heterogeneous disorder that often manifests with symptoms at the psychological, behavioural, and physiological levels. As with all diseases, reproduction of the disease and subsequent actions of corrective medications in laboratory animals are essential for the development of effective therapies. The wide spectrum of the symptoms of depression makes the task of mimicking the disorder in the laboratory especially troublesome.

Nevertheless, a number of attempts have been made to create animal models of depression, and the criteria for their evaluation. Some of the most widely used criteria were developed by McKinney and Bunney more than 30 years ago (McKinney, Jr. & Bunney, Jr., 1969). These criteria are: (1) it is "reasonably analogous" to the human disorder in its manifestations or symptomatology; (2) there is a behavioural change that can be measured objectively; (3) the behavioural changes observed should be reversed by the same treatments that are effective in humans; and (4) it should be reproducible between investigators.

Most recently, it has been suggested that, instead of attempting to reproduce the entire disease observed in humans in laboratory animals, a more useful strategy might be to model a single, clear-cut behavioural output relevant to the disease as opposed to the syndrome (Geyer & Markou, 1995). These authors have proposed that the only criteria that are necessary for the use of an animal model for a psychiatric disorder are that it has strong predictive validity and that the behavioural readout is reliable in the same laboratory as well as between laboratories.

Various models have been subsequently developed, and are widely used in the detection of antidepressant-like potential of novel compounds in preclinical settings. Many of these models have also undergone modifications from time to time to keep up with the pace of continuing advances in the development of novel drugs and insights. There is a large number of developed animal models for a range of animals, however, only three will be discussed here, namely, the rat forced swim test (FST), which is the model employed in the current study, as well as learned helplessness and a genetic model of depression.

2.5.1.2 The Rat Forced Swim Test

The rat forced swim test (FST) was developed by Porsolt and colleagues (Porsolt *et al.*, 1978) in the rat. This test is still the most widely used tool for preclinical assessment of antidepressant-like activity of drugs. The widespread use of this model is largely a result of its ease of use, reliability across laboratories, and ability to detect a broad spectrum of antidepressant agents (Borsini & Meli, 1988). The test is based on the observation that rats, following initial escape directed movements, develop an immobile posture when placed in an inescapable cylinder of water. If they are replaced in the testing apparatus 24 hours later, they resume this posture more quickly. The immobility is thought to reflect a failure in persistence in escape-directed

behaviour, and is also called behavioural despair (Lucki, 1997). If an antidepressant treatment is administered between the two exposures, the rat subjects will actively persist in escape-directed behaviour for longer periods than after vehicle treatment. However, the major drawback of the traditional FST is that it is unreliable in the detection of antidepressant activity of SSRIs (Detke *et al.*, 1995), which are the most widely prescribed group of antidepressant drugs today.

2.5.1.2.1 The modified rat forced swim test

In an effort to enhance the sensitivity of the traditional FST in the rat to be SSRI-responsive, several procedural modifications have been made (Lucki, 1997). These developments include increasing the water depth to 30 cm from the traditional depths of 15-18 cm, and/or distinguishing between different behavioural components of active behaviour, namely (1) climbing behaviour, which is defined as upward-directed movements of the forepaws along the side of the cylinder, (2) swimming behaviour, which is the movement throughout the cylinder which includes crossing into another quadrant, and (3) immobility, which is defined (as in the original Porsolt test) as when no additional activity is observed than that required to keep the rat's head above the water (Figure 2-9). This version of distinguishing between various behavioural components was also employed in the current study, while the increased water level was kept at the original 18 cm.

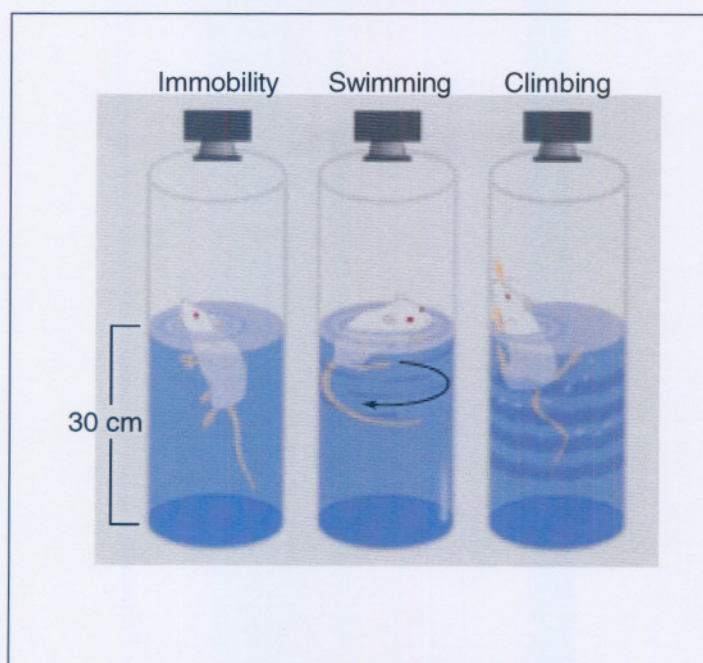


Figure 2-9: The behavioural parameters measured in the modified forced swim test. These include three distinct behavioural components, namely immobility, swimming and climbing. (Cryan *et al.*, 2002).

The major advantage of the modified FST is that it distinguishes between agents that enhance central 5-HT and *I*-NE levels. For example, agents that enhance central *I*-NE levels typically decrease immobility with a corresponding increase in climbing activity. On the other hand, agents that enhance central 5-HT levels, such as SSRIs, also decrease immobility, but increase swimming behaviour (Lucki, 1997; Cryan & Lucki, 2000).

One major drawback of the FST is the discrepancy in the drug treatment duration between the FST and the clinical treatment of humans. Acute (single/short-term) antidepressant treatments is sufficient to reverse immobility in rats in the FST, whereas the treatment of humans may require weeks for the elevation of mood. However, it has been demonstrated that doses of antidepressant drugs that are inactive acutely elicit antidepressant-like effects when administered chronically, (Detke *et al.*, 1997), and chronic treatment programmes of 2 – 4 weeks have also been evaluated with the FST (Porsolt *et al.*, 2000; Renner *et al.*, 2002a). The modified FST has also been used with much success, indicating the reliability of the modified version of this model (Renner *et al.*, 2001; Renner *et al.*, 2002a; Renner *et al.*, 2002b).

2.5.1.2.2 Receptors affecting behaviour in the forced swim test

Modulation of 5-HT function is the best documented in terms of affecting behaviour in the rat FST and will be discussed below. Much less is known about the role of dopaminergic, noradrenergic, and other more diverse classes of receptors in this regard.

2.5.1.2.2.1 Serotonin 5-HT_{1A} receptors

The 5-HT_{1A} receptor has a diverse localisation and function on serotonergic neurotransmission. The receptor is located presynaptically in the raphe nuclei where it functions as an autoreceptor, and postsynaptically in the hippocampus (Figure 2-3).

5-HT_{1A} receptor agonists have been shown to produce antidepressant-like responses in the traditional and modified versions of the FST suggesting antidepressant potential (Lucki *et al.*, 1994; De Vry, 1995), with this activity suggested to be mediated by postsynaptic 5-HT_{1A} receptors (Wieland & Lucki, 1990; Schreiber & Devry, 1993).

When given alone, 5-HT_{1A} antagonists have no effect in the FST (Lucki *et al.*, 1994; De Vry, 1995), which supports the hypothesis that postsynaptic 5-HT_{1A} receptors are involved in the mediation of antidepressant-like behaviour. An exception is pindolol, which induces a small increase in swimming scores across a narrow dose range (Cryan *et al.*, 2005). As noted in § 2.3.2.4, pindolol antagonises 5-HT_{1A} and 5-HT_{1B} receptors in addition to its β -AR blocking properties. Therefore, the ability of this drug to induce antidepressant-like behaviour in the FST may be due to blockade of presynaptic 5-HT_{1A} and 5-HT_{1B} receptors, however, it may also be

caused by the partial agonist properties of pindolol at postsynaptic 5-HT_{1A} receptors (Clifford *et al.*, 1998).

In short, the ability of a drug to activate postsynaptic 5-HT_{1A} receptors opposed to the stimulation of its presynaptic counterpart, could possibly predict its antidepressant-like effects in the FST.

2.5.1.2.2.2 Serotonin 5-HT_{1B} receptors

The most important role for this receptor subtype in the context of the FST is believed to be its inhibitory action on 5-HT release. This effect is mediated by presynaptically located 5-HT_{1B} on 5-HT terminals (Figure 2-3). However, since there are limited data on the effects of these receptors on behaviour in the FST, it can only be speculated that stimulation of 5-HT_{1B} receptors would decrease swimming and increase immobility scores by decreasing postsynaptic 5-HT availability (§ 2.2.1).

2.5.1.2.2.3 Serotonin 5-HT_{2A} receptors

As in the case of 5-HT_{1B} receptors, little is known about the role of 5-HT_{2A} receptors on behaviour in the FST. Recently, however, it has been shown that the novel 5-HT_{2A} receptor antagonist EMD-281014 increased swimming and decreased immobility in congenitally learned helpless animals (Patel *et al.*, 2004).

2.5.1.2.2.4 Serotonin 5-HT_{2C} receptors

One study has demonstrated that the 5-HT_{2C} receptor antagonist mesulergine antagonises the anti-immobility effects of fluoxetine in the FST (Cesana *et al.*, 1993). This suggests that the 5-HT_{2C} receptor may play a role in the ability of fluoxetine to reduce immobility in the FST. In support of this hypothesis, antidepressant-like effects in the FST are produced by three selective 5-HT_{2C} agonists, RO 60-0175, RO 60-0332, and WAY 161503 (Cryan & Lucki, 2000).

2.5.1.2.2.5 Serotonin 5-HT₃ receptors

It has been demonstrated that the potassium channel blockers, quinine and glyburide, potentiate the effects of antidepressants in the FST (Guo *et al.*, 1995; Guo *et al.*, 1996). This effect is thought to involve blockade of potassium ion channel-linked 5-HT₃ receptors (Bourin *et al.*, 1996). It was also found that the potassium channel activator, cromakalim, antagonises the anti-immobility effects of antidepressants in the FST (Redrobe *et al.*, 1996).

2.5.1.2.2.6 Other receptor subtypes

One group of researchers demonstrated that activation of dopaminergic D₂ and D₃, but not D₄ receptors, reduced immobility in the FST (Basso *et al.*, 2005). Other more diverse receptor subtypes suggested to be involved in the behavioural outcome of the FST include CRF₁

receptors (Chaki *et al.*, 2004), NMDA receptors (Padovan & Guimaraes, 2004), adenosine A_{2A} receptors (El *et al.*, 2001; El *et al.*, 2003), imidazoline I₂ receptors (Finn *et al.*, 2003), kappa(κ)-opioid receptors (Mague *et al.*, 2003), and GABA_B receptors (Nakagawa *et al.*, 1996).

2.5.1.3 Learned helplessness

The learned helplessness model is based in the observation that following repeated uncontrollable shocks, animals demonstrate escape deficits that are reversed by antidepressant agents (Weiss & Kilts, 1998). The behavioural deficits are sensitive to a wide range of antidepressant compounds, usually following short-term treatment. The major drawback of this model is that most of the depression-like symptoms do not persist beyond 2 to 3 days after the uncontrollable shock is stopped. A recent modification of the rat learned helplessness procedure incorporates aspects of chronic mild stress (Gambarana *et al.*, 2001). By chronic exposure to mild stressors the effects of the uncontrollable shock can be maintained for a longer period, and chronic treatment with fluoxetine and imipramine reverses these changes.

2.5.1.4 Genetic rat model of depression

The Flinders Sensitive Line (FSL) rat was originally proposed as an animal model of depression because, more analogous to depressed humans, it is supersensitive to the behavioural and hormonal effects of cholinergic (mACh receptor) agonists (Overstreet, 1993). FSL rats resemble depression in humans in that they demonstrate reduced locomotor activity, reduced body weight, increased REM sleep, and cognitive (learning) difficulties (Overstreet, 1993). A key behavioural symptom exhibited by FSL rats is the demonstration of increased immobility when exposed to stressors such as foot shock and forced swimming. This behavioural abnormality has been normalised with antidepressants such as imipramine, desipramine, sertraline, and rolipram (Overstreet, 1993).

2.5.2 Alterations in β-AR concentration following antidepressant treatment

It is generally accepted that a large number of antidepressant treatments cause a reduction in the density of cortical β-ARs of rats. This phenomenon has been reported for desipramine (Tang *et al.*, 1981; Sethy *et al.*, 1988; Paetsch & Greenshaw, 1993; Newman Tancredi *et al.*, 1996), imipramine (Asakura *et al.*, 1982; Friedman *et al.*, 1986; Sethy *et al.*, 1988), amitriptyline (Tang *et al.*, 1981; Sethy *et al.*, 1988), mirtazapine (McGrath *et al.*, 1998), clomipramine and nortriptyline (Asakura *et al.*, 1982), to name only a few. However, some clinically effective antidepressants such as bupropion and zimelidine do not cause β-AR downregulation (Suranyi-Cadotte *et al.*, 1985).

There is conflicting evidence on the ability of SSRIs to cause downregulation of β -ARs. One study has demonstrated that fluoxetine is unable to reduce β -AR density (Baron *et al.*, 1988), while another has reported that it is indeed able to reduce the density of this receptor subtype (Byerley *et al.*, 1988). Similarly, one study suggested that citalopram produces downregulation of β -ARs (Dziedzicka-Wasylewska *et al.*, 2001), while other suggested that it does not (Hyttel *et al.*, 1984; Garcha *et al.*, 1985). Similarly, some researchers demonstrated that sertraline produces a reduction in β -AR density (Byerley *et al.*, 1987; Koe & Lebel, 1995) while another group showed that sertraline does not downregulate β -ARs (Tadokoro *et al.*, 1997).

In addition to the effects of antidepressant drug treatments on β -ARs receptors, it has been reported that some effective non-drug antidepressant therapies also downregulate cortical β -ARs. These include REM sleep deprivation (Mogilnicka *et al.*, 1980) and electroconvulsive shock therapy (Green *et al.*, 1986).

The pharmacodynamic basis for the downregulation of β -ARs by antidepressants and antidepressant therapies is not clear, nor is its relationship/contribution to superior clinical antidepressant efficacy proven (Nelson *et al.*, 1991). It is unlikely that a reduction in β -ARs function contributes directly to the mood-elevating effects of antidepressant treatments, since β -ARs antagonists tend to induce or worsen depression (Baldessarini, 2001), and some antidepressants with proven clinical efficacy have been shown not to reduce β -AR density. Nevertheless, it has been postulated that a loss of inhibitory β -AR influences on 5-HT neurons may enhance the release of serotonin and contribute indirectly to antidepressant effects (Wamsley *et al.*, 1987). Taken together, the measurement of the effects of antidepressants on β -AR receptor density remains a useful screening tool for the assessment of antidepressant activity (Leonard, 1997), since downregulation of these receptors is a collective property of a remarkably wide spectrum of antidepressant treatments.

2.6 SYNOPSIS

Depression has most probably plagued humans since the origin of mankind. The incidence of the disease seems to be increasing, and the World-Health Organisation has predicted that it will be the second most frequent debilitating disease by 2020 (Murray & Lopez, 1997). However, the exact aetiology of the disease remains unknown. Although a number of factors (i.e. genetics, stress, etc.) have been implicated in depression, there is still no consensus on the relative importance of such factors. Similarly, many brain structures and pathways have been proposed as being involved in the diverse symptoms of depression. There is still only limited data on the role that these regions and systems play in the disease and its symptoms. Current research is directed towards systems such as the HPA-axis and brain reward pathways, as well

as impairment of neurotrophic mechanisms, but the clinical use of antidepressants that specifically target these systems still has to be introduced. Antidepressants on the market today are drugs that enhance monoaminergic neurotransmission, but the exact mechanisms by which these drugs ultimately alleviate depression are still not clear and there are indications that they may also influence the HPA-axis and central neurotrophic mechanisms.

An important troublesome factor in the drug treatment of depression remains the time delay of such therapies to alleviate the burdensome symptoms of this disease. Recent clinical studies have suggested that some novel antidepressants could have a more rapid onset than conventional antidepressants. These observations have renewed interest in the pathophysiology of depression and its treatment. The pharmacodynamics of these putative rapid onset compounds have become the focus in the development of novel strategies for alleviating depression more quickly. Mirtazapine is an example of one of these drugs. It is believed that a primary pharmacological mechanism of this drug is its antagonism of α_2 -AR receptors. It is therefore suggested that this mechanism could be primarily involved in the putative early onset of action of mirtazapine. In addition to this, it is possible that mirtazapine could act as an inverse agonist at α_2 -ARs, which could further potentiate its antidepressant effectiveness and/or allow it to act more rapidly.

Due to obvious ethical impediments, the primary means of developing and evaluating novel antidepressant treatments is the preclinical use of animal models. The most widely used and recognised model of depression is the rat forced swim test, developed by Porsolt and colleagues in 1978. In the current study, a modified version of this model is employed to evaluate the efficacy, and more importantly, the onset of antidepressant-like response of putative early onset strategies. In addition to the reversal of depressive-like behaviour in animals, it has been observed for decades that, in general, antidepressants cause a reduction in the concentration of β -ARs receptors in the cortical region of the brain. This effect can be used as a biochemical marker for the action of antidepressants, despite the fact that the importance of β -ARs downregulation in the alleviation of depressive symptoms is unclear.

Materials and methods

Chapter

This chapter presents and discusses the experimental methods employed in the current study, including the experimental layout, animal models, materials, drugs and dosages and assays.

The treatment of the animals and behavioural testing were conducted in the Centre for Laboratory Animals at the Potchefstroom Campus of the North West University, after approval by the Ethics Committee of the North West University (approval number 03D09) and according to the internationally accepted ethics guidelines. Preparation of drug solutions and radio-ligand receptor binding studies were carried out in the Laboratory of Applied Molecular Biology (LAMB) at the Potchefstroom Campus of the North-West University.

3.1 OVERVIEW

In brief, the study is divided into two pilot studies and an experimental study:

- **Pilot Study 1:** Lab-validation of the forced swim test.
- **Pilot Study 2:** Determination of the shortest chronic treatment period necessary for fluoxetine to exert its antidepressant-like effect in the forced swim test, as well as to down-regulate β -ARs.
- **Experimental Study:** Evaluation of the role of α_2 -lytic action in the putative early onset of antidepressant response in the rodent model, using various drugs and drug combinations.

The onset of an antidepressant-like effect was defined by two parameters:

- Behavioural changes in a rat model of depression (measuring behavioural despair in the forced swim test - FST), comparing different drug treatment groups, as well as parallel measurements of locomotor activity to confirm that changes in animal mobility in the FST is not influenced by changes in locomotor activity.
- Biochemical changes (β -AR and 5-HT_{1A} receptor concentrations) in selected brain regions of rats, comparing different drug treatment groups.

3.2 ANIMALS AND MATERIALS USED

3.2.1 Animals

All animals used in this study were male Sprague-Dawley rats, weighing ± 200 g on the day of the forced swim test and decapitation. As mentioned above, the use of the animals was approved by the Ethical Committee of the North-West University (approval no. 03D09). A total of 17 groups of rats were used in the study: 6 control groups and 11 treatment groups. Each group consisted of 21 rats that were divided into 3 replicate groups of 7 rats each. Of these, 5 rats were randomly assigned for use in the FST and 2 rats for assessment of locomotor activity. A total of 546 (FST: 390; locomotor activity: 156) rats were used in this study (see Table 3-1 and Table 3-2). All animals were housed in groups of 7 rats (FST: 5 rats; locomotor activity: 2 rats) in acrylic cages (280 x 450 x 130 mm) in a temperature (21°C) and humidity (50%) controlled animal room with a 12-hour light-dark cycle (lights 06:00 to 18:00), with food and water available *ad libitum*.

3.2.2 Drugs

Fluoxetine hydrochloride was a generous gift from Aspen (Port Elizabeth, South Africa). Mirtazapine was kindly provided by Organon (Netherlands). Idazoxan hydrochloride and yohimbine hydrochloride were obtained from Sigma-Aldrich (St. Louis, USA).

3.2.3 Radio-chemicals

[³H]-Propranolol hydrochloride (20.0 Ci/mmol) was obtained from GE Healthcare (formerly Amersham Biosciences, Buckinghamshire, England), [³H]-OH-DPAT (8-hydroxy-2-di-n-propylamino-tetralin) (106 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, USA).

3.2.4 Other chemicals

NaOH was obtained from Merck (Darmstadt, Germany), liquid N₂ was obtained from Afrox (Johannesburg, South Africa), Propranolol hydrochloride, Bradford reagent, EDTA, Ascorbic acid, Sigmacote® and fractioned BSA were obtained from Sigma-Aldrich (St. Louis, USA), Tris HCl and MgCl₂ were obtained from USB (Cleveland, USA), Filter Count LSC-cocktail was obtained from PerkinElmer (Boston, USA), serotonin creatinine sulphate was obtained from BDH Chemicals (Poole, England).

3.2.5 Instruments

Perspex cylinders (diameter 180 mm, height 400 mm), Sony Digital Video Camera Recorder (model: DCR-TRV330E), Digiscan Animal Activity Monitor (DAAM, AccuScan Instruments, Columbus, OH, USA), Consort P901 electrochemical analyzer (pH meter), Sartorius BP211D balance, Tri-Carb 2100 TR liquid scintillation analyzer (Packard, A.D.P. South Africa), Sorvall Discovery 90SE ultra-centrifuge, Consort P901 electrochemical analyzer (pH meter), Sartorius BP211D balance, 96 well plate reader and 560 nm filter (Labsystems multiskan RC), Polytron® Homogenizer (Kinematica, Switzerland), Eppendorf micropipettes, 40 well Hoefer filtration rig (locally manufactured).

3.2.6 Other materials

Whatman GF/B glass micro fibre filters were obtained from Merck (Darmstadt, Germany).

3.3 PROJECT LAYOUT

3.3.1 Pilot Study 1: Lab-validation of the rat forced swim test (FST)

The rat FST is a validated, well-described and widely used screening test for the detection of antidepressant activity of drugs, and the experimental protocol for this test was followed as described previously (Detke *et al.*, 1995; Detke & Lucki, 1995; Detke *et al.*, 1997). This test has also been previously used to assess the effects of short and long-term antidepressant treatments (Reneric *et al.*, 2002a), similar to the approach in the current study. It was important, however, to validate the rat FST as implemented under laboratory conditions. Firstly, we wanted to demonstrate that a state of behavioural despair can be induced in the animals in our laboratory, and secondly, that the development of behavioural despair can be inhibited by conventional antidepressant treatment (fluoxetine 20 mg/kg/day). Figure 3-1 illustrates schematically the experimental layout as conducted in this phase of the study.

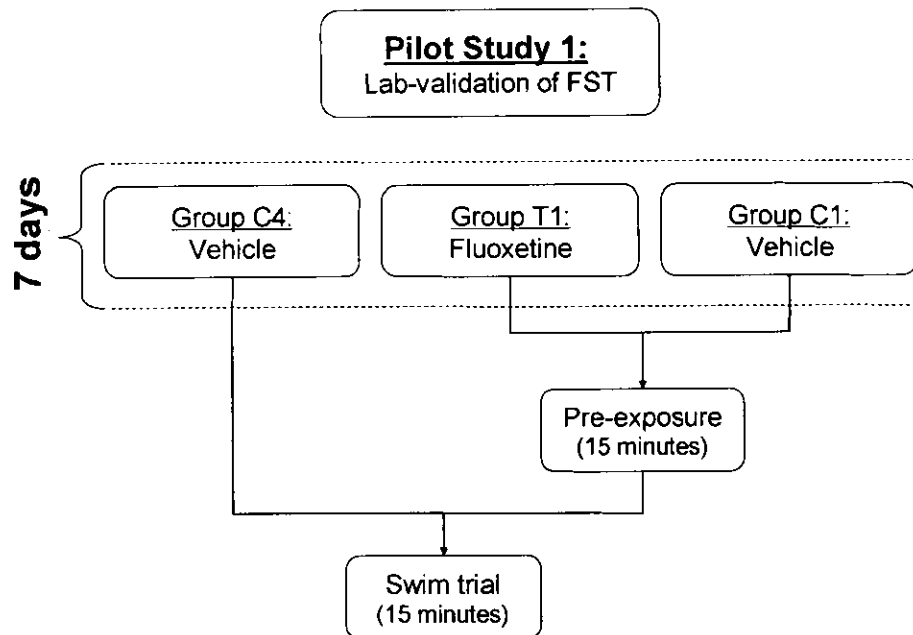


Figure 3-1 Schematic illustration of the treatment and handling of rats in Pilot Study 1.

The group numbers are defined in Table 3-1, since the groups used in this phase overlap with those used in Pilot Study 2. In brief, C denotes a control group and T denotes a testing group receiving fluoxetine. Pre-exposure refers to the 15-minute pre-conditioning swim session 24 hours prior to the 5-minute scoring swim trial.

Three treatment groups were used in the lab-validation experiment. Two groups of rats (groups C1 + C4, see Table 3-1) were treated with saline (intra-peritoneally, i.p.), while a third group (T1) was treated with fluoxetine (20 mg/kg/day, i.p.) for 7 days. The animals received their respective treatments at the same time every morning for the treatment period. On the penultimate day of the treatment period (day 6), two of the groups (C1 + T1) were exposed to 15 minutes of forced swimming to induce a state of behavioural despair, whereas the remaining group (C4) had no such experience. On the last day (day 7), and after the last administration, all treatment groups were exposed to 5 minutes of forced swimming and the behaviour of the rats recorded on video.

A comparison of C1 with C4 enabled us to evaluate whether a 15-minute pre-exposure to forced swimming 24 hours prior to the actual test can induce a state of behavioural despair. Behavioural despair would typically be associated with significantly longer immobility times (i.e. increased immobility in C1, as compared to C4).

A comparison of T1 with C4 enabled us to evaluate whether treatment with the SSRI antidepressant fluoxetine can inhibit the development of behavioural despair in the FST. Therefore, this phase of the validation process would be successful if the fluoxetine treated rats displayed shorter immobility times compared to saline treated rats.

Each treatment group consisted of 3 replicate (to verify repeatability) subgroups of 7 rats each (5 rats used in the FST, and 2 rats used for assessment of locomotor activity). In total, 15 rats were used in the FST and 6 rats for assessment of locomotor activity for each treatment group.

3.3.2 Pilot Study 2: Time-dependency of the antidepressant-like action of fluoxetine

Pilot study 2 was conducted to determine the shortest treatment period in which a conventional antidepressant (e.g. fluoxetine) could elicit antidepressant-like behavioural and biochemical changes. This treatment period, as well as a shorter period where fluoxetine was unable to produce such effects in Pilot Study 2, were then to be used in the experimental phase of the study to detect the early onset of antidepressant-like effects by mirtazapine and α_2 -lytic drugs. Figure 3-2 illustrates schematically the experimental layout, as conducted in this phase of the study.

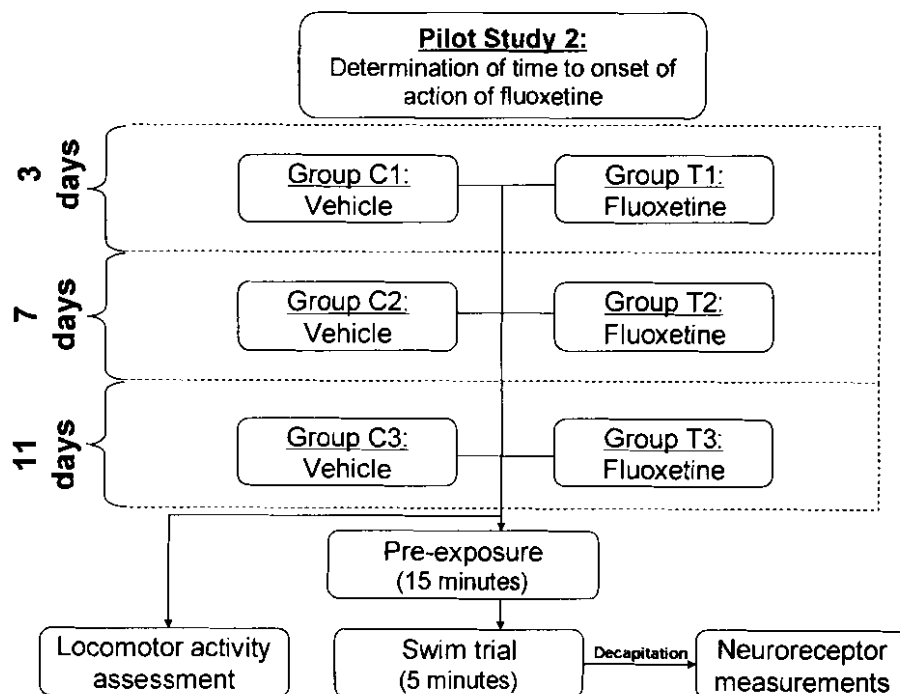


Figure 3-2: Schematic illustration of the treatment and handling of rats in Pilot Study 2. The group numbers are defined in Table 3-1. In brief, C denotes a control group and T denotes a testing group receiving fluoxetine. Pre-exposure refers to the 15-minute pre-conditioning swim session 24 hours prior to the 5-minute scoring swim trial.

Six treatment groups were used in this phase of the study (see Table 3-1). Three groups of rats (T1 – T3) were treated with fluoxetine (20 mg/kg/day) for 3, 7 or 11 days, respectively. In

addition, three control groups (C1 –C3) received vehicle for 3, 7 or 11 days respectively. All groups were allowed to swim for 15 minutes on the day prior to the assessment of behaviour. The outline of the different treatment regimes in Pilot Study 2 is presented in Table 3-1, while the treatment timescale is illustrated in Figure 3-3.

Each treatment group consisted of 3 replicate (to verify repeatability) subgroups of 7 rats each (5 rats used in the FST, and 2 rats used for assessment of locomotor activity). In total, 15 rats were used in the FST and 6 rats for assessment of locomotor activity for each treatment group.

Table 3-1: Treatment regimes in Pilot Studies

Group	Drug(s)	Days	Rats / group	Groups	Total
C1	Vehicle (i.p.)	3	7	3	21
T1	Fluoxetine (20 mg/kg/day, i.p.)	3	7	3	21
C2	Vehicle (i.p.)	7	7	3	21
T2	Fluoxetine (20 mg/kg/day, i.p.)	7	7	3	21
C3	Vehicle (i.p.)	11	7	3	21
T3	Fluoxetine (20 mg/kg/day, i.p.)	11	7	3	21
VALIDATION OF THE FST					126
C4	Vehicle (i.p.)	7	7	3	21
					21
					147

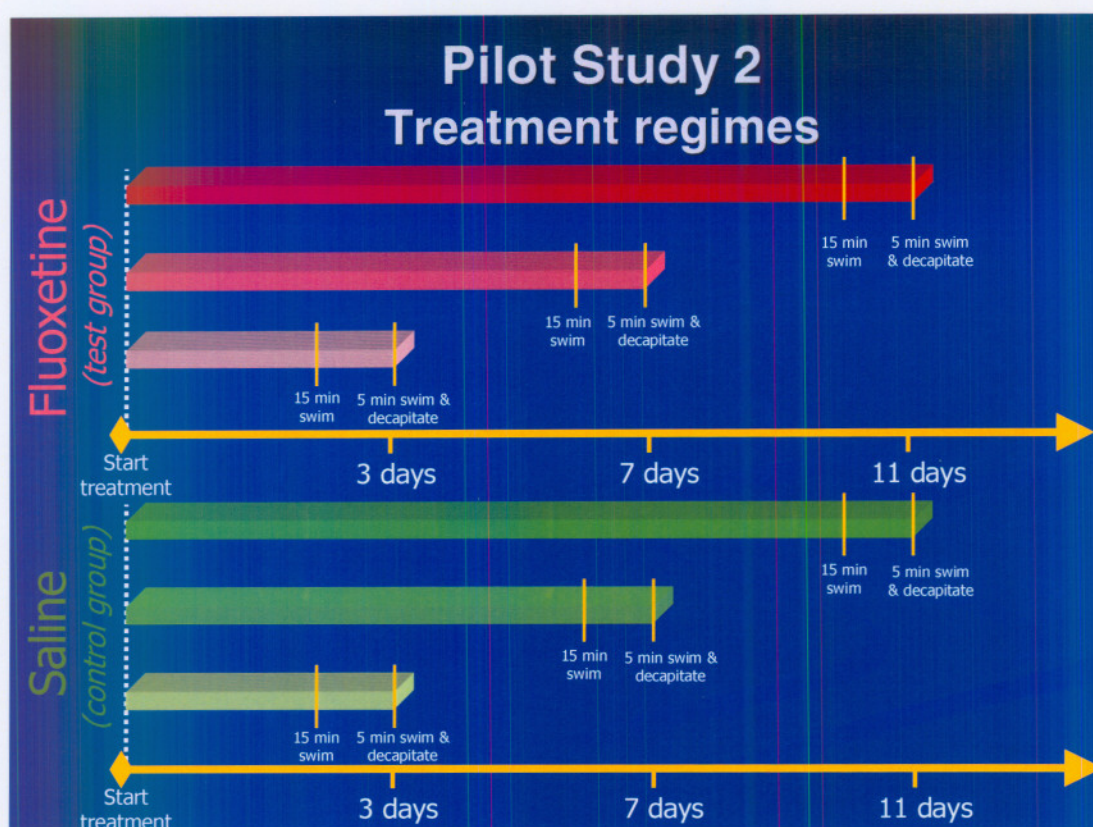


Figure 3-3: Schematic illustration of the treatment timescale for Pilot Study 2.

The animals received their respective treatments at the same time every morning for the respective treatment period. On the penultimate day of the treatment period the rats that were assigned to the FST were allowed to swim for 15 minutes. As before, the purpose of this 15 minute period was to induce a state of behavioural despair in the animals to ensure a stable, high level of immobility during the actual test. On the last day of the treatment period, and after the last dosage, the animals were allowed to swim for 5 minutes and their behaviour was recorded on video. In addition, locomotor activity was measured on the last day of the treatment period (not shown in Figure 3-3). The rats were then decapitated and the brains removed for the radio-ligand binding studies to follow.

3.3.3 Experimental Study: α_2 -AR receptor antagonism and onset of antidepressant-like responses

The treatment periods that were used in the Experimental Study were based on the data from Pilot Study 2, i.e. the shortest treatment period in which fluoxetine induced antidepressant-like behavioural and biochemical changes in Pilot Study 2 was used in the Experimental Study, as well as a shorter treatment period where no significant changes were evident. In Pilot Study 2, significant behavioural and biochemical changes were evident after 7 and 11 days, but not after 3 days (data presented in § 4.2 - discussed later). The Experimental Study was consequently

conducted using 3 day and 7 day treatment periods. In this phase the rats were treated with saline, fluoxetine, or with an α_2 -lytic drug, including mirtazapine (α_2 -lytic mode unknown), yohimbine (inverse agonist) and idazoxan (neutral antagonist), or with a combination of fluoxetine and each of the respective aforementioned α_2 -lytic drugs (see Table 3-2). This was done in order to enable us to investigate whether α_2 -AR antagonists can produce an earlier onset of antidepressant-like action than fluoxetine (known to have a delayed onset of action), or potentiate and/or hasten the onset of action of fluoxetine. If any drug treatment regime elicits antidepressant-like effects after only 3 days of treatment, it would suggest that it may indeed produce an earlier onset of antidepressant action compared to fluoxetine. Figure 3-4 illustrates schematically the experimental layout, as conducted in this phase of the study.

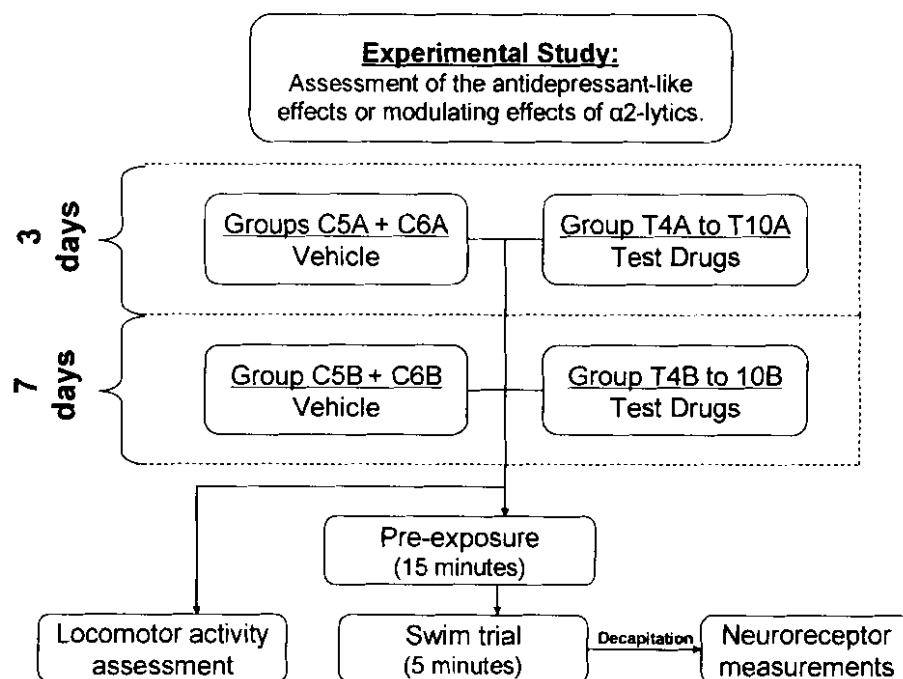


Figure 3-4: Schematic illustration of the treatment and handling of rats in the Experimental Study. The group numbers are defined in Table 3-2. In brief, C denotes a control group and T denotes a testing group, receiving test drug(s). Pre-exposure refers to the 15-minute pre-conditioning swim session 24 hours prior to the 5-minute scoring swim trial.

The same procedures were followed in the Experimental Study as in Pilot Study 2 concerning the FST, measurement of locomotor activity and radio-ligand binding studies. The outline of the different treatment regimes in the Experimental Study is presented in Table 3-2, and the treatment timescale is illustrated in Figure 3-5.

Each treatment group consisted of 3 replicate (to verify repeatability) subgroups of 7 rats each (5 rats used in the FST, and 2 rats used for assessment of locomotor activity). In total, 15 rats were used in the FST and 6 rats for assessment of locomotor activity for each treatment group.

Table 3-2: Treatment regimes for the Experimental Study

Group	Drug(s)	Days	Rats / group	Groups	Total
C5A	vehicle	3 days	7	3	21
T4A	fluoxetine	3 days	7	3	21
T5A	mirtazapine	3 days	7	3	21
T6A	yohimbine	3 days	7	3	21
T7A	idazoxan	3 days	7	3	21
C6A	vehicle	3 days	7	3	21
T8A	fluoxetine + mirtazapine	3 days	7	3	21
T9A	fluoxetine + yohimbine	3 days	7	3	21
T10A	fluoxetine + idazoxan	3 days	7	3	21
C5B	vehicle	7 days	7	3	21
T4B	fluoxetine	7 days	7	3	21
T5B	mirtazapine	7 days	7	3	21
T6B	yohimbine	7 days	7	3	21
T7B	idazoxan	7 days	7	3	21
C6B	vehicle	7 days	7	3	21
T8B	fluoxetine + mirtazapine	7 days	7	3	21
T9B	fluoxetine + yohimbine	7 days	7	3	21
T10B	fluoxetine + idazoxan	7 days	7	3	21
					378

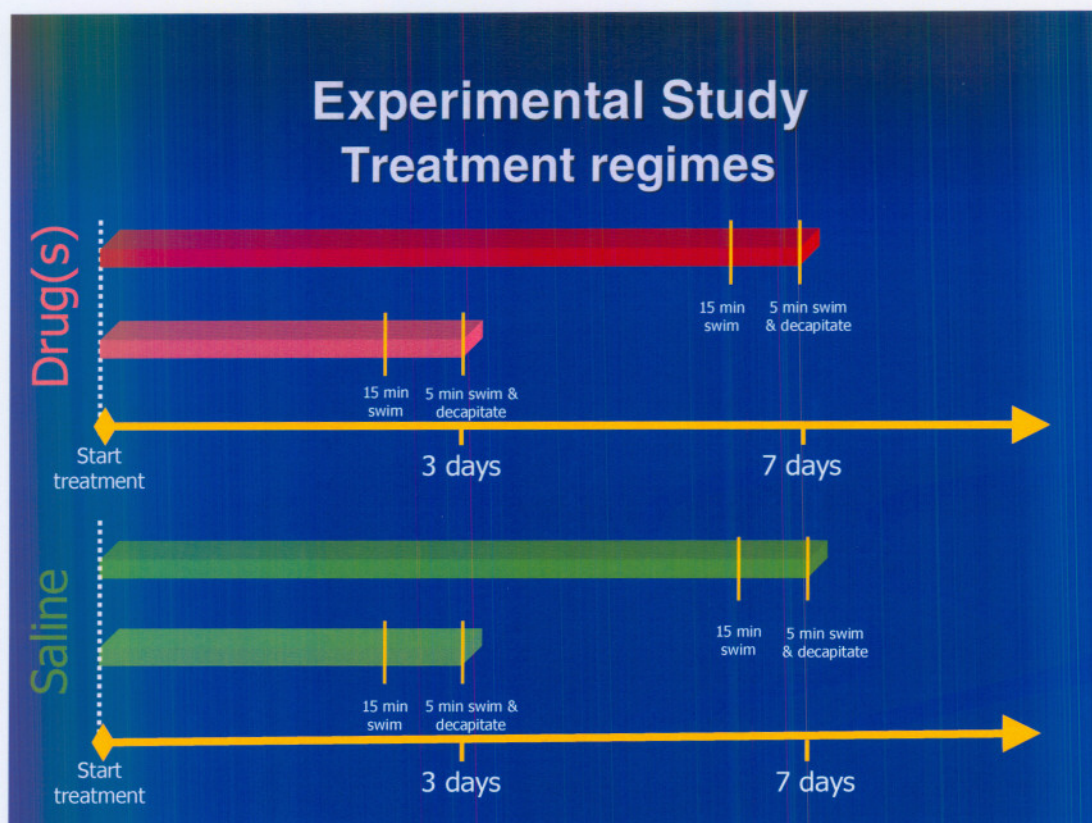


Figure 3-5: Schematic illustration of the treatment timescale for the Experimental Study.

3.4 DOSAGE CHOICES FOR THE DRUGS EMPLOYED

3.4.1 Principles of dosage selection in animal models

The dosage choices for the drugs that were used in the current study were based on two criteria. Firstly a comprehensive review was done to get an overview of the doses used in rats for earlier studies where these drugs were used, especially studies where similar effects of these drugs were required. Secondly the pharmacodynamic profiles of the drugs (in terms of the affinity for a series of receptors and receptor subtypes) were considered, in particular those sites that have been suggested to be involved in depression, as well as other sites that may influence the outcome of the results. Subsequently, the dosages were selected to ensure the most selective action on the receptors involved in the putative antidepressant action(s) of the drugs. The affinity is expressed in terms of the K_i -value (nM).

3.4.2 Fluoxetine

3.4.2.1 Doses in earlier studies

A dose of 20 mg/kg was used in a study that distinguished between serotonergic and noradrenergic effects in the rat FST (Detke & Lucki, 1995). In another study fluoxetine was administered at a dose of 10 mg/kg to rats for investigating the modifying effect of idazoxan and 8-OH-DPAT on antidepressant-mediated behavioural responses in the rat FST (Reneric *et al.*, 2001). A dose of 10 mg/kg was also used in a study that examined the interactions of noradrenergic and serotonergic antidepressants in the rat FST (Reneric *et al.*, 2002b).

3.4.2.2 Receptor binding profile

The affinity values of fluoxetine for a series of receptors and receptor subtypes are presented in Table 3-3.

The major site of antidepressant action of fluoxetine is thought to be the inhibition of the 5-HT transporter, with its affinity for the 5-HT transporter usually about 10-fold to 1000-fold greater than for other relevant targets. The affinity values of fluoxetine for the *L*-NE transporter and adrenergic receptors are much lower than that for the 5-HT transporter, so that its influence at therapeutic doses is negligible. The affinity for other types of receptors is also low and need not influence the selection of the dosage for fluoxetine within the normal range.

3.4.2.3 Dose selection

Early experiments with a dose of 10 mg/kg/day in the current study failed to produce significant decreases in immobility in the forced swim test (data not shown), and consequently a dose of 20 mg/kg/day was chosen.

Table 3-3: Receptor binding profile of fluoxetine

Receptor		K _i -value (nM)	Tissue	Reference
5-HT transporter		6.8	Rat brain	(Sanchez & Hyttel, 1999)
		10.8	Rat cortex	(Shank <i>et al.</i> , 1988)
		2.0	Rat cortex	(Owens <i>et al.</i> , 1997)
I-DA transporter		3 160	Rat striatum	(Shank <i>et al.</i> , 1988)
		5 000	Rat striatum	(Sanchez & Hyttel, 1999)
I-NE transporter		794.12	Rat frontal cortex	(Cordi <i>et al.</i> , 2001)
		119	Rat brain	(Beique <i>et al.</i> , 1998)
5-HT	5-HT _{1A}	11 481	Rat cortex	(Wong <i>et al.</i> , 1991)
	5-HT _{2A}	710	Rat cortex	(Sanchez & Hyttel, 1999)
	5-HT _{2C}	50	Rat cloned cells	(Rothman <i>et al.</i> , 2000)
α-AR	α ₁	14 000	Rat cortex	(Sanchez & Hyttel, 1999)
	α ₂	2 800	Rat cortex	(Sanchez & Hyttel, 1999)
H ₁		3200	Rat brain	(Sanchez & Hyttel, 1999)

3.4.3 Mirtazapine

3.4.3.1 Doses in earlier studies

A dose of 16 mg/kg/day was used in a study that showed that mirtazapine increases I-DA release in the rat frontal cortex via indirect 5-HT_{1A} activation (Nakayama *et al.*, 2004). In another study that compared the chronic and sub-acute effects of dual 5-HT and I-NE antidepressants to selective drugs in the rat FST, a dose of 20 mg/kg was used (Reneric *et al.*, 2002a).

In some cases lower doses were used. For example, 5 and 10 mg/kg/day doses were used in a study that examined the mirtazapine-induced co-release of I-DA and I-NE from neurons in the

medial frontal and occipital cortex (Devoto *et al.*, 2004). Such low doses may be insufficient to investigate behavioural effects in rodents, and will be discussed below.

3.4.3.2 Receptor binding profile

The affinity values of mirtazapine for a series of receptors and receptor-subtypes are presented in Table 3-4.

Table 3-4: Receptor binding profile of mirtazapine

Receptor		K _i -value (nM)	Tissue	Reference
5-HT	5-HT _{1A}	> 18 000	Human	(Wikstrom <i>et al.</i> , 2002)
	5-HT _{2A}	2.0	Rat cortex	(Wikstrom <i>et al.</i> , 2002)
	5-HT _{2C}	5.5	Rat tissue	(Wikstrom <i>et al.</i> , 2002)
	5-HT ₃	6.31	Mouse neuroblastoma	(de Boer, 1996)
α -ARs	α_1	372	Rat frontal cortex	(de Boer <i>et al.</i> , 1988)
		1 050	Rat whole brain	(Wikstrom <i>et al.</i> , 2002)
	α_2	112	Rat frontal cortex	(de Boer <i>et al.</i> , 1988)
		58	Rat cortex	(Wikstrom <i>et al.</i> , 2002)
mACh		794	Rat whole brain	(Wikstrom <i>et al.</i> , 2002)
H ₁		0.5	Rat frontal cortex	(de Boer <i>et al.</i> , 1988)
		5.1	Guinea pig lung	(Wikstrom <i>et al.</i> , 2002)

Since the role of α_2 -AR antagonism in antidepressant action is the focus of the current study, the affinity of mirtazapine for the α_2 -AR, relative to other receptors, is important for considering an appropriate dose.

The affinity of mirtazapine at α_2 -ARs is moderate compared to the affinity for other receptor subtypes presented in Table 3-4. Mirtazapine displays a very low affinity at 5-HT_{1A} receptors, but a very high affinity at 5-HT_{2A}, 5-HT_{2C} and 5-HT₃ receptors. Although these receptors are thought to have relevance for the antidepressant action of mirtazapine, they may also be relevant to the advantageous tolerability of the drug (Nutt, 1997). Affinity of mirtazapine at α_1 -ARs is lower than at α_2 -ARs and these receptors are not thought to be involved in its antidepressant action. Mirtazapine also binds to mACh receptors with low affinity and this

receptor subtype is thought to be involved in the mechanism of antidepressant action by mirtazapine.

In contrast, the antihistaminergic effects of mirtazapine are especially important when deciding on an appropriate dose. The affinity of mirtazapine at H_1 -receptors is the highest for all receptor subtypes. Therefore, at low doses, antihistaminergic effects would predominate, leading to significant sedation which could influence immobility as well as locomotor activity in the behavioural tests of the current study. However, at higher doses, the α_2 -lytic effects of mirtazapine would become more significant, leading to increased *L*-NE neurotransmission, counteracting the sedative side effect of this drug.

3.4.3.3 Dose selection

A dosage of 15 mg/kg/day was ultimately selected.

3.4.4 Idazoxan

3.4.4.1 Doses in earlier studies

A dose of 5 mg/kg produced significant antidepressant-like behavioural changes in the rat FST in a study that investigated the effects of idazoxan and 8-OH-DPAT on noradrenergic and serotonergic antidepressants (Reneric *et al.*, 2001). A dose of 1 mg/kg was used in a study that investigated the modulatory role of 5-HT on the firing activity of locus coeruleus *L*-NE neurons (Szabo & Blier, 2001). At a dose of 1.5 mg/kg, idazoxan increased the levels of *L*-DA and DOPAC in the rat medial frontal cortex (Hertel *et al.*, 1999).

3.4.4.2 Receptor binding profile

The affinity values of idazoxan for a series of receptors and receptor subtypes are presented in Table 3-5:

The most probable site of antidepressant action of idazoxan involves its antagonism at α_2 -ARs, since this is the only property it shares with known effective antidepressants, such as mianserin and mirtazapine (Grossman *et al.*, 1999). According to the receptor binding profile, idazoxan has the highest affinity for α_2 -ARs, some affinity for 5-HT_{1A}-receptors and relatively low affinity for α_1 -ARs.

Table 3-5: Receptor binding profile of idazoxan

Receptor		Ki-value (nM)	Tissue	Reference
5-HT	5-HT _{1A}	162.89	Rat cortex	(Kwong <i>et al.</i> , 1986)
	5-HT _{2C}	> 10 000	Rat cloned cells	(Toll <i>et al.</i> , 1998)
α -ARs	α_1	91	Rat cortex	(Doxey <i>et al.</i> , 1984)
		1 000	Rat forebrain	(Megens <i>et al.</i> , 1986)
	α_2	3.16	Rat cortex	(Millan <i>et al.</i> , 2000)
		7.6	Rat cortex	(Megens <i>et al.</i> , 1986)

3.4.4.3 Dose selection

A moderate dosage should provide effects which are primarily α_2 -AR mediated, and a dose of 3 mg/kg/day was eventually selected.

3.4.5 Yohimbine

3.4.5.1 Doses in earlier studies

In one study a dose of 2 mg/kg was injected twice daily to investigate the effect of yohimbine on β -ARs and cAMP production in the rat brain (Campbell *et al.*, 1984). In another study that investigated the effect of yohimbine on motor behaviour in rats, a dose of 2 mg/kg was used (Bowes *et al.*, 1992). A dose of 3 mg/kg was used in a study that investigated the yohimbine-induced anti-conflict effect in rats (Soderpalm *et al.*, 1995).

3.4.5.2 Receptor binding profile

The affinity values of yohimbine for a series of receptors and receptor subtypes are presented in Table 3-6.

Table 3-6: Receptor binding profile of yohimbine

Receptor		K _i -value (nM)	Tissue	Reference
5-HT	5-HT _{1A}	125.89	Rat hippocampus	(Millan <i>et al.</i> , 2000)
α-ARs	α ₁	251.18	Rat frontal cortex	(Millan <i>et al.</i> , 2000)
		230.00	Rat brain	(Doxey <i>et al.</i> , 1984)
	α ₂	2.67	CHO-K1 cells	(Bodenstein <i>et al.</i> , 2005)
		8.67	CHO-K1 cells	(Brink <i>et al.</i> , 2000)
		14.3	Rat brain	(Boyajian & Leslie, 1987)
		31.62	Rat cortex	(Millan <i>et al.</i> , 2000)
Dopamine D ₂		501.18	Rat striatum	(Millan <i>et al.</i> , 2000)

Similar to idazoxan and mirtazapine, the most probable site of antidepressant action of yohimbine is the antagonism of α_2 -ARs (Cappiello *et al.*, 1995). This is reflected in the high affinity of yohimbine for this receptor, and the relatively low affinity of this drug for other receptor subtypes that may play a role in alleviating the symptoms of depression.

3.4.5.3 Dose selection

A moderate dose of yohimbine should produce effects which are mainly α_2 -AR-mediated, and a dose of 3 mg/kg/day was ultimately selected.

3.5 EXPERIMENTAL PROTOCOLS

3.5.1 Drug administration

The animals were weighed each morning, the respective dosages calculated and drug solutions prepared. No drug (control), fluoxetine (20 mg/kg/day), mirtazapine (15 mg/kg/day), idazoxan (3 mg/kg/day) and yohimbine (3 mg/kg/day), or drug combinations of the aforementioned, were dissolved in a 0.9% saline solution containing 3% v/v Tween 80. All drugs and drug combinations were administered intra-peritoneally (i.p.) in a volume of 0.5 ml.

3.5.2 The rat Forced Swim Test (FST)

3.5.2.1 Validation of the rat FST in our laboratory

First of all, the rat FST had to be validated under the conditions in our laboratory. Three groups of rats were used in this experiment, groups C1, T1 and C4 in Table 3-1. Groups C1 and C4 were both treated with vehicle for 7 days, but group C1 was exposed to 15 minutes of swimming on the penultimate day of the treatment period (to induce behavioural despair), whereas group C4 had no such previous experience. On the other hand, group T1 was treated with fluoxetine (20 mg/kg/day) for 7 days, and was exposed to 15 minutes of swimming 24 hours prior to the actual test.

To demonstrate that the 15 minute period of forced swimming 24 hours prior to the assessment of behaviour can induce a state of behavioural despair in the animals, the immobility times of groups C1 and C4 were compared (see Figure 4-1). This component of the validation process would be perceived as successful if group C1 (pre-exposed to forced swimming) presented with significant more immobility compared to group C4 (no pre-exposure to forced swimming).

The second component of the validation process was aimed at demonstrating that antidepressant treatment (i.e. fluoxetine 20 mg/kg/day) can inhibit the development of behavioural despair. This part of the validation experiment would be successful if group T1 (fluoxetine treated) presented with significantly less immobility compared to group C1 (vehicle treated) (see Figure 4-2).

When both parts of the validation process proved to be successful, we continued to the next stage of the study.

3.5.2.2 Detection of antidepressant-like responses

3.5.2.2.1 Induction of behavioural despair

On the penultimate day of the specific treatment period (24 hours prior to the assessment of behaviour), the group of rats to be tested was placed in the room where the FST was to take place and left for 60 minutes to habituate. After the habituation period the rats were placed in the cylinders containing 18 cm clean water (at 25°C) for 15 minutes. The cylinders were separated by opaque screens and the water was deep enough to keep the rats from touching the bottom of the cylinder with their hind paws. After this period the rats were placed and kept in heated cages to dry for 15 minutes before returning them to their home cages.

3.5.2.2.2 Recording of behaviour

On the last day of the specific treatment period, the group of rats to be tested was allowed to habituate in the test room for 60 minutes. After habituation, the animals were replaced in the cylinders containing 18 cm of clean water (at 25°C) for 5 minutes. During this period, the behaviour of the rats was recorded with a video camera. After the test, the animals were placed into heated cages for 15 minutes to dry, before they were taken to the dissection room to be decapitated.

3.5.2.2.3 Scoring technique

The scoring of behaviour was done by a person blind to the treatment group that was being scored.

Three specific behavioural components were distinguished and measured from the videotapes (see Figure 2-9):

- Immobility is defined when no additional activity is observed other than that required to keep the rat's head above the water,
- Climbing behaviour (also known as thrashing) is defined as upward directed movements of the forepaws along the side of the cylinder; and
- Swimming behaviour is defined as swimming movements (usually horizontal) throughout the cylinder.

Each rat was scored separately while the scorer was blind to the treatment of the rats. The behavioural components were identified and measured in terms of the amount of time (in seconds) the specific behavioural component was observed, for a total period of 5 minutes (the combined time of the three components added up to 300 seconds).

3.5.3 Decapitation and dissection

When the FST was completed, the rats were decapitated and their brains removed. The hippocampus and frontal cortex regions were rapidly dissected on ice and snap-frozen. The tissue was stored at -86°C for subsequent radio-ligand binding studies.

3.5.4 Measurement of locomotor activity

On the last day and after the final treatment, the rats that were assigned to the assessment of locomotor activity were placed in the locomotor measurement cages for 20 minutes to habituate. After the habituation period the locomotor activity of the animals was measured for 10 minutes

and digitally recorded. The parameter that was used to define locomotor activity was horizontal activity (expressed in the number of movements per 10 minutes).

3.5.5 Radio-ligand saturation binding studies

3.5.5.1 Preparation of membrane suspensions from brain tissue

The objective of this experiment was to prepare, separate and purify membrane proteins from rat brain tissue for the purpose of determining receptor concentration by using appropriate radio-ligand saturation binding studies.

- The following buffers were used for the membrane preparations:

Table 3-7: Buffers used for radio-ligand saturation binding assays

	β-ARs	5-HT_{1A}-receptors
Wash buffer	50 mM Tris HCl	50 mM Tris HCl 2.5 mM MgCl ₂ 2 mM EDTA
Assay buffer	50 mM Tris HCl	50 mM Tris HCl 2.5 mM MgCl ₂ 2 mM EDTA 0.001% Ascorbic acid

Note: The pH of all buffers was adjusted to 7.4 with NaOH.

- The frontal cortex (for β-ARs) and hippocampus (for 5-HT_{1A} receptors) brain regions were removed from the -86°C freezer and thawed on ice (five brains were pooled for one experiment).
- A beaker containing 25 ml of ice-cold wash buffer was weighed, the tissue added and the beaker weighed again to calculate the wet weight of the tissue.
- The tissue was homogenised with a Polytron homogenizer on setting 7 for 10 seconds.
- The homogenate was centrifuged in an ultra-centrifuge at 48,000 × g (4°C) for 10 minutes, and the supernatant fluid decanted.

- The pellet was resuspended in 25 ml of ice-cold wash buffer, and the homogenising and centrifugation repeated, and the supernatant decanted.
- The final pellet was resuspended in an appropriate volume ice-cold assay buffer to give a concentration of 16 mg wet weight per ml. The resuspension of the pellet was done using a Polytron homogeniser on setting 7 for 10 seconds.
- The membrane suspension was kept on ice until use.

3.5.5.2 Measurement of protein concentration: The Bradford method (Bradford, 1976)

The objective of this experiment was to quantify the protein concentration in the prepared membrane suspension in order to prepare brain tissue suspensions with an appropriate protein concentration for radio-ligand saturation binding studies.

- The Bradford reagent was gently shaken in the bottle; 5 ml withdrawn and allowed to reach room temperature in a dark environment.
- Protein standards were prepared by dissolving 2 mg bovine serum albumin (BSA) in 1 ml double-distilled water (to produce a 2 mg/ml solution) and then making a series of 100 μ l dilutions as indicated in Table 3-8:

Table 3-8: Preparation of protein standards

Protein concentration	Dilution procedure	
	Volume of 2 mg/ml BSA	Volume of 50 mM Tris buffer
0 mg/ml	0 μ l	100 μ l
0.1 mg/ml	5 μ l	95 μ l
0.4 mg/ml	20 μ l	80 μ l
0.7 mg/ml	35 μ l	65 μ l
1.0 mg/ml	50 μ l	50 μ l
1.4 mg/ml	70 μ l	30 μ l

- 2 $\times$ 5 μ l of each protein standard dilution, as well as 2 $\times$ 5 μ l of the membrane suspension were added to separate wells of a 96-well plate, i.e. all in duplicate.
- 250 μ l of Bradford reagent was added to each well, and immediately shaken on the mixing facility of the plate reader for 30 seconds. The plate was incubated for 15 minutes at room temperature.

- The absorbance in each well was determined in a 96-well plate reader using a 560 nm filter. The protein concentration of the membrane suspension was calculated using the mathematical equation obtained when net absorbance was plotted against the protein concentration of the standards.
- The membrane suspension was diluted with ice-cold assay buffer to yield a protein concentration of 500 µg/ml.

3.5.5.3 Measurement of β -AR density

The aim of this experiment was to determine the concentration of β -ARs ($B_{\max}$) in the frontal cortex region of the rats, by using membrane suspensions (prepared as described above). An eight point concentration series that ranged from 0.5 nM to 25 nM of [^{3} H]-propranolol ("hot propranolol") was used to define total binding, while the same series, but also containing 60 µM propranolol ("cold propranolol"), was used to define non-specific binding. Specific binding was determined by subtracting non-specific binding from total binding, which was then plotted to calculate $B_{\max}$.

- Sigmacote® was used to coat 8 polypropylene test tubes with silica atoms to eliminate adsorption of the radio-ligand to the tube (in early experiments, we detected that adsorption of the ligand to the untreated tubes was significant). These tubes were used to prepare the radio-ligand concentration series.
- Eight dilutions of [^{3} H]-propranolol were prepared in assay buffer to produce final assay concentrations of: 0, 0.375, 0.75, 1.5, 3, 6, 12 and 25 nM.
- Another 32 polypropylene test tubes were placed on ice (8 tubes, in duplicate, for measurement of total binding; 8 tubes, in duplicate, for measurement of non-specific binding), and appropriately marked. To the 16 tubes for measurement of total binding, 960 µl of membrane suspension was added, as well as 20 µl of assay buffer, whereas to the 16 tubes for measurement of non-specific binding 960 µl of membrane suspension was added, as well as 20 µl of a 3 mM propranolol solution. The final assay volume in the binding study was 1 ml, therefore each tube contained a total of 500 µg protein.
- 20 µl of each radio-ligand concentration was added to the 4 corresponding test tubes (2 tubes for measurement of total binding, and 2 tubes for measurement of non-specific binding). The total assay volume for all tubes added up to 1 ml.
- Each tube was vortexed and placed in a shaking water bath for 15 minutes at 25°C for the binding of the ligand to the receptors to reach equilibrium.

- The contents of the tubes were transferred to Whatman GF/B filters on the manifold and washed twice with 5 ml ice-cold assay buffer.
- The filters were placed into scintillation bottles, 3 ml scintillation cocktail added, and capped.
- The bottles were left to stand overnight, whereafter they were gently shaken and placed in a Tri-Carb TR liquid scintillation counter to be counted.

3.5.5.4 Measurement of 5-HT_{1A} receptor density

The aim of this experiment was to determine the concentration of 5-HT_{1A} receptors (B_{max}) in the hippocampus region of the rat brains, by using membrane suspensions (prepared as described above). An eight point concentration series that ranged from 0.9 nM to 12 nM of [³H]-8-OH-DPAT was used to define total binding, while the same series, but also containing 20 μ M serotonin, was used to define non-specific binding. Specific binding was determined by subtracting non-specific binding from total binding, which was then plotted to calculate B_{max} .

- Sigmacote® was used to coat 8 polypropylene test tubes with silica atoms to eliminate adsorption of the radio-ligand to the test tubes. These tubes were used to prepare the radio-ligand concentration series.
- Eight dilutions of [³H]-8-OH-DPAT were prepared in assay buffer to produce final assay concentrations of: 0.09, 0.19, 0.4, 0.8, 1.5, 3, 6 and 12 nM.
- Another 16 polypropylene test tubes were placed on ice (8 tubes for measurement of total binding and 8 tubes for measurement of non-specific binding), and appropriately marked. To the 8 tubes for measurement of total binding, 400 μ l of membrane suspension was added, as well as 50 μ l of assay buffer, and to the 8 tubes for measurement of non-specific binding 400 μ l of membrane suspension was added, as well as 50 μ l of a 200 μ M serotonin solution (containing 0.02% ascorbic acid). The final assay volume in the binding study was 500 μ l. Therefore, each tube contained a total of 250 μ g protein.
- 50 μ l of each radio-ligand concentration was added to the two corresponding test tubes (One tube for measurement of total binding and one tube for measurement of non-specific binding).
- Each tube was vortexed and placed in a shaking water bath for 60 minutes at 25°C for the binding of the ligand to the receptors to reach equilibrium.
- The contents of each tube were transferred to a Whatman GF/B filter on the manifold and washed twice with 5 ml ice-cold assay buffer.

- The filters were placed in scintillation bottles, 3 ml scintillation cocktail added, and capped.
- The bottles were left to stand overnight, whereafter they were gently shaken and placed in the Tri-Carb TR liquid scintillation counter to be counted.

3.5.5.5 Calculations

All data were plotted, and raw $B_{\max}$ and K_i values determined using GraphPad Prism[®] (Version 4.02). The raw $B_{\max}$ values derived from the plotted graphs are in terms of cpm/250 μ g protein (since each tube contained 500 μ l of a protein concentration of 500 μ g/ml). The standard curve was also plotted for each experiment (10 μ l for [³H]-8-OH-DPAT binding, and 20 μ l for [³H]-propranolol binding, of each ligand concentration was added to a Whatman GF/B filter, 3 ml scintillation cocktail added, capped, and counted), and the slope was calculated in terms of cpm/nM. The slope was converted to yield a value expressed in terms of cpm/mol ligand, and this value was used to convert the raw $B_{\max}$ value to units of mol receptors/mg protein.

Example:

This is an example of the calculations for a single saturation binding study of [³H]-8-OH-DPAT binding to 5-HT_{1A} receptors. Figure 3-6 illustrates the data obtained from one such experiment.

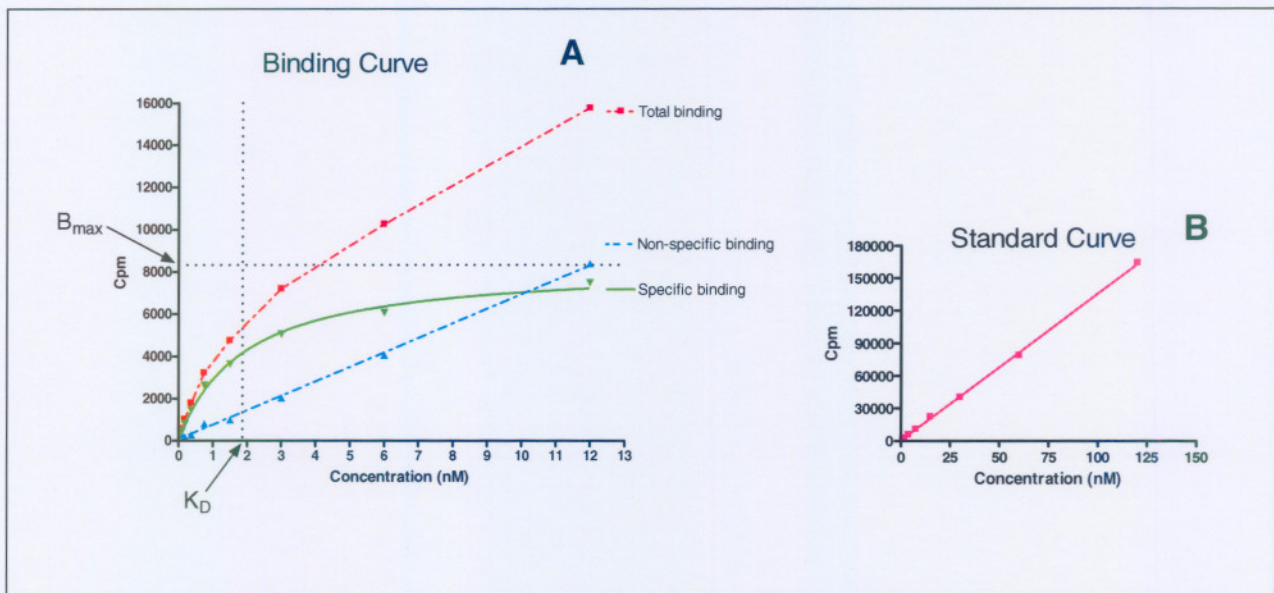


Figure 3-6: Example of results obtained with a single saturation binding study of [³H]-8-OH-DPAT to 5-HT_{1A} receptors. The (A) binding curve depicts total binding, non-specific binding (defined with 10 μ M serotonin), and specific binding (calculated by subtracting non-specific binding from total binding). The raw values of $B_{\max}$ and K_D are indicated on the graph. The (B) standard curve was obtained by measuring the total radioactivity of 10 μ l of each radio-ligand concentration.

The computer software subtracts the curve of non-specific binding from the curve of total binding, and subsequently fits and plots the curve of specific binding using non-linear regression (in this example $r^2 = 0.9951$). In the example, $B_{\max}$ is calculated as 8326 ± 261.9 cpm/250 μ g protein (Figure 3-6A) and the K_D value as 1.9 ± 0.17 nM. The K_D value compares well with published affinity values of [^{3} H]-8-OH-DPAT for 5-HT_{1A} receptors (Gozlan *et al.*, 1988; Boess & Martin, 1994; Schoeffter & Hoyer, 1988). In addition, the software plots the standard curve obtained from the total radioactivity of the radio-ligand concentrations, using linear regression ($r^2 = 0.9994$), and calculates the slope of the straight line to be 1359 ± 13.6 cpm/nM (Figure 3-6B).

The slope of the standard curve is converted to units of cpm/mol by using the volume of the radio-ligand added to the filter (in this case, 10 μ l). As a result, the value of the slope becomes 1.359×10^{17} cpm/mol. The raw $B_{\max}$ (in terms of cpm/250 μ g protein) is divided by the slope of the standard curve to yield a value of 6.127×10^{-14} mol/250 μ g protein. This value is then converted to represent a final value in terms of mol/mg protein. This is done by dividing the $B_{\max}$ value by the total amount of protein in the final assay volume (0.25 mg, since a total assay volume of 500 μ l was used with a protein concentration of 500 μ g/ml). The 5-HT_{1A} receptor concentration ($B_{\max}$), for this experiment, was subsequently calculated to be 2.451×10^{-13} mol/mg protein (245.1 fmol/mg protein).

3.6 DATA ANALYSIS

As mentioned in the study layouts above, data from all FST studies with rats were from 3 separate, comparable experiments, each with 5 rats. For behavioural data (i.e. FST) observations were made individually for each rat, while the tissue from indicated brain regions of 5 rats was pooled for membrane preparation for radio-ligand binding studies. Parallel locomotor control studies were routinely evaluated from 3 separate, comparable experiments, each with 2 rats (i.e. 2 additional rats added to each treatment group).

GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analysis of data. For comparison of two values, the nonparametric Student's *t* test (two-tailed) was implemented. For multiple comparisons, the one-way ANOVA was performed followed by either the Dunnett's post-test (for comparing experimental groups to the control) or the Tukey-Kramer post-test (for comparing experimental groups to each other). For all reported statistical probability values, $p < 0.05$ was regarded as statistically significant.

Results and discussion

Chapter

This chapter will present and discuss all experimental results obtained in the current study, including the validation of the forced swim test (FST) for rats in our laboratory (development of behavioural despair), determination of the time-dependency for the inhibition of development of behavioural despair by the conventional antidepressant fluoxetine, and lastly the effect of mirtazapine and other α_2 -AR antagonists or combinations thereof with fluoxetine on the development of behavioural despair. By measuring behavioural despair, the results describe the antidepressant-like properties of the drugs.

Since the standard FST, an animal model of depression, has not been performed on rats in the current laboratory setup and conditions, it was important to validate and optimise the procedure. This was done by illustrating that behavioural despair, a depression-like state in rats, can be induced. In addition it was important to show that the development of behavioural despair can be inhibited by antidepressant treatments.

After validation and optimisation (Pilot Study 1), the time-dependency of the onset of an antidepressant-like response with the conventional antidepressant and prototype SSRI, fluoxetine, was determined in Pilot Study 2 of the study. The aim was to determine the shortest treatment period required for an antidepressant-like response. The results depicted in this chapter show the effect of a 3, 7 and 11-day treatment of rats with fluoxetine on the development of behavioural despair compared to vehicle treated (control) rats, as well as changes in β -AR density in the frontal cortex (since β -AR downregulation is commonly accepted as being indicative of antidepressant action).

When the time-dependency of the development of an antidepressant-like response by fluoxetine was established, the primary objectives of the study were investigated in the experimental phase. Treatment periods with the test drugs and combinations thereof were chosen as the shortest period that yielded response with fluoxetine, and the shortest period that did not yield response. Rats were treated with vehicle (control), fluoxetine, a series of α_2 -AR antagonists (i.e. mirtazapine, yohimbine and idazoxan) or a combination of fluoxetine with each of the α_2 -AR antagonists for both treatment periods. The α_2 -AR antagonists were also specifically chosen to include an inverse agonist (yohimbine) and a neutral antagonist (idazoxan) at α_2 -ARs. This was to investigate whether an α_2 -AR antagonist can hasten the onset of action of a conventional antidepressant (such as fluoxetine), and also to investigate whether an inverse agonist at α_2 -

ARs is superior to a neutral antagonist in this regard. We defined the onset of an antidepressant-like response as behavioural changes in the FST, as well as β -AR downregulation in the frontal cortex. We also measured 5-HT_{1A} receptor density in the hippocampus of the animals, since these receptors are thought to play an important role in the onset of action of antidepressants.

4.1 PILOT STUDY 1: VALIDATION OF THE RAT FORCED SWIM TEST (FST)

4.1.1 Development of behavioural despair

To establish whether 15 minutes of pre-exposure to forced swimming 24 hours prior to the 5-minute testing period produces a state of behavioural despair in the rats, two groups of 5 rats each were treated with vehicle for 7 days. One of the groups was exposed to 15 minutes of swimming 24 hours prior to recording of behaviour, while the control group had no previous such experience. The experiment was repeated twice (i.e. 3 trials in total), so that 15 rats were used per treatment group ($n = 15$). Figure 4-1 illustrates the effect of prior exposure to swimming on the display of immobility during the 5-minute test period.

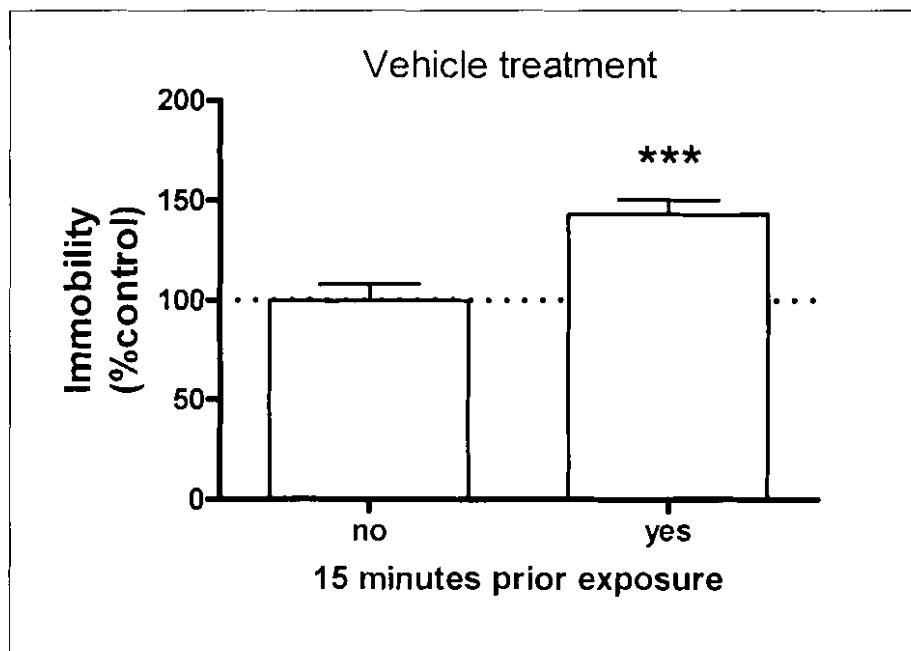


Figure 4-1: Measurement of immobility (behavioural despair) during a 5-minute trial, 24 hours after 0 or 15 minutes of pre-exposure to forced swimming. All groups were treated with vehicle for 7 days. Data are from 3 independent experiments of 5 rats each ($n = 15$) and are expressed as percentage of control, calculated as mean $\pm$ standard error of the mean, where *** represents $p < 0.001$.

It can be seen in Figure 4-1 that a 15-minute prior exposure to forced swimming induces significant behavioural despair, with immobility increasing from $100 \pm 8.2\%$ to $143 \pm 7.1\%$ ($p = 0.0005$). It is therefore clear that the current experimental conditions and setup are sufficient to induce behavioural despair in rats, rendering them suitable for further experimentation.

Secondly it was investigated whether development of behavioural despair can be inhibited by fluoxetine. Another group of rats were treated in parallel for 7 days with fluoxetine (20 mg/kg/day), and the results are illustrated in Figure 4-2.

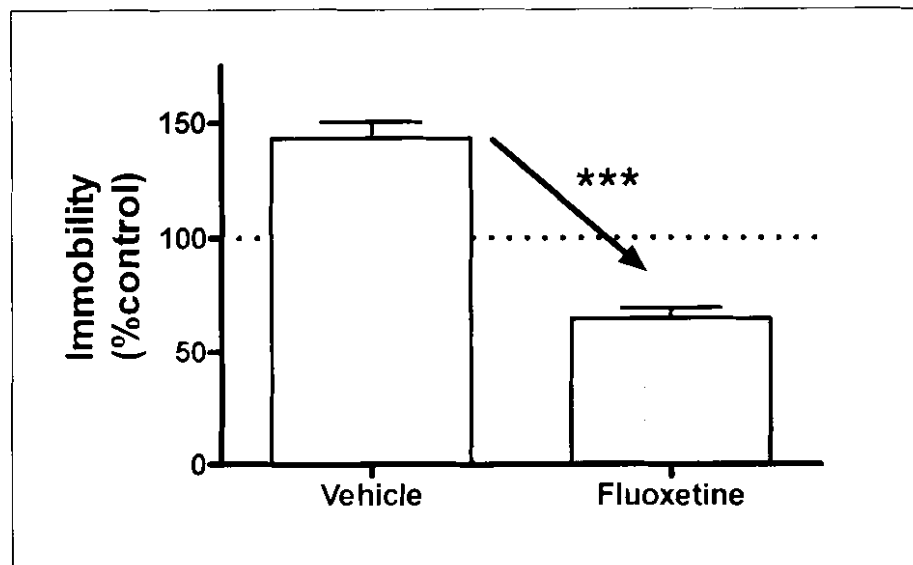


Figure 4-2: Measurement of immobility (behavioural despair) during a 5-minute trial following 7 day vehicle or fluoxetine (20 mg/kg/day) administration. Data are from 3 independent experiments of 5 rats each ($n = 15$) and are expressed as percentage of control, calculated as mean $\pm$ standard error of the mean, where *** represents $p < 0.001$.

As depicted in Figure 4-2, treatment with fluoxetine for 7 days significantly reduces immobility from $143 \pm 7.1\%$ to $64 \pm 4.6\%$ ($p < 0.0001$) in the FST. Since reduced immobility is associated with the antidepressant-like properties of a drug, the data suggest that fluoxetine is able to exert its antidepressant action in the current model, under the experimental conditions and setup employed.

These data therefore confirm the validity of the FST under the current experimental conditions and setup in our laboratory for testing the onset of response of antidepressant treatments.

4.2 PILOT STUDY 2: Time-dependency of the antidepressant-like action of fluoxetine

4.2.1 The Forced Swim Test

To establish the time-dependency of the onset of the inhibition of behavioural despair (antidepressant-like behavioural effect) by fluoxetine in the FST, six groups consisting of 7 rats each were treated with either vehicle (control) or with fluoxetine (20 mg/kg/day) for 3, 7 or 11 days. Of each treatment group 5 rats were used for the FST and 2 rats were used for the assessment of locomotor activity. The experiment (including all treatment groups) was repeated twice (3 trials in total), so that 15 rats per treatment group were used for the FST ($n = 15$) and 6 rats per treatment group for assessment of locomotor activity ($n = 6$). Figure 4-3 illustrates the effects of fluoxetine (20 mg/kg/day) administered for 3, 7, or 11 days on behaviour in the FST and on locomotor activity.

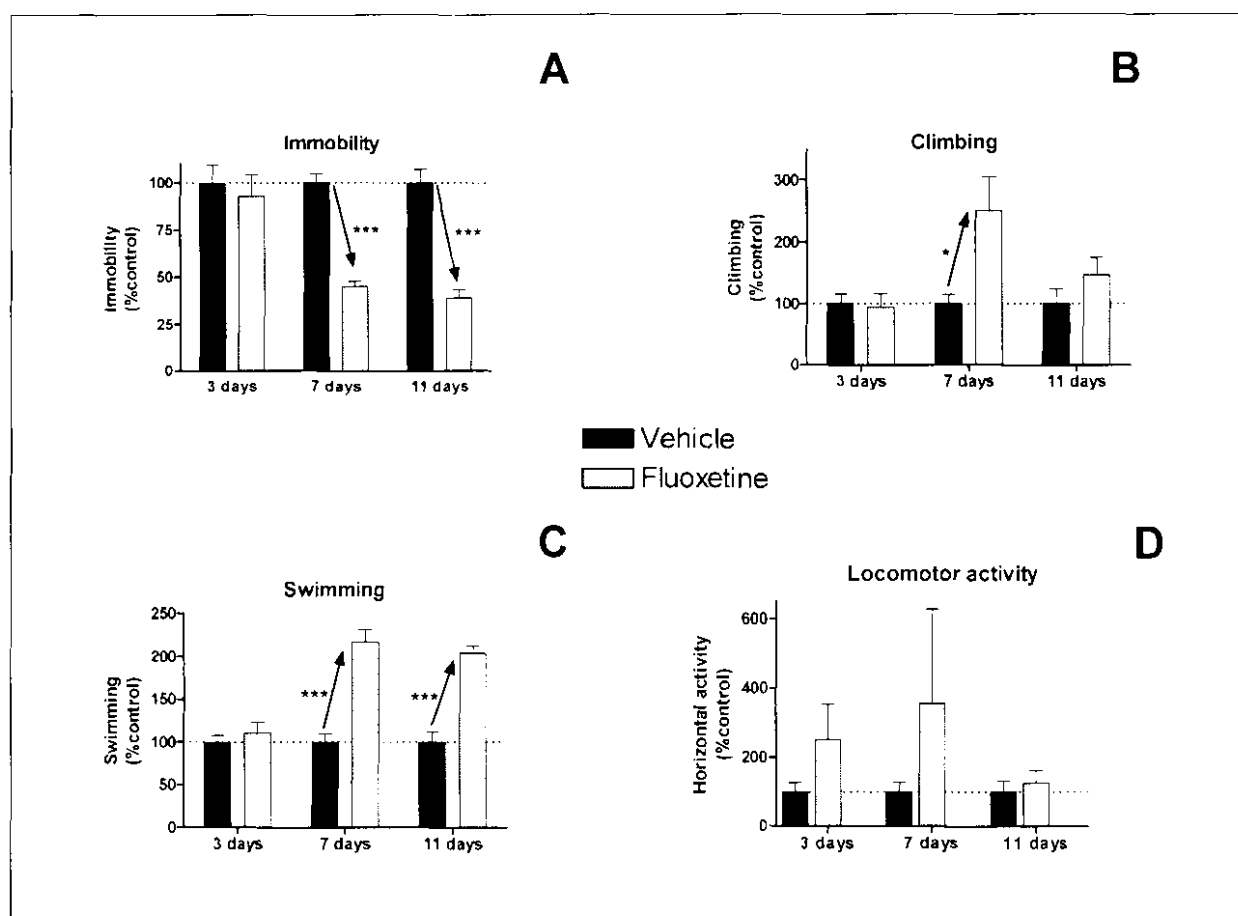


Figure 4-3: Behavioural effects in the FST and effects on locomotor activity produced by administration of vehicle or fluoxetine (20 mg/kg/day) for 3, 7 or 11 days. Parameters measured in the FST include (A) immobility, (B) climbing and (C) swimming. (D) Locomotor activity, measured as horizontal activity, was measured for all groups. Data in all graphs are from 3 independent experiments with data in graphs A, B and C from 5 rats per treatment group ($n = 15$) and data in graph D from 2 rats per treatment group ($n = 6$). All data are expressed as percentage of control, calculated as mean $\pm$ standard error of the mean, where * represents $p < 0.05$ and *** represents $p < 0.001$.

It can be seen in Figure 4-3 that, compared to control, fluoxetine significantly reduces immobility in the FST after 7 ($100 \pm 4.8\%$ versus $45 \pm 2.9\%$ with $p < 0.001$) and 11 days (100 ± 7.4 versus $39 \pm 4.4\%$ with $p < 0.0001$) but not after 3 days ($100 \pm 9.7\%$ versus $93 \pm 11.3\%$ with $p > 0.05$). In accord with fluoxetine being a serotonergic drug, swimming behaviour is significantly increased compared to control after 7 ($100 \pm 9.5\%$ versus $216 \pm 14.5\%$ with $p < 0.0001$) and 11 days ($100 \pm 11.8\%$ versus $203 \pm 8.5\%$ with $p < 0.0001$). Climbing behaviour is not affected, except after the 7-day treatment period, after which it is significantly increased compared to control ($100 \pm 14.0\%$ versus $249 \pm 55.2\%$ with $p = 0.0136$). Fluoxetine does not produce any antidepressant-like behavioural changes after 3 days of treatment, coinciding with the drug's inability to present with early onset of action. There are no significant alterations in locomotor activity, which indicates that any reduction of immobility in the FST is not a result of a primary decrease in locomotor activity.

These data stipulate that for an antidepressant to have a more rapid onset of antidepressant-like action in the current study, it would have to significantly inhibit the development of behavioural despair after a 3-day treatment period. Consequently, in the experimental phase, rats were treated with antidepressant treatments for 3 and 7 days to investigate whether any of the treatments could inhibit the development of behavioural despair after only 3 days of treatment.

Since almost all previous studies used the FST treated rats with 3 administrations 24 hours prior to the 5-minute testing period, their data cannot be directly compared to the data from this study, where the animals received only one administration each day. However, there is a recent study which demonstrated that fluoxetine (5 mg/kg/day) administration for 7 and 14 days, but not 1 day, reduced immobility and increased swimming behaviour in the FST (Vazquez-Palacios *et al.*, 2004).

Furthermore, the data in the current study are reliable and repeatable since each experiment consisted of 3 replicate trials spaced weeks apart.

4.2.2 β -AR concentration

The same rats that were used in the FST were also used for determination of β -AR concentration. After the FST was completed, the animals were decapitated and the frontal cortex removed for receptor binding experiments. Figure 4-4 illustrates the effect of fluoxetine (20 mg/kg/day) administered for 3, 7, or 11 days on β -AR density in the frontal cortex of the animals.

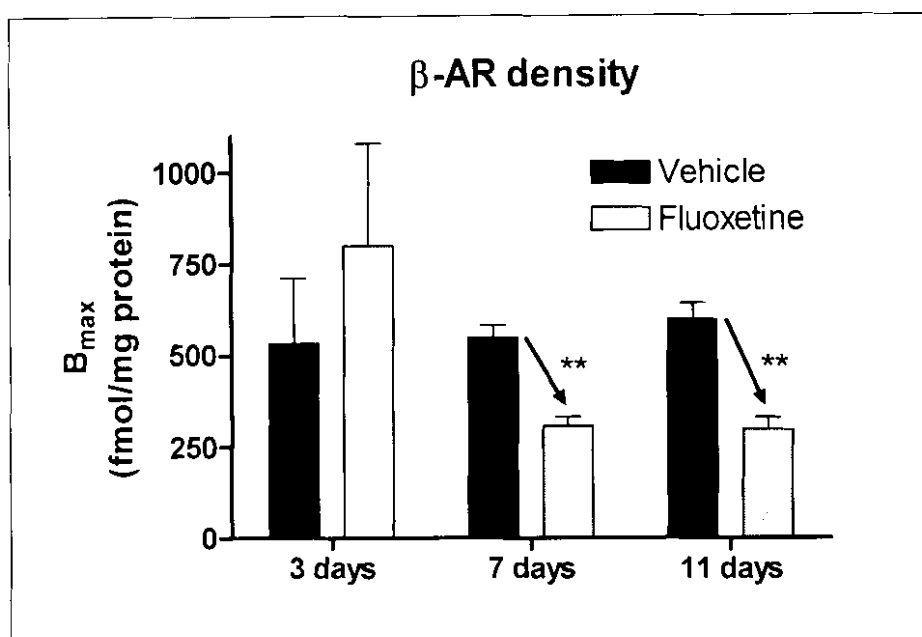


Figure 4-4: Effects on β -AR density in the frontal cortex produced by administration of vehicle or fluoxetine for 3, 7, or 11 days. Data are from 3 independent experiments ($n = 3$) and 5 frontal cortices from each treatment group were pooled for one experiment. β -AR density (B_{max}) is expressed in terms of fmol receptors per mg protein, and as the mean $\pm$ standard error of the mean where ** represents $p < 0.01$.

In Figure 4-4, and in agreement with the behavioural data in the Pilot Study 2, β -AR density is significantly reduced after 7-day (547 ± 33.2 fmol/mg versus 305 ± 26.2 fmol/mg with $p = 0.0046$) and 11-day (596 ± 44.1 fmol/mg versus 296 ± 33.1 fmol/mg with $p = 0.0055$) treatment periods with fluoxetine compared to control groups, but not after 3 days (532 ± 178.7 fmol/mg versus 800 ± 277.9 fmol/mg with $p = 0.5850$). These data support the behavioural data in this phase which specify that antidepressant-like effects will be recognised as early effects if they exist after 3 days of treatment with a specific drug or combination of drugs.

4.3 EXPERIMENTAL STUDY: α_2 -AR receptor antagonism and onset of antidepressant-like responses

4.3.1 The Forced Swim Test (FST)

In this phase of the study, a series of α_2 -AR antagonists were administered alone as well as in combination with fluoxetine. This was done in order to investigate whether the putative early onset of action of mirtazapine is related to its α_2 -lytic properties. This would be the case if α_2 -lytic drugs in general (including mirtazapine) showed an earlier onset of action, if such earlier action existed.

In addition to this, a second element was investigated in this study, which is whether the mode of antagonism has any effect on onset of action and/or the magnitude of an antidepressant-like response. To address this, a series of α_2 -lytic drugs was selected to include a neutral antagonist (idazoxan) as well as an inverse agonist (yohimbine) (Wade *et al.*, 2001). Recent data from our laboratory indicated that mirtazapine could act either as a neutral antagonist or as an inverse agonist at these receptors (Khoza, 2004). This selection enabled us to investigate whether inverse agonists at α_2 -ARs could act faster and/or more efficiently than neutral antagonists at these receptors, regarding their antidepressant response.

Based on the outcome of Pilot Study 2, animals were treated for 3 or 7 days in the Experimental Study. Two control groups consisting of 30 animals ($n = 30$) each were injected with vehicle for the respective time periods. Another 14 groups consisting of 15 animals each ($n = 15$) were injected with fluoxetine, mirtazapine, yohimbine or idazoxan alone, or fluoxetine combined with each of the other drugs for the respective period. In parallel with this experiment, another two groups consisting of 12 animals ($n = 12$) each received vehicle as a control, and two groups consisting of 6 animals ($n = 6$) each receiving antidepressant treatment were treated for the respective periods and were used for assessment of locomotor activity. See Table 3-2 for a detailed outline of the various treatment groups.

Figure 4-5 illustrates the behavioural effects in the FST and effects on locomotor activity produced by treatment with antidepressants for 3 or 7 days.

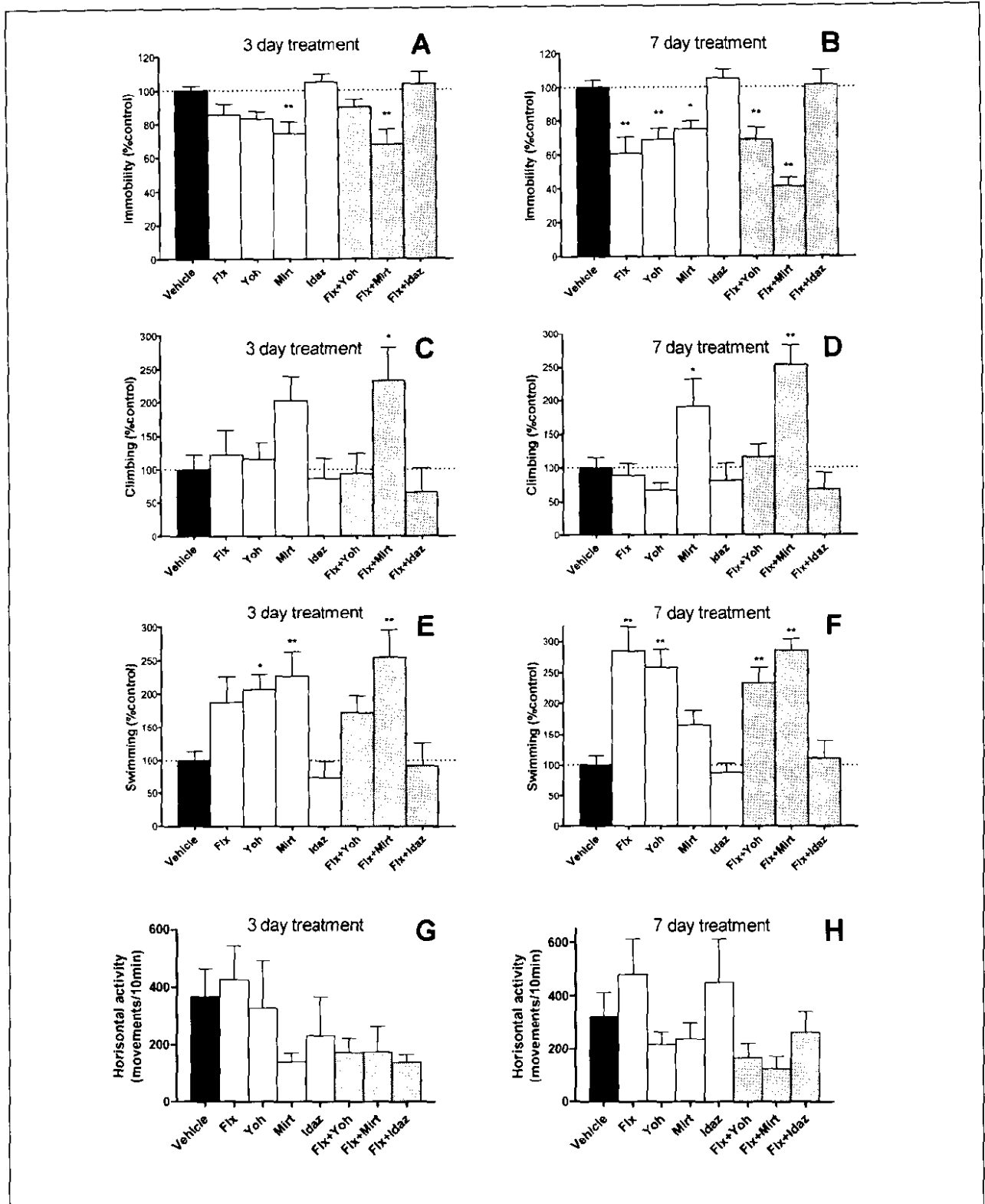


Figure 4-5: Behavioural effects in the FST and effects on locomotor activity produced by administration of antidepressants. Immobility after (A) 3 days and (B) 7 days, climbing behaviour after (C) 3 days and (D) 7 days, and swimming behaviour after (E) 3 days and (F) 7 days were measured separately for 5 rats in 3 independent experiments ($n = 15$) except the controls which were measured separately for 5 rats in 6 independent experiments ($n = 30$) where * represents $p < 0.05$ and ** represents $p < 0.01$. Locomotor activity after (G) 3 days and (H) 7 days of treatment was measured separately for 2 rats in 3 individual experiments ($n = 6$) except for the controls which were measured for 2 rats in 6 independent experiments and measured in terms of horizontal activity. All data are expressed as percentages of the respective controls and calculated as the mean $\pm$ standard error of the mean.

Consistent with data from Pilot Study 2, fluoxetine alone produces a significant reduction in immobility ($100 \pm 4.1\%$ versus $60 \pm 9.8\%$ with $p < 0.01$) and significantly increases swimming behaviour ($100 \pm 14.8\%$ versus $284 \pm 39.4\%$ with $p < 0.01$) after 7 days of treatment compared to control. Fluoxetine does not produce any significant change in climbing behaviour after any treatment period, which is consistent with the drug's ability to selectively inhibit the reuptake of serotonin (Cryan *et al.*, 2002). The α_2 -AR inverse agonist yohimbine significantly reduces immobility ($100 \pm 4.1\%$ versus $69 \pm 6.4\%$ with $p < 0.01$) and increases swimming behaviour ($100 \pm 14.8\%$ versus $258 \pm 28.8\%$ with $p < 0.01$) after a 7-day treatment period compared to control, but has no significant anti-immobility effect after 3 days ($100 \pm 2.7\%$ versus $83 \pm 4.1\%$ with $p > 0.05$). It does however significantly increase swimming behaviour after 3 days ($100 \pm 13.3\%$ versus $206 \pm 22.14\%$ with $p < 0.05$) compared to control. The α_2 -AR neutral antagonist idazoxan does not produce any antidepressant-like effects per se in the FST after any treatment period, but it has previously been demonstrated that idazoxan is non-responsive in the FST when it is administered alone (Cervo *et al.*, 1990). On the other hand, mirtazapine significantly reduces immobility after as short a period as 3 days of treatment ($100 \pm 2.7\%$ versus $74 \pm 7.0\%$ with $p < 0.01$) compared to control. It also significantly increases swimming behaviour after this short treatment period ($100 \pm 13.3\%$ versus $226 \pm 37.6\%$ with $p < 0.01$) compared to control. After 7 days immobility is still significantly reduced ($100 \pm 4.1\%$ versus $75 \pm 4.8\%$ with $p < 0.05$), but swimming behaviour is no longer significantly elevated ($100 \pm 14.8\%$ versus $165 \pm 22.4\%$ with $p > 0.05$) compared to control. This is caused by a significant increase in climbing behaviour ($100 \pm 15.1\%$ versus $190 \pm 41.7\%$ with $p < 0.05$) which reduces the total available time for swimming, since immobility is practically constant for both treatment periods. Yohimbine combined with fluoxetine produces significant anti-immobility effects only after 7 days of treatment ($100 \pm 4.1\%$ versus $69 \pm 6.9\%$ with $p < 0.01$), produces no change in swimming behaviour after any treatment period, and induces a significant increase in swimming behaviour after 7 days ($100 \pm 14.8\%$ versus $232 \pm 25.7\%$ with $p < 0.01$) compared to control. Mirtazapine combined with fluoxetine significantly reduces immobility after 3 (100 ± 2.7 versus 68 ± 8.9 with $p < 0.01$) and 7 days ($100 \pm 4.1\%$ versus $41 \pm 4.9\%$ with $p < 0.01$), significantly increases climbing behaviour after 3 ($100 \pm 21.7\%$ versus $233 \pm 48.9\%$ with $p < 0.05$) and 7 days ($100 \pm 15.1\%$ versus $253 \pm 28.7\%$ with $p < 0.01$), and significantly increases swimming behaviour after 3 days ($100 \pm 13.3\%$ versus $255 \pm 39.7\%$ with $p < 0.01$) and 7 days ($100 \pm 14.8\%$ versus $285 \pm 18.3\%$ with $p < 0.01$) compared to control. Idazoxan combined with fluoxetine produces no significant change in any behavioural component after any treatment period.

Neither yohimbine nor idazoxan significantly increases climbing behaviour after any treatment period. This is surprising since both these drugs antagonise α_2 -autoreceptors and reportedly increase synaptic availability of *L*-NE (Thomas *et al.*, 1994; Broderick, 1997). It can only be

speculated that the failure of these drugs to increase climbing may be due to the antagonism of postsynaptic α_2 -ARs, and by the reported inability of idazoxan to give any effects per se in the FST (Cervo *et al.*, 1990). In contrast, mirtazapine increases climbing behaviour after 7 days compared to control. This suggests an increase in *I*-NE transmission, most probably caused by antagonism of α_2 -autoreceptors on *I*-NE terminals. Mirtazapine combined with fluoxetine increases climbing behaviour after 3 and 7 days compared to control. There is currently no evidence supporting the stimulation of the *I*-NE system by increased 5-HT tone in the hippocampus, thus the early increase in climbing behaviour induced by the mirtazapine-fluoxetine combination is most probably due to the sole effect of mirtazapine on *I*-NE neurotransmission. A possible explanation for the ability of the FST to distinguish between mirtazapine and the other α_2 -lytic drugs employed here regarding climbing behaviour, is the reported capability of mirtazapine to bind to, and stimulate postsynaptic α_1 -ARs (de Boer, 1996), which would simulate an increased *I*-NE tone.

Fluoxetine causes no significant alteration in immobility or swimming behaviour after 3 days, which is most probably due to stimulation of presynaptic somatodendritic 5-HT_{1A} autoreceptors caused by the acute increase in 5-HT availability (Blier, 2003). This is consistent with the observation that the antagonism of presynaptic 5-HT_{1A} autoreceptors shortens the onset time of SSRIs (Bordet *et al.*, 1998). However, after 7 days of treatment immobility is decreased and swimming is increased by fluoxetine compared to control. It is suggested that the delay in onset of action of fluoxetine is dependent on the desensitisation of presynaptic 5-HT_{1A} autoreceptors, which has been reported to occur with this drug (Newman *et al.*, 2004). Yohimbine increases swimming behaviour after 3 days of treatment compared to control. This observation should however be questioned since no anti-immobility effects are present with yohimbine after 3 days of treatment, and since a yohimbine-fluoxetine combination does not produce a significant change in swimming behaviour after 3 days compared to control. After 7 days of treatment, yohimbine decreases immobility and increases swimming behaviour compared to control. This could be explained by the drug's reported ability to cause an increase in 5-HT levels via indirect stimulation of α_1 -heteroreceptors on serotonergic cell bodies in the dorsal raphe, secondary to an increased availability of *I*-NE (Broderick, 1997). These effects are unaltered when yohimbine is combined with fluoxetine. The observation that an idazoxan-fluoxetine combination fails to alter immobility or swimming behaviour will be further discussed in § 4.3.1.1.2.

The most prominent result from this experiment is the ability of mirtazapine alone, as well as in combination with fluoxetine to decrease immobility and increase swimming behaviour after as short a period as 3 days of treatment. This early effect of mirtazapine coincides with clinical evidence suggesting a putative earlier onset of antidepressant action for this drug (Wheatley *et al.*, 1998; Leinonen *et al.*, 1999; Benkert *et al.*, 2006). Mirtazapine antagonises α_2 -autoreceptors located on the cell bodies of *I*-NE neurons in the locus coeruleus, leading to

increased cell firing, which includes projections to the dorsal raphe. This, in turn, causes increased stimulation of α_1 -heteroreceptors on serotonergic cell bodies, leading to an increased serotonergic tone. Both these systems project to the hippocampus, specifically leading to enhanced stimulation of pyramidal CA₃ neurons. The increase in serotonergic neurotransmission would normally be dampened by the stimulation of α_2 -heteroreceptors on serotonergic terminals caused by the increased release of *I*-NE by mirtazapine. However, mirtazapine also blocks these receptors permitting the increase in 5-HT release. The resulting effect would be enhanced stimulation of postsynaptic 5-HT_{1A} receptors and α_1 -ARs located on pyramidal CA₃ neurons in the hippocampus (see § 2.2.1.3.3).

Since yohimbine and idazoxan also antagonise α_2 -ARs they should theoretically exhibit the same mechanism presented here. However, no early antidepressant-like response is evident with either of these drugs in the current study. It seems that α_2 -AR antagonism is not the major mechanism involved in producing an early antidepressant-like response. In fact, it appears that mirtazapine possesses some properties that render it superior to these two drugs regarding onset of antidepressant-like response. These may include postsynaptic effects like antagonism of 5-HT₂ and 5-HT₃ receptors, and weak, but not negligible direct 5-HT_{1A} agonistic properties (de Boer, 1996). This profile would most probably lead to a more rapid effect in the FST, since several authors have suggested a functional link between 5-HT_{1A} and 5-HT₂ receptors in relation to depression (Deakin, 1988; Borsini, 1994; Berendsen, 1995), and, more significantly, it has been suggested that a compound, BIMT 17, which activates 5-HT_{1A} receptors and concurrently antagonises 5-HT₂ receptors, produces acute effects similar to those seen only after chronic antidepressant treatment (Borsini, 1994; Borsini *et al.*, 1997). In addition to this, there is some evidence suggesting behavioural antidepressant-like effects induced by 5-HT₃ receptor antagonists (Greenshaw, 1993; Redrobe & Bourin, 1997).

There is no significant alteration in locomotor activity for any treatment after any treatment period compared to control, suggesting that anti-immobility effects induced in the FST by antidepressant treatment are not secondary to a primary increase in locomotor activity.

4.3.1.1 Interactions of α_2 -AR antagonists with fluoxetine in the FST

In this section, some results were selected and extracted from Figure 4-5, to illustrate in more detail the effects of the different combinations of α_2 -lytic drugs with fluoxetine on behaviour in the FST. More specifically, this was to investigate more closely the mechanisms of interaction of the three α_2 -AR antagonists with a serotonin reuptake inhibitor, to give us an opportunity to speculate on putative synergistic mechanisms of antidepressant action, which could possibly be exploited in future augmentation strategies. Comparisons were made between all treatment groups, using a one-way ANOVA statistical analysis, followed by a Tukey-Kramer post-test.

4.3.1.1.1 Interaction of mirtazapine and fluoxetine in the FST

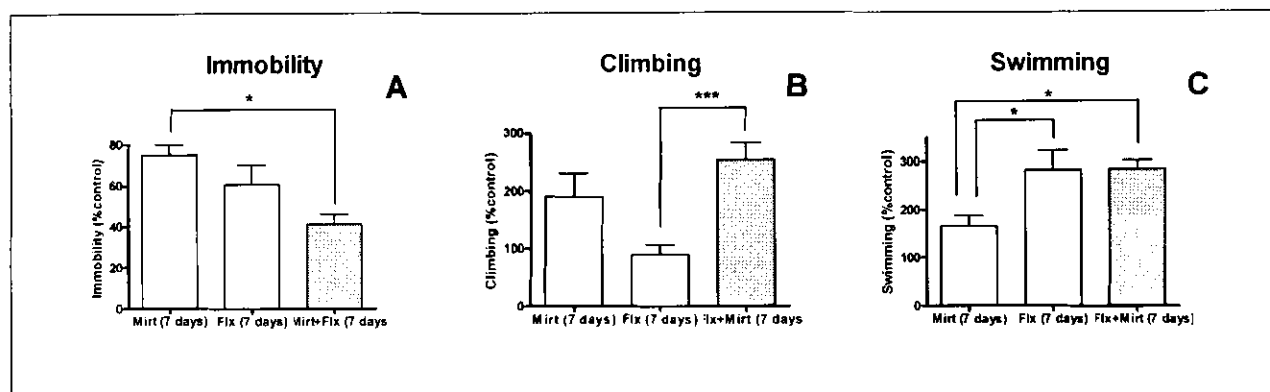


Figure 4-6: The interactions of mirtazapine and fluoxetine in the FST when administered for 7 days. Parameters measured in the FST include (A) immobility, (B) climbing, and (C) swimming. All data are from 3 independent experiments consisting of 5 rats each ($n = 15$) and are expressed as percentage of control and calculated as mean $\pm$ standard error of the mean, where * represents $p < 0.05$, and *** represents $p < 0.001$ (Tukey, see Figure 4-5).

Figure 4-6 depicts that a mirtazapine-fluoxetine combination treatment for 7 days significantly reduces immobility ($75 \pm 4.8\%$ versus $41 \pm 4.9\%$ with $p < 0.05$) and increases swimming behaviour ($165 \pm 22.4\%$ versus $285 \pm 18.3\%$ with $p < 0.05$) compared to mirtazapine administered alone for 7 days. However, the abovementioned effects may be attributed to the effects of fluoxetine alone, since no significant difference were observed between the effects elicited by a mirtazapine-fluoxetine combination and fluoxetine alone on immobility ($61 \pm 9.8\%$ versus $41 \pm 4.9\%$ with $p > 0.05$) and swimming (284 ± 39.4 versus $285 \pm 18.3\%$ with $p > 0.05$). Also, although a mirtazapine-fluoxetine combination increased climbing significantly compared to fluoxetine alone ($89 \pm 17.3\%$ versus $253 \pm 28.7\%$ with $p < 0.001$), the effect of the combination treatment did not differ significantly from the effect produced by mirtazapine alone ($190 \pm 41.7\%$ versus $253 \pm 28.7\%$ with $p > 0.05$).

Therefore, it can be speculated that mirtazapine and fluoxetine may have additive effects in the FST, but there is not conclusive evidence from this data that this is indeed the case.

4.3.1.1.2 Interaction of idazoxan and fluoxetine in the FST

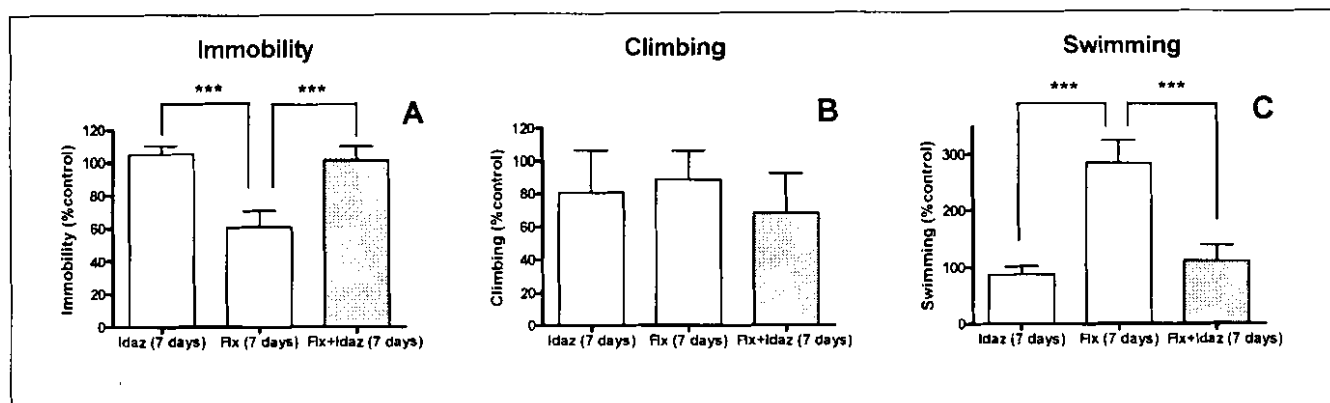


Figure 4-7: The interactions of idazoxan and fluoxetine in the FST when administered for 7 days. Parameters measured in the FST include (A) immobility, (B) climbing and (C) swimming. All data are from 3 independent experiments consisting of 5 rats each ($n = 15$) and are expressed as percentage of control and calculated as mean $\pm$ standard error of the mean, where *** represents $p < 0.001$ (Tukey, see Figure 4-5).

Figure 4-7 illustrates that idazoxan actually attenuates the antidepressant-like effects of fluoxetine in the FST when they are administered simultaneously for 7 days. Idazoxan significantly increases immobility ($61 \pm 9.8\%$ versus $101 \pm 8.5\%$ with $p < 0.001$) and decreases swimming behaviour ($284 \pm 39.4\%$ versus $111 \pm 28.4\%$ with $p < 0.001$) when it is combined with fluoxetine for 7 days, compared to fluoxetine administered alone for 7 days. This is an interesting observation since idazoxan antagonises α_2 -autoreceptors thereby increasing noradrenergic tone (Tao & Hjorth, 1992; de Boer *et al.*, 1996) and should indirectly (via α_1 -heteroreceptors) increase serotonergic tone, which would theoretically lead to increased antidepressant-like behaviour in the FST. There are, however, some explanations for this phenomenon. Firstly, it has been demonstrated that idazoxan is an agonist at 5-HT_{1A} autoreceptors, causing a decrease in 5-HT synthesis in the cortex and hippocampus regions of rats (Llado *et al.*, 1996). This would antagonise the serotonergic effects induced by fluoxetine in the FST, thereby decreasing swimming behaviour and increasing immobility. Secondly, it has been reported that idazoxan exhibits a 250-fold greater affinity for α_2 -autoreceptors than for α_2 -heteroreceptors on serotonergic terminals (Preziosi *et al.*, 1989). This would cause the idazoxan-induced increase in noradrenergic outflow (Tao & Hjorth, 1992; de Boer *et al.*, 1996) to negatively modulate the release of 5-HT, which would antagonise the serotonergic effects caused by fluoxetine in the FST, thereby decreasing swimming behaviour and increasing immobility.

4.3.1.1.3 Interaction of yohimbine and fluoxetine in the FST

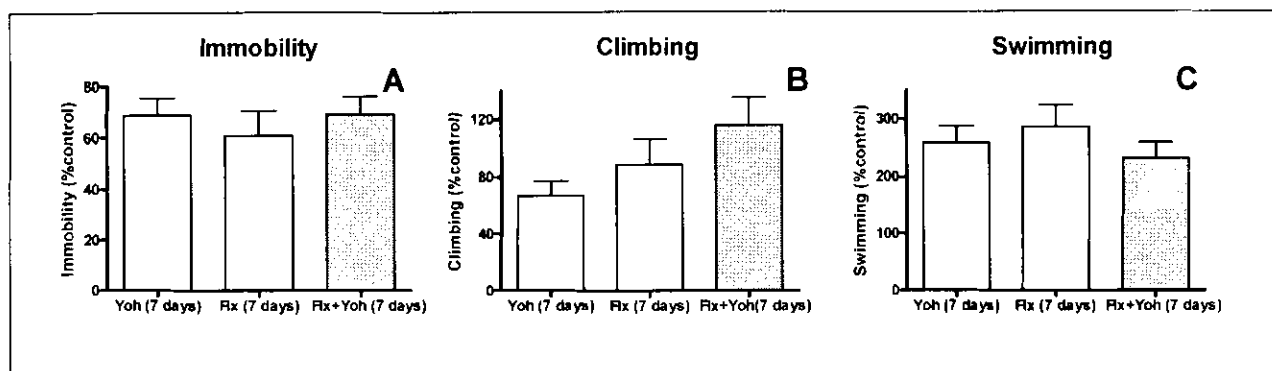


Figure 4-8: The interactions of yohimbine and fluoxetine in the FST when administered for 7 days. Parameters measured in the FST include (A) immobility, (B) climbing, and (C) swimming. All data are from 3 independent experiments consisting of 5 rats each ($n = 15$) and are expressed as percentage of control and calculated as mean $\pm$ standard error of the mean.

As depicted in Figure 4-8, there are no significant alterations in immobility ($61 \pm 9.8\%$ versus $69 \pm 6.9\%$ with $p > 0.05$), climbing ($89 \pm 17.3\%$ versus $115 \pm 19.6\%$ with $p > 0.05$), or swimming ($284 \pm 39.4\%$ versus $232 \pm 25.7\%$ with $p > 0.05$) in the FST when yohimbine is combined with fluoxetine for 7 days compared to fluoxetine administered alone for 7 days. This suggests that yohimbine lacks certain specific properties of mirtazapine that allow it to seemingly interact additively with fluoxetine in the FST (see § 4.3.1.1.1), and it does not possess over the specific properties of idazoxan that allow it to antagonise the effects of fluoxetine in the FST (see § 4.3.1.1.2). These results also imply that although yohimbine is an inverse agonist at α_2 -ARs (Wade *et al.*, 2001), antagonism at these receptors alone is neither enough to potentiate the effects of fluoxetine, nor is it sufficient to produce a more rapid onset of action, regardless of the mode of antagonism.

4.3.2 Changes in β -AR and serotonin 5-HT_{1A} receptor concentration

4.3.2.1 β -AR concentration

The frontal cortex regions of the animals used in the FST were removed and used in receptor binding experiments for measurement of β -AR density.

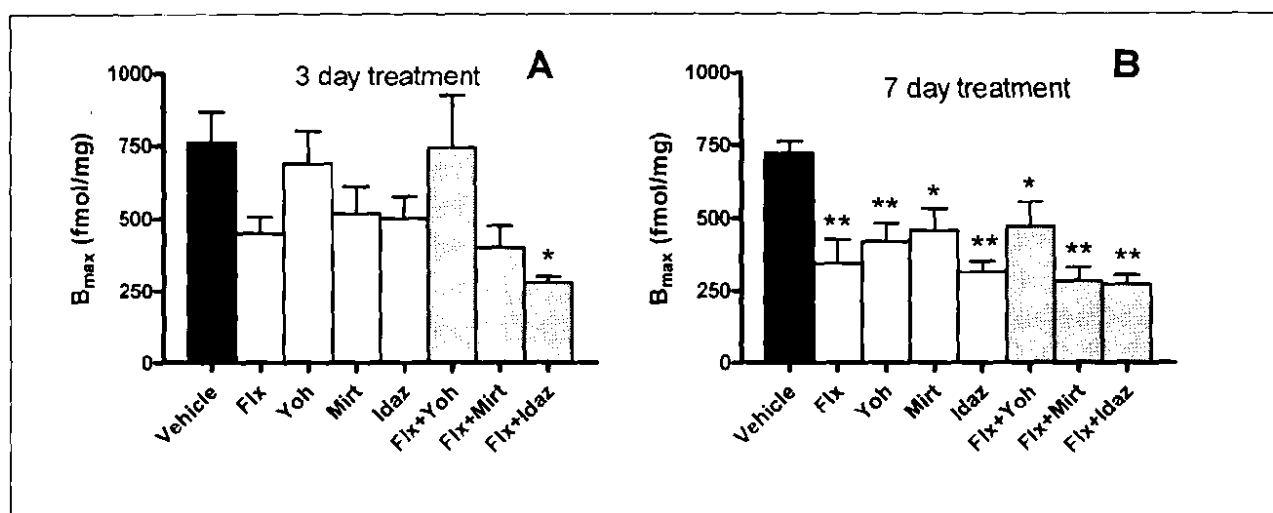


Figure 4-9: Effects on β -AR density in frontal cortex areas produced by administration of antidepressants for (A) 3 days, or (B) 7 days. Five frontal cortex regions were pooled for one experiment. All experiments were carried out in triplicate ($n = 3$), except the control groups which were carried out 6 times ($n = 6$). β -AR density (B_{max}) is expressed in terms of fmol receptors per mg protein, and as mean $\pm$ standard error of the mean, where * represents $p < 0.05$ and ** represents $p < 0.01$.

It is evident in Figure 4-9 that none of the treatments produce significant downregulation of β -ARs after 3 days of treatment compared to control, except an idazoxan-fluoxetine combination (760 ± 103.5 fmol/mg versus 278 ± 24.0 fmol/mg with $p < 0.05$). The relevance of this result should however be questioned, since this treatment produces no behavioural antidepressant-like effects after any treatment period. In contrast, all treatments produce significant downregulation of β -ARs after 7 days of treatment compared to control. This supports the general acceptance that chronic antidepressant treatment leads to a decrease in cortical β -AR density.

Taken together, changes in β -AR density do not correlate with early behavioural changes in the FST. The observation that neither mirtazapine nor a mirtazapine-fluoxetine combination produces downregulation of β -ARs after 3 days of treatment compared to control suggests that β -AR downregulation is not an accurate indication of onset of early antidepressant-like response, since both these treatments produce antidepressant-like behavioural changes in the FST after 3 days of treatment. Another discrepancy between β -AR downregulation and behavioural changes in the current model is that idazoxan significantly downregulated these receptors after 7 days, but failed to produce behavioural antidepressant-like responses in the FST after this treatment period.

4.3.2.2 Serotonin 5-HT_{1A}-receptor concentration

The hippocampus regions of the animals used in the FST were removed and used in receptor binding experiments for measurement of 5-HT_{1A} receptor density.

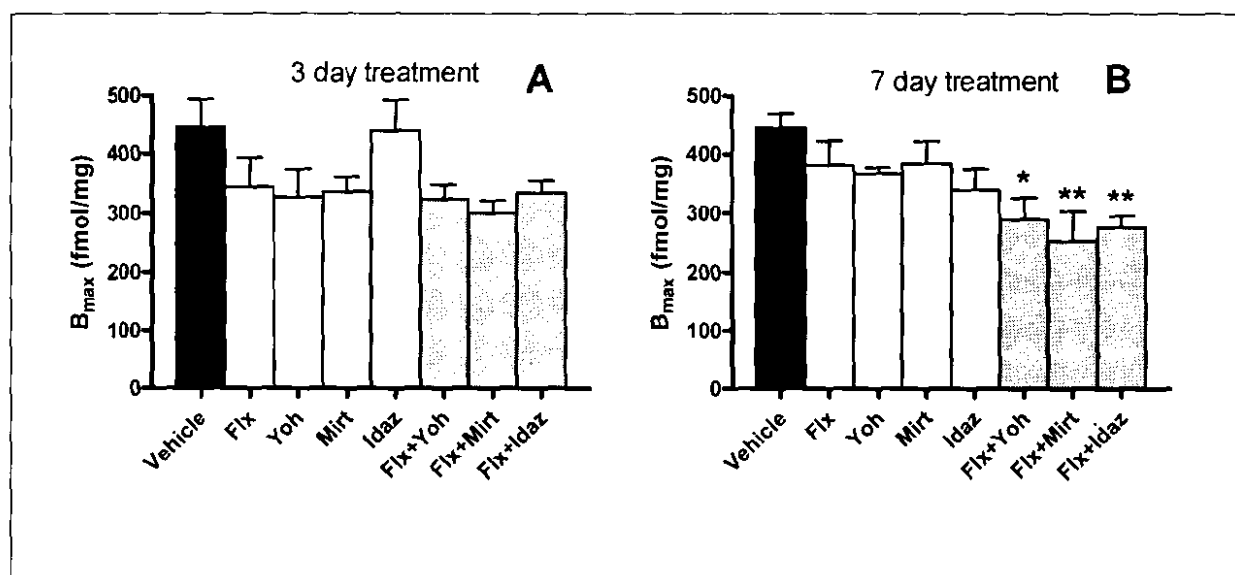


Figure 4-10: Effects on serotonin 5-HT_{1A}-receptor density in the hippocampus produced by administration of antidepressants for (A) 3 days, or (B) 7 days. Five frontal cortex regions were pooled for one experiment and all experiments were carried out in triplicate (n=3), except the control groups which were carried out 6 times (n = 6). Serotonin 5-HT_{1A}-receptor density (B_{max}) is expressed in terms of fmol receptors per mg protein, and as mean $\pm$ standard error of the mean, where * represents $p < 0.05$ and ** represents $p < 0.01$.

As illustrated in Figure 4-10, none of the treatments produce downregulation of 5-HT_{1A} receptors after 3 days of treatment compared to control. After 7 days of treatment, however, all combination treatments, i.e. yohimbine-fluoxetine (446 ± 23.9 fmol/mg versus 290 ± 36.6 fmol/mg with $p < 0.05$), mirtazapine-fluoxetine (446 ± 23.9 fmol/mg versus 254 ± 50.5 fmol/mg with $p < 0.01$) and idazoxan-fluoxetine (446 ± 23.9 fmol/mg versus 277 ± 19.13 fmol/mg with $p < 0.01$), produce significant downregulation of 5-HT_{1A} receptors in the hippocampus.

The values for 5-HT_{1A} receptor density that were obtained in this experiment include both presynaptic and postsynaptic 5-HT_{1A} receptors, while the presynaptic 5-HT_{1A} receptor is implemented to be restrictive in the onset of action of antidepressants, since stimulation of these receptors leads to decreased serotonergic cell firing and serotonin synthesis. The sensitivity of this experiment to reveal downregulation of presynaptic 5-HT_{1A} receptors is therefore impaired. Nonetheless, it seems that combinations of fluoxetine with α_2 -AR antagonists in general are more effective in decreasing 5-HT_{1A} receptor density (presynaptically and/or postsynaptically) in the hippocampus. Another deficiency in this data is that it does not expose desensitisation of receptors, which is thought to be a major alteration occurring presynaptically in serotonergic neurons following antidepressant treatment (Li *et al.*, 1997; Hervas *et al.*, 2001; Newman *et al.*, 2004).

4.3.2.2 Serotonin 5-HT_{1A}-receptor concentration

The hippocampus regions of the animals used in the FST were removed and used in receptor binding experiments for measurement of 5-HT_{1A} receptor density.

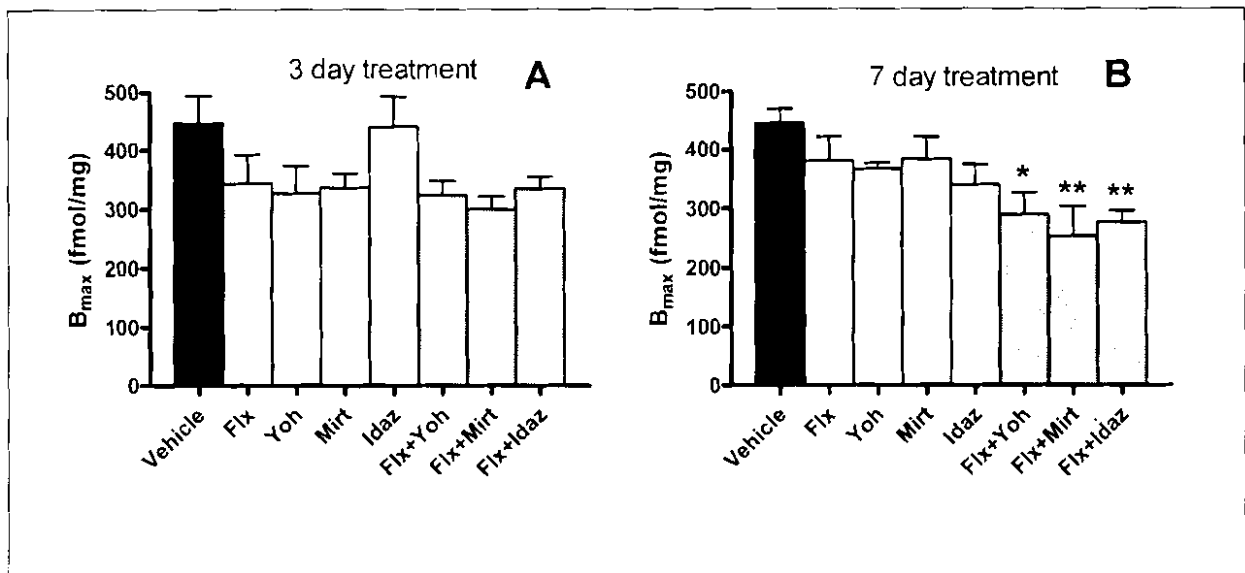


Figure 4-10: Effects on serotonin 5-HT_{1A}-receptor density in the hippocampus produced by administration of antidepressants for (A) 3 days, or (B) 7 days. Five frontal cortex regions were pooled for one experiment and all experiments were carried out in triplicate (n=3), except the control groups which were carried out 6 times (n = 6). Serotonin 5-HT_{1A}-receptor density (B_{max}) is expressed in terms of fmol receptors per mg protein, and as mean $\pm$ standard error of the mean, where * represents $p < 0.05$ and ** represents $p < 0.01$.

As illustrated in Figure 4-10, none of the treatments produce downregulation of 5-HT_{1A} receptors after 3 days of treatment compared to control. After 7 days of treatment, however, all combination treatments, i.e. yohimbine-fluoxetine (446 ± 23.9 fmol/mg versus 290 ± 36.6 fmol/mg with $p < 0.05$), mirtazapine-fluoxetine (446 ± 23.9 fmol/mg versus 254 ± 50.5 fmol/mg with $p < 0.01$) and idazoxan-fluoxetine (446 ± 23.9 fmol/mg versus 277 ± 19.13 fmol/mg with $p < 0.01$), produce significant downregulation of 5-HT_{1A} receptors in the hippocampus.

The values for 5-HT_{1A} receptor density that were obtained in this experiment include both presynaptic and postsynaptic 5-HT_{1A} receptors, while the presynaptic 5-HT_{1A} receptor is implemented to be restrictive in the onset of action of antidepressants, since stimulation of these receptors leads to decreased serotonergic cell firing and serotonin synthesis. The sensitivity of this experiment to reveal downregulation of presynaptic 5-HT_{1A} receptors is therefore impaired. Nonetheless, it seems that combinations of fluoxetine with α_2 -AR antagonists in general are more effective in decreasing 5-HT_{1A} receptor density (presynaptically and/or postsynaptically) in the hippocampus. Another deficiency in this data is that it does not expose desensitisation of receptors, which is thought to be a major alteration occurring presynaptically in serotonergic neurons following antidepressant treatment (Li *et al.*, 1997; Hervas *et al.*, 2001; Newman *et al.*, 2004).

Conclusion

Chapter

5.1 SUMMARY OF RESULTS

In the current study male Sprague-Dawley rats were treated with vehicle or fluoxetine, mirtazapine, yohimbine, idazoxan or a combination of fluoxetine with each of these α_2 -lytic drugs, whereafter the rats were exposed to the FST and cortical α_2 -AR and hippocampal 5-HT_{1A} receptor concentration. With this study design the aim was to investigate the role of the α_2 -lytic action of mirtazapine in its putative earlier onset of antidepressant action.

The effects, as found with the rat FST, produced by all drug treatment regimes in the current study are summarised in Table 5-1.

Table 5-1: Effects of antidepressants in the rat FST after 3 and 7 days of treatment

Treatment	Effect elicited in the FST					
	3-day treatment			7-day treatment		
	Immobility	Climbing	Swimming	Immobility	Climbing	Swimming
Fluoxetine	↔	↔	↔	↓↓	↔	↑↑
Mirtazapine	↓↓	↔	↑↑	↓	↑	↔
Yohimbine	↔	↔	↑	↓↓	↔	↑↑
Idazoxan	↔	↔	↔	↔	↔	↔
Fluoxetine + Mirtazapine	↓↓	↑	↑	↓↓	↑↑	↑↑
Fluoxetine + Yohimbine	↔	↔	↔	↓↓	↔	↑↑
Fluoxetine + Idazoxan	↔	↔	↔	↔	↔	↔

Note: In the table, a horizontal arrow denotes no effect, a downward arrow denotes a reduction while an upward arrow denotes an increase in a specific behavioural component. Two arrows indicate a more significant effect than one arrow.

In the current study, mirtazapine produced a more rapid onset of antidepressant-like effect than fluoxetine in an animal model of depression. In contrast, the α_2 -AR inverse agonist yohimbine alone produced antidepressant-like behavioural changes within a similar period as fluoxetine. Yohimbine was, however, not superior to fluoxetine, neither did it enhance the effect of fluoxetine. The α_2 -AR neutral antagonist idazoxan did not produce antidepressant-like effects after any treatment, but rather antagonised the antidepressant-like effect produced by fluoxetine.

The interactions of the α_2 -AR antagonists used in the current study in combination with fluoxetine are depicted in Table 5-2.

Table 5-2: Interactions of α_2 -lytic drugs with fluoxetine in the rat FST

Drug	Interactions with fluoxetine		
	Immobility	Climbing	Swimming
Mirtazapine	Possible additive effect	Possible additive effect	Possible additive effect
Yohimbine	No interaction	No interaction	No interaction
Idazoxan	Antagonism	No interaction	Antagonism

In the current study, mirtazapine and fluoxetine showed a possible additive relationship in the rat FST for all behavioural parameters, but the current study did not provide conclusive evidence for this deduction. No interaction was evident with the combination of yohimbine and fluoxetine in the rat FST. These include effects on immobility, climbing and swimming. In contrast, idazoxan attenuated the antidepressant-like behavioural effects induced by fluoxetine in the rat FST by increasing immobility and decreasing swimming behaviour elicited by fluoxetine alone.

The effects of the drugs employed in the current study on β -AR density in the frontal cortex of the rats are summarised in Table 5-3:

Table 5-3: Effects on cortical β -AR density

Treatment	Effect on β -AR density	
	3 days	7 days
Fluoxetine	↔	↓↓
Mirtazapine	↔	↓
Yohimbine	↔	↓↓
Idazoxan	↔	↓↓
Fluoxetine + Mirtazapine	↔	↓↓
Fluoxetine + Yohimbine	↔	↓
Fluoxetine + Idazoxan	↓	↓↓

Note: In the table, a horizontal arrow denotes no effect, while a downward arrow indicates a reduction in β -AR density. Two arrows indicate a more significant effect than one arrow.

β -ARs in the frontal cortex of rats were downregulated by all treatments after the longer treatment period. However, after 3 days of treatment, neither mirtazapine nor mirtazapine in combination with fluoxetine (which did produce early behavioural responses) were able to significantly decrease β -AR concentration in the rats.

The effects of the drugs employed in the current study on 5-HT_{1A} receptor density in the hippocampus of the rats are summarised in Table 5-4:

Table 5-4: Effects on 5-HT_{1A} receptor density

Treatment	Effect on 5-HT _{1A} density	
	3 days	7 days
Fluoxetine	↔	↔
Mirtazapine	↔	↔
Yohimbine	↔	↔
Idazoxan	↔	↔
Fluoxetine + Mirtazapine	↔	↓
Fluoxetine + Yohimbine	↔	↓↓
Fluoxetine + Idazoxan	↔	↓↓

Note: In the table, a horizontal arrow denotes no effect, while a downward arrow indicates a reduction in 5-HT_{1A} density. Two arrows indicate a more significant effect than one arrow.

5-HT_{1A} receptor density in the hippocampus regions of the rats was significantly reduced only by the combination treatments after the longer treatment period, whereas after the shorter period, none of the treatments produced significant downregulation of 5-HT_{1A} receptors.

5.2 CONCLUSIONS

The current study has provided useful and novel information regarding the role of the α_2 -lytic activity of mirtazapine in its putative earlier onset of antidepressant action. Moreover, it comments on the hypothesis related to potential mechanisms involved in earlier onset of action.

- ***Mirtazapine presents with an earlier onset in an animal model of depression***

Firstly, in the animal model of depression employed in the current study (the rat FST), we confirmed a more rapid onset of antidepressant-like effects by mirtazapine compared to fluoxetine. This is in support of recent clinical trials suggesting an earlier onset of antidepressant action for this drug in humans (Wheatley *et al.*, 1998; Leinonen *et al.*, 1999; Benkert *et al.*, 2000; Vester-Blokland & Van Oers, 2002), although the data are inconclusive. While the current data from a rodent model of depression supports an earlier onset of action, this warrants further investigation into the mechanisms involved. However, it should be noted that the results obtained in the current study does not imply that mirtazapine relieves depression more rapidly in humans. However, further clinical trials investigating this prospect are warranted.

- ***The α_2 -lytic property of mirtazapine is not important for its earlier onset of action***

Moreover, the mode(s) of action of mirtazapine may provide valuable insight into future strategies for the development of novel antidepressants. In the current study, and in contrast to the hypothesis that α_2 -AR antagonism plays an important role in the early onset of action of mirtazapine, we found that neither the α_2 -AR inverse agonist yohimbine, nor the α_2 -AR neutral antagonist idazoxan (alone, or in combination with fluoxetine), displayed a more rapid onset than fluoxetine alone. This implies that, regardless of whether mirtazapine acts as an inverse agonist or a neutral antagonist, its α_2 -lytic property *per se* may not be as critical for its more rapid onset of action as previously suggested. This finding may encourage future research into novel mechanisms involved in earlier onset of antidepressant action. Care should however be taken to extrapolate the data in the current study to circumstances in humans, especially since rats express the α_{2D} -AR, an orthologue of the human α_{2A} -AR (Lanier *et al.*, 1991; Harrison *et al.*, 1991). While the pharmacological profiles of the α_{2A} - and α_{2D} -AR are mostly comparable (Naselsky *et al.*, 2001; Lanier *et al.*, 1991), differences in binding affinity have been demonstrated for a few ligands (Eltze *et al.*, 2002; Lanier *et al.*, 1991; Harrison *et al.*, 1991). It is reasonable, however, to assume that the binding profiles determined in the current study with the porcine α_{2A} -AR will match the profiles in humans and closely resemble that for the rat α_{2D} -AR.

- ***Other properties of mirtazapine may be responsible for its earlier onset of action***

Furthermore, of all the drugs used in the current study, only mirtazapine acted more rapidly than fluoxetine, and presented with possible additive effects with fluoxetine in the rat FST. This suggests that mirtazapine possesses a unique pharmacological profile (with its α_2 -lytic property playing a less important role than previously anticipated) that permits it to act in this distinct manner. However, the essential mechanisms rendering mirtazapine superior to the other drugs used in the current study, regarding onset of response or a possible additive interaction with fluoxetine, remains elusive. These properties most likely include 5-HT₂ and 5-HT₃ receptor antagonism, which allow mirtazapine to selectively (albeit indirectly) enhance postsynaptic 5-HT_{1A} receptor stimulation, or may include unidentified properties of mirtazapine.

Evidence for other hypotheses to explain the earlier onset of action of mirtazapine

Evidence for the involvement of 5-HT₂ receptors (specifically the 5-HT_{2C} subtype) in the onset of action of antidepressants originates from previous studies (Dremencov *et al.*, 2004; Dremencov *et al.*, 2005). In these studies it was suggested that the onset of an antidepressant-like response is dependent on the restoration of the serotonin-induced release of *D*-DA in the nucleus accumbens (NAC), which has been found to be impaired in rats in an animal model of depression. Furthermore it was demonstrated that the atypical antidepressant nefazodone (5-HT_{2C} receptor antagonist) is able to produce a more rapid onset of behavioural antidepressant-like changes in rats than the tricyclic antidepressant desipramine. In addition, the abovementioned studies suggest that the time for the onset of antidepressant-like effects of nefazodone correlates directly with the ability of this drug to restore the serotonin-induced release of *D*-DA in the NAC to basal state. This ability of nefazodone has been attributed to its 5-HT_{2C} receptor antagonistic property, since this receptor plays an inhibitory role in the release of central *D*-DA (Di Matteo *et al.* 2001), and is a property it shares with mirtazapine (de Boer, 1996). Therefore, the earlier onset of antidepressant-like action of mirtazapine may be explained by the abovementioned hypothesis.

Differences regarding postsynaptic 5-HT_{1A} receptor stimulation between early onset antidepressants (e.g. mirtazapine) and drugs that display a more delayed onset of action may also be of interest. Since 5-HT_{1A} receptor activation is suggested to be essential for an antidepressant response (Blier *et al.*, 1990; Haddjeri *et al.*, 1998; Blier & de Montigny, 1999; Blier & Ward, 2003; Cryan *et al.*, 2005), discrepancies in the magnitude and promptness of the activation of these receptors by antidepressants may explain the differences in the onset of action of these drugs. In other words, mirtazapine may produce an earlier and/or more pronounced stimulation of 5-HT_{1A} receptors, and thereby produce a more rapid onset of antidepressant action. Such a comparison of 5-HT_{1A} receptor stimulation between antidepressants has not been done as yet.

Another possible mechanism of action of mirtazapine that was not explored in the current study is its potential neuroprotective effects, since these effects are associated with antidepressant action (discussed in § 2.2.2). These may include effects on BDNF and/or CREB levels, alterations in apoptotic and/or anti-apoptotic pathways, and modulating effects on the immune system (given the proven neurodegenerative effects of activation of the immune system during depression (Maes, 1995; Tuglu *et al.*, 2003; Wichers *et al.*, 2006)). Furthermore, and in correlation with the previous paragraph, stimulation of 5-HT_{1A} receptors has been shown to have neuroprotective effects (Gould, 1999). Therefore, mirtazapine may have a neuroprotective action via indirect enhancement of 5-HT_{1A} receptor stimulation or diverse mechanisms. Lastly, although there is no evidence that mirtazapine interacts with NMDA receptors, the possibility is

not excluded that this drug may indeed elicit neuroprotective effects is via the NMDA/NO/cGMP pathway.

Secondary conclusions

The secondary conclusions of the current study relate to β -AR and 5HT_{1A} receptor downregulation as markers of earlier onset of antidepressant-like activity. The downregulation of cortical β -ARs produced by the drugs in the current study after 7 days concur with the general consensus that these receptors are downregulated following antidepressant treatment. However, in the current study we suggest that downregulation of cortical β -ARs is not a reliable marker for the onset of an antidepressant-like response, and therefore there is a need for other, more accurate markers of antidepressant action. Furthermore, although 5-HT_{1A} receptors are involved in the putative mechanism of antidepressant action of antidepressant drugs, downregulation of these receptors does not correlate well with antidepressant administration in general.

5.3 RECOMMENDATIONS AND PROSPECTIVE STUDIES

In the current study we provide evidence for a more rapid onset of antidepressant-like action for mirtazapine in an animal model of depression. These findings warrant further investigation using different animal models of depression, as well as other preclinical and clinical studies to clarify the putative early onset of antidepressant action of mirtazapine.

Based on the outcome of the current study, several strategies for future studies can be deduced:

- Animal models of chronic depression may be useful for generating more evidence for the putative early onset of mirtazapine. These models would simulate more closely the chronic disease and may reflect in a more reliable fashion the delayed onset of action of antidepressants and any earlier onset of action. Such models include olfactory bulbectomy (Song & Leonard, 2005) and a genetic model of depression, namely the Flinders Sensitive Line (FSL) rats (Overstreet, 1993) (see § 2.5.1.4).
 - The importance of the 5-HT₂ and 5-HT₃ receptor antagonistic properties of mirtazapine in the putative earlier onset of action of this drug may be an interesting focus for future studies. Several prospective strategies can be proposed to investigate this hypothesis.
 - Mirtazapine may be combined with selective antagonists and/or agonists at 5-HT₂ and 5-HT₃ receptors, and the effects of these combinations evaluated in an animal model of depression. If agonists or antagonists at one or both of these receptors respectively
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abolish or bolster the earlier onset of action of mirtazapine, one or both of these receptors may be involved in the more rapid onset of action of this drug.

- Combinations of 5-HT₂ and 5-HT₃ receptor antagonists with drugs that do not present with an early onset (e.g. fluoxetine) may be employed to investigate whether these drugs can accelerate the onset of an antidepressant response.
- Another strategy may be the utilization of 5-HT₂ and 5-HT₃ receptor “knock out” mice. If these receptors are indeed involved in the time for onset of action of antidepressants, this would accelerate the onset of action of antidepressants that present with a delayed onset of action.
- The effects of mirtazapine on pathways involved in neurogenesis may also be of interest. These may include effects on neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and cAMP response element binding protein (CREB). The mRNA levels for these factors may be measured, as well as quantification of the associated protein expressions. Discrepancies in the promptness of mirtazapine-induced changes in the expression of these proteins compared to antidepressants with a delayed onset of antidepressant action may provide insight into the importance of these effects in the earlier onset of action of mirtazapine.
- Lastly, diverse receptor interactions of mirtazapine, which are less well explored, may also play important roles in its putative earlier onset of action. These include weak, but not negligible α_1 -AR and 5-HT_{1A} receptor agonistic properties (de Boer, 1996). The importance of the roles of such diverse receptor activities may also be further explored.

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Appendix

Article submitted for publishing

The data and opinions in this study form part of an article submitted for publishing in the Journal of Pharmaceutical and Experimental Therapeutics (JPET). The *Instructions to authors* for this journal is supplied (as presented on <http://jpet.aspetjournals.org/misc/ifora.shtml>) and the article, as submitted for publishing, is attached. By the time of print of this dissertation there was still no reply on whether the article is accepted.

INSTRUCTIONS TO AUTHORS (JPET)

Organization of the Manuscript

Manuscripts should contain the following sections in the order listed. Each section should begin on a new page and all pages should be numbered consecutively.

1. Title page. This should contain the complete title of the article, the names of all authors, and the primary laboratory of origin. Affiliation should be indicated by author initials only. Financial support for the research should not be on this page but indicated as an unnumbered footnote to the title and included with other footnotes on a separate page following the References section.

2. Running title page. The running title page should contain the following:

- A running title, which conveys the sense of the full title (not to exceed 60 characters, including spaces and punctuation). Commonly used abbreviations (e.g., "ATP", "Ach", etc.) may be used.
- The name, address, telephone and fax numbers, and e-mail address of the corresponding author. Only one author may be designated as the corresponding author. The e-mail address will also be used as a hypertext link in the online version of the paper.
- The number of text pages, number of tables, figures, and references, and the number of words in the Abstract, Introduction, and Discussion (each item should be placed on a separate line).

- A list of non-standard abbreviations used in the paper. Standard abbreviations are those listed in the Instructions to Authors of the Journal of Biological Chemistry, 272:1–4 (1997). The use of abbreviations should be minimized to enhance readability and comprehension of the text.
- e) A recommended section assignment to guide the listing in the table of contents. Section options are:
 - Cardiovascular
 - Cellular and Molecular
 - Chemotherapy, Antibiotics, and Gene Therapy
 - Endocrine and Diabetes
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 - Neuropharmacology
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3. Abstract. The abstract should concisely present the hypothesis being tested, general methods, results, and conclusions. Abstracts of more than 250 words will not be accepted. A word is one or more characters bounded by white space. The abstract must be a single paragraph. IMPORTANT: If your manuscript is accepted, the abstract entered into the online metadata form will appear online EXACTLY the way you enter it during the submission process. Please make sure to code all special characters, including sub- and superscripts, using the codes available from the online submission system. For revisions, update the abstract, if changed from the original.

4. Introduction. This section, which has no heading, must contain a clear statement of the aims of the work or of the hypotheses being tested. A brief account of the relevant background that supports the rationale of the study should also be given. The length of the Introduction should not exceed 750 words.

5. Methods. This section should contain explicit, concise descriptions of all new methods or procedures employed. Whereas modifications of previously published methods must be described, commonly used procedures require only a citation of the original source. Descriptions of methods must be sufficient to enable the reader to judge the accuracy,

reproducibility, and reliability of the experiment(s). The name and location (city and state or country) of commercial suppliers of chemicals, reagents, and equipment must be given. Sources of compounds, reagents, and equipment not available commercially should be identified by name and affiliation here or in the Acknowledgments section.

6. Results. Contained in this section are the experimental data, with no discussion of their significance. Results are typically presented in figures or tables, with no duplication of information in the text. If a table or figure includes less than four values, the data should be presented in the text rather than as a separate table or figure. Magnitudes of variables reported should be expressed in numerals. Generally, units are abbreviated without punctuation and with no distinction between singular and plural forms (e.g., 1 mg, 25 mg). Sufficient data should be presented to allow for judgment of the variability and reliability of the results. Statistical probability (p) in tables, figures, and figure legends should be expressed as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. For second comparisons, one, two, or three daggers may be used. For multiple comparisons within a table, footnotes italicized in lower case, superscript letters are used and defined in the table legend.

7. Discussion. Conclusions drawn from the results presented are included in this section. Whereas speculative discussion is allowed, it must be identified as such and be based on the data presented. The Discussion must be as concise as possible and should not exceed 1,500 words.

8. Acknowledgments. The Acknowledgments section is placed at the end of the text. Personal assistance is noted here. Financial support is acknowledged as an unnumbered footnote to the title.

9. References. References are cited in the text by giving the first author's name (or the first and second if they are the only authors) and the year of publication (e.g., Ruth and Gehrig, 1929; McCarthy, 1952; or Kennedy et al., 1960). In the reference list, the references should be arranged alphabetically by author and not numbered. The names of all authors should be given in the reference list. If reference is made to more than one publication by the same author(s) in the same year, suffixes (a, b, c, etc.) should be added to the year in the text citation and in the references list. Journal titles should be abbreviated as given in the abbreviation list linked to the online Instructions to Authors. The total number of references is limited to 40. References to personal communications, unpublished observations, and papers submitted for publication are given in parentheses at the appropriate location in the text, not in the list of references. Only papers that have been officially accepted for publication may be cited as "in press" in the reference list. The authors are responsible for the accuracy of the references. The format for journal article, chapter and book references is as follows:

Griffiths RR, Bigelow GE and Liebson IA (1986) Human coffee drinking: Reinforcing and physical dependence producing effects of caffeine. *J Pharmacol Exp Ther* 239:416-425.

Chernow B and O'Brian JT (1984) Overview of catecholamines in selected endocrine systems, in *Norepinephrine* (Ziegler MG and Lake CR eds) pp 439-449, Williams & Wilkins, Baltimore.

Tallarida RJ and Murray RB (1987) *Manual of Pharmacologic Calculations with Computer Programs*. Springer-Verlag, New York.

10. Footnotes. Footnotes should be listed on a separate page and presented in the following order:

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13. Figures. Submit illustrations prepared as specified at <http://cjs.cadmus.com/da/>. Each figure must be uploaded as a separate file in a 600+ dots per inch .tif, .eps, or .jpg format and scaled to fit an 8.5 x 11 inch page. Authors are advised to avoid submitting .ppt files; they do not reproduce as clearly as other formats. Label the front of every figure with the figure number. Lettering on figures should be large enough to be legible after reduction to single-column width of 21 picas (about 3½ inches or 9 cm). Type sizes after reduction should be 6–8 points. Do not use varying letter type sizes within a single figure; use the same size or similar sizes throughout the drawing. Figures should be ready, in all respects, for direct reproduction. All panels of a multipart figure should be provided in the same file. If symbols are not explained on the face of the figure, only standard print characters may be used. Include figure titles in the legend and not

on the figure itself. Photomicrographs and electron micrographs must be labeled with a magnification calibration in micrometers or Angstrom units. A statement concerning the magnification must appear in the figure legend.

ARTICLE AS SUBMITTED FOR PUBLISHING

TITLE PAGE

Title of article

The α_2 -lytic properties of mirtazapine may not be critical for a putative earlier onset of antidepressant action. Evidence from rat behavioral and neuro-receptor studies.

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RUNNING TITLE PAGE**Running title**

α_2 -Adrenoceptor antagonism and mirtazapine onset of action

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List of non-standard abbreviations: 5-HT, 5-hydroxytryptamine; CHO-K1, Chinese hamster ovary cell line; α_{2A} -H, Chinese hamster ovary cell line expressing the porcine α_{2A} -adrenoceptor at relative high numbers; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EMEM, minimum essential medium (Earle's base); FBS, Fetal bovine serum; FST, forced swim test; PBS, phosphate-buffered saline; SSRI, serotonin reuptake inhibitor.

Recommended section: Neuropharmacology

Abstract

While delayed onset of antidepressant action remains a shortcoming of current antidepressants, preliminary clinical data with newer antidepressants, including mirtazapine, show promise of hastened onset. Several potential mechanisms have been postulated and investigated. Mirtazapine displays antidepressant serotonin 5-HT₂ receptor blocking properties, while its putative earlier onset of action is thought to be related to its α_2 -adrenoceptor lytic action (at α_2 -adrenergic autoreceptors and heteroreceptors), thereby modulating central serotonergic and noradrenergic neurotransmission. The current study investigated the role of α_2 -adrenoceptor antagonism for earlier onset of action in cultured cell lines and a rat model of depression. In cultured cells the binding affinity of mirtazapine, mianserin, yohimbine and idazoxan at α_2 -adrenoceptors were determined, whereafter their mode of antagonism were evaluated with functional studies. Results suggest that mirtazapine is a neutral antagonist or inverse agonist at α_2 -adrenoceptors. Rats were treated for 3 and 7 days with fluoxetine, mirtazapine, yohimbine (α_2 -adrenoceptor strong inverse agonist), idazoxan (α_2 -adrenoceptor neutral antagonist), or combinations with fluoxetine, whereafter the forced swim test (FST) were employed and cortical β -adrenoceptor and hippocampal 5-HT_{1A} receptor densities were measured. Results with the FST support an earlier onset of action by mirtazapine, but do not support an important role for α_2 -lytic action in this regard. β -Adrenoceptor density generally decreased with antidepressant action, but is not a good marker of hastened onset. 5-HT_{1A} receptor density is not a good marker for antidepressant action. It was concluded that a property different from its α_2 -adrenoceptor lytic action may be important for the earlier onset of action by mirtazapine.

Mood disorders are amongst the most prevalent forms of mental illnesses, also being recurrent, life threatening (risk of suicide), and a major cause of morbidity worldwide (Akiskal, 2000). The introduction of the selective serotonin reuptake inhibitors (SSRIs) has significantly improved the treatment of depression as far as tolerability is concerned, and represent the currently most widely prescribed group of antidepressants. However, the delay in onset of action of antidepressants, typically longer than two weeks, continues to plague the effective treatment of this disease (Bymaster, et al., 2003; Leonard, 2003).

Some preliminary clinical studies suggest a putatively more rapid onset of antidepressant action for some recently introduced antidepressants, including mirtazapine (Benkert, et al., 2000; Leinonen, et al., 1999; Wheatley, et al., 1998; Vester-Blokland and Van Oers, 2002), venlafaxine (Guelfi, et al., 1995; Benkert, et al., 1996; Entsuah, et al., 1998), as well as SSRI augmentation with pindolol (Perez, et al., 1997; Tome, et al., 1997; Zanardi, et al., 2001). There has subsequently been a rekindling of interest in the mechanisms responsible for this putative rapid onset of action.

While mirtazapine also displays anti-serotonergic action, its α_2 -lytic properties have been implicated as having an important role in its earlier onset of action, rendering a dual action on central serotonergic and noradrenergic neurotransmission in the hippocampus (Blier, 2003).

If one assumes that an α_2 -lytic action would facilitate hastened onset of antidepressant action, one would predict that an inverse agonist at these receptors will be superior to a neutral antagonist. Many antagonists at G protein coupled receptors, such as α_2 -adrenoceptors, have been reclassified as inverse agonists (Wade *et al.*, 1999), so it is of relevance that the undetermined mode of action of mirtazapine in this regard may have bearing on understanding its reported earlier onset of action.

The forced-swim test (FST), a validated and well-established animal model of depression (Porsolt, et al., 1978), is effective in detecting a wide spectrum of antidepressant treatments in rodents (Borsini and Meli, 1988). A modification to the FST (Detke, et al., 1995) has enabled the test to effectively identify antidepressant responses produced by SSRIs, as well as to distinguish between serotonergic and noradrenergic effects produced by antidepressants, manifesting as increases swimming and climbing behavior, respectively. This therefore may allow the FST to be used to investigate preferential 5-HT and *I*-NE mechanisms for a given antidepressant (Borsini, 1995). While the FST has been developed originally for acute drug treatments (administered between two swim sessions 24 hours apart), chronic drug treatment programs of 2 to 4 weeks have also been evaluated with the FST (Porsolt, et al., 2000; Renner, et al., 2002a).

Another useful and acknowledged marker for the screening of antidepressant action is a reduction in cortical β -adrenoceptor density, being a collective property of a remarkably wide spectrum of antidepressant treatments (Tang, et al., 1981; Sethy, et al., 1988; Asakura, et al., 1982; Asakura and Tsukamoto, 1985; Tang, et al., 1981; Sethy, et al., 1988; Paetsch and Greenshaw, 1993; Asakura, et al., 1982; Asakura and Tsukamoto, 1985; Byerley, et al., 1987; Byerley, et al., 1988). The pharmacodynamic basis for this effect, however, is not clear, nor is its contribution to superior clinical antidepressant efficacy proven (Nelson, et al., 1991). It is unlikely that a reduction in β -adrenoceptor function contributes directly to the mood-elevating effects of antidepressant treatments, since β -adrenoceptor antagonists tend to induce or worsen depression (Baldessarini, 2001), and some antidepressants with proven clinical efficacy have been shown not to reduce β -adrenoceptor density (Suranyi-Cadotte, et al., 1985).

The objectives of the present study was to first determine the mode of action of mirtazapine at α_2 -adrenoceptors, and then to investigate the importance of this property in its possible earlier onset of antidepressant action. The mode of action of mirtazapine at α_2 -adrenoceptors was investigated in cultured cell lines, while the onset of antidepressant-like response was investigated in rats by means of behavioral changes in the FST, as well as changes in the density of cortical β -adrenoceptors. In addition, the concentration of hippocampal 5-HT_{1A}-receptors was also measured, since these receptors are implicated in the neuropathology of depression, as well as in the mechanism of antidepressant action of mirtazapine (Blier, 2003). Various drugs and drug combinations, including a strong α_2 -adrenoceptor inverse agonist (yohimbine), an α_2 -adrenoceptor neutral antagonist (idazoxan), mirtazapine, together with the typical antidepressant fluoxetine, were studied on the aforementioned behavioral and neurochemical parameters.

METHODS

Drugs, Chemicals, Radioligands & Culture Media. Mirtazapine was a kind gift from Organon (Netherlands). Fluoxetine hydrochloride was a generous gift from Aspen (Port Elizabeth, South Africa). Mianserin was obtained from Tocris (Ellisville, USA). Idazoxan hydrochloride, brimonidine (UK-14,304 or 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine) and yohimbine hydrochloride were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

Trichloroacetic acid (TCA), 3-isobutyl-1-methylxanthine (IBMX), adenosine 3',5'-cyclic monophosphate (cAMP), alumina type WN-3, 1,3-diaza-2,4-cyclopentadiene (imidazole), adenosine triphosphate (ATP), forskolin, propranolol hydrochloride, Bradford reagent, EDTA, ascorbic acid and fractioned BSA were obtained from Sigma Aldrich (St. Louis, USA). 2-amino-2-hydroxymethyl-propan-1,3,-diol (Tris) was obtained from Acros (Geel, Belgium). Tris HCl and MgCl₂ were obtained from USB (Cleveland, USA). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). Filter count LSC-cocktail was obtained from PerkinElmer Life and Analytical Sciences (Boston, USA). Serotonin creatinine sulphate was obtained from BDH Chemicals (Poole, England).

[2-³H]-adenine (23.0 Ci/mmol) and [³H]-propranolol hydrochloride (20.0 Ci/mmol) was obtained from GE Healthcare (formerly Amersham Biosciences, Buckinghamshire, England). [methyl-³H]-yohimbine (85.0 Ci/mmol) and [³H]-OH-DPAT (8-hydroxy-2-di-*n*-propylamino-tetralin) (106 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, USA).

Ham's F-12 medium, minimum essential medium (Earle's base) (EMEM), Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 (1:1 ratio mixture) and G-418 was obtained from Bio Whittaker (Walkersville, USA). Trypsin-versine, bovine serum albumin (BSA), and DMEM were obtained from Highveld Biological (Johannesburg, South Africa). Foetal bovine serum (FBS) and penicillin-streptomycin mixture were obtained from Gibco™ Invitrogen Life Technologies (California, USA).

Cell lines. Two Chinese hamster ovary (CHO-K1) cell lines, one stably transfected to express the wild-type porcine α_{2A} -adrenoceptor at high numbers (cell line denoted α_{2A} -H) and the other mock-transfected (control cell line containing the selection plasmid but no α_{2A} -adrenoceptor vector, denoted neo), were kindly provided by Dr. Richard Neubig (Department of Pharmacology, University of Michigan, Ann Arbor, MI, U.S.A.). The pharmacological profiles and receptor expression characteristics of the α_{2A} -H cell line have been previously characterized, where the determined α_{2A} -adrenoceptor concentration was reported as 19 ± 2 pmol/mg membrane protein (Brink, et al., 2000). We confirmed the high receptor expression for α_{2A} -H in our laboratory, determined as 46 ± 5 pmol/mg membrane protein (Bodenstein, et al.,

2005). These cell lines were used to investigate the α_2 -adrenoceptor binding and signaling properties of the test drugs.

Seeding cultured cells for experiments. The α_{2A} -H and neo cells were seeded at a density of approximately 2×10^6 cells/ml in 24-well plates and maintained for at least 18 hours before initiation of experiments (ligand-binding or [^{3} H]cAMP assays as described below) to allow adequate attachment of a monolayer of cells to the plate bottom surfaces.

Preparing membranes from α_{2A} -H cells. Membranes were prepared from α_{2A} -H cells essentially as described before (Bodenstein, et al., 2005). After the appropriate pre-treatment of whole α_{2A} -H cells (see above), the cells were washed twice with phosphate-buffered saline (PBS), the cell monolayer was detached with ethylenediaminetetraacetic acid (EDTA) in PBS (0.02% w/v), and the cells scraped from the culture flask surface with a cell scraper. The cell suspension was centrifuged in a bench top centrifuge ($5,411 \times g$, 4 °C, 15 minutes), the supernatant discarded and the pellet washed twice with ice-cold PBS, where after the pellet was re-suspended in 1 mM Tris buffer (pH 7.4). The cell suspension was tumbled for 15 minutes at 4 °C, homogenized with a Teflon homogenizer, and centrifuged at $1,000 \times g$ in a Beckman ultracentrifuge at 4 °C for 15 minutes. The supernatant was collected and kept on ice, while the pellet was re-suspended in the Tris buffer and the preceding procedure repeated to collect all protein. The resulting supernatants were centrifuged at $40,000 \times g$ in a Beckman ultracentrifuge at 4 °C for 60 minutes. The resulting pellet was re-suspended and homogenized in TME buffer (50 mM Tris, 10 mM $MgCl_2$ and 1 mM EDTA, pH 7.4). Protein concentrations were determined with the Bradford method (Bradford, 1976), using bovine serum albumin as standard and determining absorbance with a 96-well plate reader and a 560 nm filter (Labsystems Multiskan RC). Snap-frozen aliquots were stored at -86 °C for up to 4 weeks.

Radioligand binding assays in cultured cell membranes. We determined the K_d value of [O-methyl- ^{3} H]yohimbine at α_{2A} -adrenoceptors from radioligand saturation-binding experiments in whole α_{2A} -H cells, defining non-specific binding with 10 μ M yohimbine, as described before (Bodenstein, et al., 2005). In preparation for the competition binding experiments the cells were plated and incubated as described above. Cells were then rinsed once with EMEM, where after the assay was initiated by adding EMEM with different concentrations of 5 nM [O-methyl- ^{3} H]yohimbine, with or without 10 μ M yohimbine. After a 30 minute incubation at 37 °C in 5% CO_2 , the medium was aspirated, the cells rinsed twice with ice-cold PBS and the reaction terminated with 1 ml of 5% (w/v) TCA and let to stand for at least 30 minutes to allow the cells to lyse. The TCA from each well was then transferred directly into scintillation vials and the radioactivity counted.

To determine the pK_i value of the α_{2A} -adrenoceptor ligands at α_{2A} -adrenoceptors in α_{2A} -H cells we performed competition binding assays in whole α_{2A} -H cells against 5 nM [O-methyl- ^3H]yohimbine. In preparation for the competition binding experiments the cells were plated and incubated as described above. Cells were then rinsed once with EMEM, where after the assay was initiated by adding EMEM with 5 nM [O-methyl- ^3H]yohimbine and different concentrations of the indicated test ligand. After a 30 minute incubation at 37 °C in 5% CO₂, the medium was aspirated, the cells rinsed twice with ice-cold PBS and the reaction terminated with 1 ml of 5% (w/v) TCA and let to stand for at least 30 minutes to allow the cells to lyse. The TCA from each well was then transferred directly into scintillation vials and the radioactivity (bound [O-methyl- ^3H]yohimbine) counted.

Measurement of whole-cell [^3H]cAMP accumulation. [^3H]cAMP accumulation was determined in whole α_{2A} -H cells in 24-well plates as described previously (Wade, et al., 1999; Wong, 1994; Bodenstein, et al., 2005). Briefly, cells were radiolabeled by adding 1 μ Ci per well [2- ^3H]adenine (19-23 Ci/mmol; Amersham Pharmacia Biotech, U.K.) at least 18 hours before the assay. After the rinsing the cells once with DMEM, the assay was initiated by adding DMEM with 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical, St Louis, MO, U.S.A.) and 30 μ M forskolin (Sigma Chemical, St Louis, MO, U.S.A.) and the appropriate concentration of the indicated ligand to establish the dose-response relationships (for semilogarithmic dose-response curves, when response was elicited). After a 20 minute incubation time at 37 °C in 5% CO₂, the medium was aspirated and the reaction terminated with 1 ml ice-cold 5% (w/v) trichloroacetic acid (TCA; Sigma Chemical, St Louis, MO, U.S.A.) containing 1 mM ATP (Sigma Chemical, St Louis, MO, U.S.A.) and 1 mM cAMP (Sigma Chemical, St Louis, MO, U.S.A.) and allowed to stand for 30 minutes at 4 °C to lyse the cells. The acid soluble nucleotides were separated on Dowex and Alumina columns as described before (Salomon, et al., 1974) and radioactivity determined by liquid scintillation counting. The cAMP accumulation was normalized by dividing the [^3H]cAMP counts by the total [^3H]nucleotide counts. This value was then divided by the corresponding value obtained in the presence of IBMX and forskolin, without agonist (to calculate percentage of control).

Animals. Male Sprague-Dawley rats weighing $\pm$ 200g on the day of the FST were used in the behavioral experiments. The use of the animals was approved by the Ethical Committee of the North-West University (approval no. 03D09) and all experiments have been carried out in accordance with the Declaration of Helsinki. Suffering and discomfort to animals were minimized and the number of animals per group was the minimum needed for assays and meaningful statistical evaluation of the data. Animals were housed in groups of seven rats in acrylic cages at a controlled temperature (21 °C) and humidity (50%). They were maintained on

a 12 hour light-dark cycle (lights on at 06:00 hours) and food and water provided *ad libitum*. These conditions were maintained constant throughout the experiments.

Drug treatment of animals. Fifteen rats per treatment group (five rats per experiment, repeated twice to ensure repeatability) were assigned for behavioral and neuro-receptor analyses, while an additional six rats per treatment group (two rats per experiment, repeated twice to ensure repeatability) were assigned for the locomotor activity measurements. The animals were weighed each morning where after the dosages were calculated and drug solutions prepared. Fluoxetine (20 mg/kg/day), mirtazapine (20 mg/kg/day), idazoxan (3 mg/kg/day), yohimbine (3 mg/kg/day) and the indicated drug combinations, or no drug (control), were dissolved in a 0.9% saline solution containing Tween 80 (3% v/v). The dosages used for the drugs in the current study are based on previous studies where similar central effects were required for fluoxetine (Detke and Lucki, 1995; Reneric, et al., 2001; Reneric, et al., 2002b), mirtazapine (Nakayama, et al., 2004; Reneric, et al., 2002a), yohimbine (Campbell, et al., 1984; Bowes, et al., 1992; Soderpalm, et al., 1995) and idazoxan (Reneric, et al., 2001; Szabo and Blier, 2001; Hertel, et al., 1999). All drugs were administrated intra-peritoneally in a volume of 0.5 ml between 08:00 and 09:00 each morning. Depending on the treatment group, animals were treated with drug(s) or vehicle for 3, 7 or 11 days.

Forced Swim Test. The FST essentially followed that described by Porsolt and co-workers (Porsolt, et al., 1978) with the pre-conditioning swim instated on the penultimate day of the designated chronic treatment period, an approach that has been successfully deployed for chronic drug studies (Harvey, et al., 2002; Xu, et al., 2005). On the penultimate day of the designated treatment period the animals were placed in the testing room for 60 minutes to habituate, where after they were placed for 15 min in inescapable Perspex cylinders (diameter 18 cm, height 40 cm) containing 18 cm of clean water and maintained at a constant temperature of 25 °C by immersion in a surrounding water bath (157 x 26 x 25 cm). The cylinders were separated by opaque screens and the water deep enough to keep the rats from touching the bottom of the cylinder with their hind paws. Following the pre-conditioning swim session the rats were placed in heated cages to dry for 15 minutes before returning them to their home cages.

On the last day of the treatment period the animals were placed in the testing room for 60 minutes to habituate, where after they were replaced in the above-described cylinders containing 18 cm of clean water (25 °C) for 5 minutes and their behavior recorded on video from the side. After the test the rats were placed into heated cages to dry for 15 minutes.

The scoring of behavior was performed by a person blind to the treatment group. The following specific behavioral components were distinguished and assessed: immobility, defined when no

additional activity is observed other than that required to keep the rat's head above the water; climbing behavior (also known as thrashing), defined as upward directed movements of the forepaws along the side of the cylinder; and swimming behavior, defined as swimming movements (usually horizontal) throughout the cylinder. The animals were scored separately and the individual behavioral components were identified and measured in terms of the amount of time (seconds) the specific behavioral component was observed, for a total period of 5 minutes.

Locomotor behavior. On the last day, after the final dose, rats were placed in automated locomotor measurement cages (Accuscan Instruments, Columbus, U.S.A.) for 20 minutes to habituate, where after the locomotor activity (horizontal activity, defined as number of movements per 10 minutes) of the animals was digitally recorded and measured for 10 minutes.

Preparing membranes from rat brain tissue. When the FST was completed, the rats were decapitated and the brains rapidly dissected on ice. The hippocampus and frontal cortex regions were then snap-frozen and stored at -86°C for a maximum of 3 months. On the day of the radioligand binding study the desired brain regions were removed and allowed to thaw on ice.

Preparing membranes for measurement of β -adrenoceptor density. The frontal cortex regions of five animals were pooled for one experiment and the weight of the tissue recorded. The tissue was homogenized for 10 seconds with a Polytron homogenizer in 25 ml ice cold 50 mM Tris HCl buffer (pH = 7.4). The homogenate was centrifuged at $48,000 \times g$ (4°C) for 10 minutes and the supernatant decanted. The pellet was resuspended in 25 ml ice cold 50 mM Tris HCl buffer, and the homogenation and centrifugation repeated. The final pellet was resuspended in an appropriate volume ice cold 50 mM Tris HCl buffer to yield a concentration of 16 mg wet weight per ml using a Polytron homogenizer for 10 seconds. Protein concentrations were determined with the Bradford method (Bradford, 1976), using bovine serum albumin as standard and determining absorbance with a 96-well plate reader and a 560 nm filter (Labsystems Multiskan RC), where after the suspension was diluted with ice cold 50 mM Tris HCl buffer to yield a protein concentration of 500 $\mu\text{g}/\text{ml}$. The membrane suspension was kept on ice until use.

Preparing membranes for measurement of 5-HT_{1A}-R density: The same procedure was used as for β -adrenoceptor density, with appropriate modifications. The hippocampus regions of the animals were utilized for measurement of 5-HT_{1A}-R densities. Ice cold 50 mM Tris HCl buffer containing 2.5 mM MgCl_2 and 2 mM EDTA (pH = 7.4) was used for the homogenation and centrifugation steps, while the same buffer, but also containing 0.001% ascorbic acid, was used for the suspension of the final pellet and dilution to the final protein concentration.

Radioligand binding assays using membranes from rat brain.

Measurement of β -adrenoceptor density. Eight concentrations of [^{3} H]-propranolol ranging from 0.375 to 25 nM (in the final assay volume) were prepared in 50 mM Tris HCl buffer (pH = 7.4) and added in duplicate to membrane suspensions to yield a final assay volume of 1 ml to determine total binding. The same series, but also containing 60 μ M propranolol, was used to define non-specific binding. After a 15 minute incubation period in a shaking water bath at 25 °C, the contents of the tubes were transferred to Whatman GF/B filters and rinsed twice with 5 ml ice cold 50 mM Tris HCl buffer. The filters were placed in scintillation vials, scintillation cocktail added, and allowed to stand overnight where after radioactivity was counted.

Measurement of 5-HT_{1A}-R density. An eight point concentration series of [^{3} H]-8-OH-DPAT that ranged from 0.9 nM to 12 nM (in the final assay volume) were prepared in 50 mM Tris HCl buffer containing 2.5 mM MgCl₂, 2 mM EDTA and 0.001% ascorbic acid (pH = 7.4) and added in duplicate to membrane suspensions to yield a final assay volume of 1 ml to determine total binding. The same series, but also containing 20 μ M serotonin (in 0.02% ascorbic acid), was used to define non-specific binding. After a 60 minute incubation period at 25 °C in a shaking water bath, the contents of the tubes were transferred to Whatman GF/B filters and rinsed twice with 5 ml ice cold 50 mM Tris HCl buffer containing 2.5 mM MgCl₂ and 2 mM EDTA (pH = 7.4). The filters were placed in scintillation vials, scintillation cocktail added, and allowed to stand overnight where after radioactivity was counted.

Data analysis. Data from all studies with cultured cells were obtained from triplicate observations from at least 3 separate, comparable experiments, and results are expressed as average $\pm$ standard error of the mean (SEM). Semilogarithmic one-site competition-binding or dose-response curves were constructed as least square non-linear fits, utilizing the computer software GraphPad Prism (version 4.01 for Microsoft Windows, GraphPad Software, San Diego, CA, U.S.A.). The K_i values were calculated from the IC_{50} values of competition-binding curves, applying the K_d value of the radioligand into the built-in Cheng-Prusoff correction of the software. Data from all studies with rats were from 3 separate, comparable experiments, each with 5 rats. For behavioral data (i.e. forced swim test) observations were made individually for each rat, while the tissue from indicated brain regions of 5 rats was pooled for membrane preparation for radioligand binding studies. Parallel locomotor control studies were routinely evaluated from 3 separate, comparable experiments, each with 2 rats (i.e. 2 additional rats added to each treatment group).

GraphPad Prism was also utilized for all statistical analyses of data. For comparison of two values, the nonparametric Student's *t* test (two-tailed) was implemented. For multiple comparisons the one-way ANOVA comparison was performed followed by either the Dunnett's

post-test (for comparing experimental groups to the control) or the Tukey-Kramer post-test (for comparing experimental groups to each other). For all reported statistical probability values, $p < 0.05$ was regarded as statistically significant.

RESULTS

Radioligand binding at α_{2A} -adrenoceptors. The K_d value of the inverse agonist [methyl- ^3H]-yohimbine at α_{2A} -adrenoceptors in the α_{2A} -H cells (expressing the wild type α_{2A} -adrenoceptor at relatively high numbers) was calculated from saturation binding curves as 6.72 ± 2.05 nM and the B_{max} value was calculated as 46.18 ± 5.23 pmol/mg protein (data not shown). Figure 11 depicts the competition-binding curves and calculated pK_i values of the α_{2A} -adrenoceptor ligands brimonidine (agonist; $pK_i = 6.39 \pm 0.06$, i.e. $K_i \approx 407$ nM), idazoxan (neutral antagonist; $pK_i = 7.48 \pm 0.03$, i.e. $K_i \approx 33$ nM), mirtazapine (atypical antidepressant; $pK_i = 6.50 \pm 0.07$, i.e. $K_i \approx 316$ nM) and mianserin (atypical antidepressant; $pK_i = 6.94 \pm 0.14$, i.e. $K_i \approx 115$ nM), against 5 nM [methyl- ^3H]-yohimbine in α_{2A} -H cells. The affinities of these ligands for α_{2A} -adrenoceptors are therefore in the mid to high nanomolar range, with the affinity values of mirtazapine, mianserin and brimonidine being comparable and idazoxan displaying approximately a 10-fold and yohimbine approximately a 100-fold higher affinity for α_{2A} -adrenoceptors.

Dose-response curves of α_{2A} -adrenoceptor ligands. Figure 12 depicts dose-response-relationships of the α_{2A} -adrenoceptor ligands brimonidine, yohimbine, idazoxan, mirtazapine and mianserin in neo cells (negative control, mock-transfected) and in α_{2A} -H cells (transfected to express the porcine α_{2A} -adrenoceptor at relatively high numbers). It is important to note that only brimonidine displayed a dose-response curve in α_{2A} -H cells ($pEC_{50} = 9.27 \pm 0.19$; $E_{max} = 69.0 \pm 5.8\%$ inhibition of [^3H]-cAMP accumulation), while all other drugs did not elicit any significant α_{2A} -adrenoceptor-mediated inhibition of cAMP production in α_{2A} -H cells. None of the drugs displayed any response in neo cells.

Lab validation of the forced swim test. A 15 minute pre-exposure, as compared to no pre-exposure, of drug-naïve (vehicle treated control) rats to forced swimming 24 hours before, increased immobility during the second (scoring) swim session from $100 \pm 8.2\%$ to $143 \pm 7.1\%$ ($p = 0.0005$, data not presented graphically). In addition, 7 days fluoxetine-treated rats demonstrated significant anti-immobility effects compared to drug-naïve rats ($64 \pm 4.6\%$ versus $143 \pm 7.1\%$, $p < 0.0001$, data not presented graphically). The data therefore confirms that the FST, as outlined in the methodology and applied under our laboratory conditions, induced behavioral despair that was reversible after chronic treatment program with fluoxetine. The FST therefore presents with sufficient sensitivity to detect the antidepressant-like actions of the positive control, the SSRI fluoxetine.

Time to onset of antidepressant-like response with fluoxetine. To investigate the role of α_2 -adrenoceptor antagonism in the time to onset of antidepressant-like responses, it was important to first determine the nominal treatment period required for the prototype antidepressant

fluoxetine to induce a measurable antidepressant-like response in rats in the FST, and a shorter treatment period where a significant response is absent.

Forced swim test: As depicted in Figure 13A, immobility was significantly reduced by fluoxetine as compared to vehicle treated control in the FST after 7 days ($100 \pm 4.8\%$ versus $45 \pm 2.9\%$, $p < 0.001$) and 11 days (100 ± 7.4 versus $39 \pm 4.4\%$, $p < 0.0001$), but not after 3 days ($100 \pm 9.7\%$ versus $93 \pm 11.3\%$, $p > 0.05$). Conversely, as shown in Figure 13C, swimming behavior was significantly increased compared to control after 7 days ($100 \pm 9.5\%$ versus $216 \pm 14.5\%$, $p < 0.0001$) and 11 days ($100 \pm 11.8\%$ versus $203 \pm 8.5\%$, $p < 0.0001$), but not after 3 days ($100 \pm 8.1\%$ versus $110 \pm 12.5\%$, $p > 0.05$). Data in Figure 13B show that climbing behavior was significantly increased after 7 days with fluoxetine as compared to control ($100 \pm 14.0\%$ versus $249 \pm 55.2\%$, $p = 0.0136$), while no significant changes were observed after 3 days ($100 \pm 15.5\%$ versus $94 \pm 21.2\%$, $p > 0.05$) or 11 days ($100 \pm 23.2\%$ versus $145 \pm 28.2\%$, $p > 0.05$).

Locomotor activity: There were no significant changes in locomotor activity after any treatment period with fluoxetine as compared to control (data not shown).

β -Adrenoceptor density: As depicted in Figure 13D β -adrenoceptor density was significantly reduced (roughly 45 to 50%) with fluoxetine treatment as compared to control after 7 days (547 ± 33.2 fmol/mg versus 305 ± 26.2 fmol/mg, $p = 0.0046$) and 11 days (596 ± 44.1 fmol/mg versus 296 ± 33.1 fmol/mg, $p = 0.0055$), but not after 3 days (532 ± 178.7 fmol/mg versus 800 ± 277.9 fmol/mg, $p = 0.5850$).

Taken together, the data in Figure 13 confirm that 7 days treatment with fluoxetine is sufficient to induce antidepressant-like responses in rats (i.e. changes in the measured parameters of the FST) and in β -adrenoceptor density in the frontal cortex, while the drug is unable to illicit this response within 3 days of treatment. No measurement of 5-HT_{1A} receptor concentration was done during this initial phase of establishing the time of onset of antidepressant-like response with the prototype antidepressant fluoxetine, since it is not a well distinguished marker of the onset of antidepressant-like action.

α_2 -Adrenoceptor antagonism and onset of antidepressant-like responses. *Forced swim*

test: It is evident from the data in Figure 14A that, as compared to vehicle treated control, immobility was reduced after 3 days of treatment only by mirtazapine alone ($100 \pm 2.7\%$ versus $74 \pm 7.0\%$, $p < 0.01$) and mirtazapine in combination with fluoxetine (100 ± 2.7 versus 68 ± 8.9 , $p < 0.01$). As depicted in Figure 14B, immobility was significantly reduced after 7 days treatment with all drugs or drug combinations, with the exception of idazoxan or idazoxan in combination with fluoxetine. In this regard it can be seen that a reduction in immobility, as compared to control, was induced by 7 days of treatment with fluoxetine ($100 \pm 4.1\%$ versus $61 \pm 9.8\%$, $p < 0.01$), yohimbine ($100 \pm 4.1\%$ versus $69 \pm 6.4\%$, $p < 0.01$), mirtazapine ($100 \pm 4.1\%$

versus $75 \pm 4.8\%$, $p < 0.05$), a combination of yohimbine with fluoxetine ($100 \pm 4.1\%$ versus $69 \pm 6.9\%$, $p < 0.01$), and a combination of mirtazapine with fluoxetine ($100 \pm 4.1\%$ versus $41 \pm 4.9\%$, $p < 0.01$). Importantly, a combination of mirtazapine with fluoxetine was superior to mirtazapine alone ($75 \pm 4.8\%$ versus $41 \pm 19.0\%$, $p < 0.05$) but not statistically significantly to fluoxetine alone ($61 \pm 9.8\%$ versus $41 \pm 4.9\%$, $p > 0.05$). A similar potentiation of effect was not seen with the combination of yohimbine with fluoxetine, which was not superior to yohimbine ($69 \pm 6.4\%$ versus $69 \pm 6.9\%$, $p > 0.05$) or fluoxetine ($61 \pm 9.8\%$ versus $69 \pm 6.9\%$, $p > 0.05$) alone. Interestingly, the combination of idazoxan with fluoxetine reversed reduction in immobility induced by fluoxetine alone ($61 \pm 9.8\%$ versus $101 \pm 8.5\%$, $p < 0.001$).

It is evident in Figure 14C that only the combination of mirtazapine and fluoxetine significantly increased climbing behavior after 3 days of treatment ($100 \pm 21.7\%$ versus $233 \pm 48.9\%$, $p < 0.05$) compared to control, while all other treatments failed to do so. Furthermore, it can be seen in Figure 14D that after 7 days of treatment, mirtazapine alone ($100 \pm 15.1\%$ versus 190 ± 41.7 , $p < 0.05$) and mirtazapine in combination with fluoxetine ($100 \pm 15.1\%$ versus $253 \pm 28.7\%$, $p < 0.01$) significantly increased climbing behavior compared to control. The combination of mirtazapine with fluoxetine was also superior to fluoxetine alone ($89 \pm 17.3\%$ versus $253 \pm 28.7\%$, $p < 0.001$) in increasing climbing behavior after 7 days.

It is evident from the data in Figure 14E that 3 days of treatment with mirtazapine ($100 \pm 13.3\%$ versus $226 \pm 37.6\%$, $p < 0.01$), mirtazapine in combination with fluoxetine ($100 \pm 13.3\%$ versus $255 \pm 39.7\%$, $p < 0.01$), as well as yohimbine ($100 \pm 13.3\%$ versus $207 \pm 22.1\%$, $p < 0.05$) increased swimming behavior compared to control. As depicted in Figure 14F, several treatments induced increases in swimming scores after 7 days of treatment compared to control. These include fluoxetine ($100 \pm 14.8\%$ versus $284 \pm 39.4\%$, $p < 0.01$), yohimbine ($100 \pm 14.8\%$ versus $258 \pm 28.8\%$, $p < 0.01$), yohimbine in combination with fluoxetine ($100 \pm 14.8\%$ versus $232 \pm 25.7\%$, $p < 0.01$), and mirtazapine in combination with fluoxetine ($100 \pm 14.8\%$ versus $285 \pm 18.2\%$, $p < 0.01$). In addition, the combination of mirtazapine with fluoxetine was superior to mirtazapine alone ($165 \pm 22.4\%$ versus $285 \pm 18.3\%$, $p < 0.05$), but not to fluoxetine alone ($284 \pm 39.4\%$ versus $285 \pm 18.3\%$, $p > 0.05$), regarding increased swimming after 7 days of treatment. Interestingly, the combination of idazoxan and fluoxetine suppressed the increase in swimming induced by fluoxetine alone ($284 \pm 39.4\%$ versus $111 \pm 28.4\%$, $p < 0.001$) after 7 days of treatment.

Locomotor activity: There were no significant alterations in locomotor activity (data not presented graphically) with any drug treatment or any treatment period compared to vehicle treated control. After 3 days treatment the locomotor activity was measured as 368.1 ± 94.5 (control); 426.0 ± 117.6 (fluoxetine); 328.2 ± 164.0 (yohimbine), 140.3 ± 30.9 (mirtazapine), 230.0 ± 137.1 (idazoxan); 173.0 ± 48.1 (fluoxetine + yohimbine); 174.7 ± 88.8 (fluoxetine +

mirtazapine); 139.0 ± 26.8 (fluoxetine + idazoxan). After 7 days treatment the locomotor activity was measured as 322.5 ± 88.7 (control); 478.7 ± 134.2 (fluoxetine); 216.7 ± 47.6 (yohimbine), 236.5 ± 63.3 (mirtazapine), 449.8 ± 162.5 (idazoxan); 167.2 ± 54.1 (fluoxetine + yohimbine); 126.5 ± 46.6 (fluoxetine + mirtazapine); 262.8 ± 79.3 (fluoxetine + idazoxan). When locomotor activity was numerically changed (not statistically significant), it was usually increased (rather than reduced) relative to control, thus excluding a role for decreased locomotor activity in any observed reduction of immobility in the FST.

β -Adrenoceptor density: As presented in Figure 15A, none of the treatments produced significant down-regulation of β -adrenoceptors in the frontal cortex after 3 days of treatment compared to control, except a combination of idazoxan with fluoxetine (760 ± 103.5 fmol/mg protein for control versus 278 ± 24.0 fmol/mg protein for the drug combination; $p < 0.05$). In contrast, all treatments produced significant down-regulation of β -adrenoceptors after 7 days of treatment compared to control (Figure 15B). These include control (722 ± 37.6 fmol/mg protein) versus fluoxetine (346 ± 81.9 fmol/mg protein; $p < 0.01$), yohimbine (420 ± 63.0 fmol/mg protein; $p < 0.01$), mirtazapine (457 ± 75.1 fmol/mg protein; $p < 0.05$), idazoxan (313 ± 39.1 fmol/mg protein; $p < 0.01$), yohimbine in combination with fluoxetine (472 ± 85.2 fmol/mg protein; $p < 0.05$), mirtazapine in combination with fluoxetine (283 ± 50.4 fmol/mg protein; $p < 0.01$), and idazoxan in combination with fluoxetine (273 ± 30.9 fmol/mg protein; $p < 0.01$).

5-HT_{1A} receptor density: As depicted in Figure 15C, none of the treatments produced a significant down-regulation of 5-HT_{1A} receptors after 3 days of treatment compared to control. After 7 days of treatment (Figure 15D), however, all drug combination treatments produced significant down-regulation of 5-HT_{1A} receptors in the hippocampus, while mono treatments still did not induce any significant changes in 5-HT_{1A} receptor density. These include control (446 ± 23.9 fmol/mg protein) versus yohimbine combined with fluoxetine (290 ± 36.6 fmol/mg protein; $p < 0.05$), mirtazapine combined with fluoxetine (254 ± 50.5 fmol/mg protein; $p < 0.01$) and idazoxan combined with fluoxetine (277 ± 19.13 fmol/mg protein; $p < 0.01$).

Discussion

Mirtazapine binds to α_2 -adrenoceptors with an affinity in the high nanomolar range. This measured affinity (K_i value) of mirtazapine at α_{2A} -adrenoceptors (Figure 11) corresponds well with that reported in the literature (de Boer, et al., 1988). This affinity value is also comparable with that of mianserin and brimonidine, but lower than seen with yohimbine and idazoxan (i.e. mirtazapine < idazoxan < yohimbine). The reported potency of (+)-mirtazapine for inhibiting the release of *I*-NE ($pA_2 = 8.35$) and 5-HT ($pA_2 = 8.13$) (de Boer, et al., 1988) suggests α_2 -adrenergic autoreceptor selectivity. However, these reported pA_2 values do not necessarily reflect drug-receptor dissociation constants (K_D), which requires specific kinetic conditions to be met (Neubig, et al., 2003).

Mirtazapine is a neutral antagonist or inverse agonist at α_2 -adrenoceptors. The lack of responses of any of the ligands in mock-transfected control neo cells (Figure 12) indicates an absence of endogenous mechanisms for cAMP inhibition in CHO-K1 cells. In transfected α_2 -H cells, only the agonist UK-14-304 (brimonidine) elicits a full dose-response curve. α_{2A} -H cells express a high concentration of α_{2A} -adrenoceptors (46.18 ± 5.23 pmol/mg protein) such that even partial agonists should yield a full response in these cells (Brink, et al., 2000). Since yohimbine, idazoxan, mirtazapine and mianserin did not elicit any responses in these cells, these ligands are not partial agonists at α_{2A} -adrenoceptors, but rather are neutral antagonists or inverse antagonists. It has been shown before in CHO-K1 cells expressing constitutively activated mutant α_{2A} -adrenoceptors, that yohimbine is a strong inverse agonist and idazoxan a neutral antagonist at α_{2A} -adrenoceptors (Wade, et al., 2001).

Fluoxetine induces antidepressant-like responses after 7 days, but not after 3 days in rats. Fluoxetine exerts an antidepressant-like effect in the FST in a time-dependent fashion (Figure 13A), significantly decreasing immobility after 7 days, but not after 3 days of treatment. The same time-dependency was seen with changes in climbing and swimming behavior (Figure 13B & Figure 13C). No changes in locomotor activity were observed at any time, excluding a possible role for increased locomotor activity in the FST response, and thus in agreement of earlier efficacy studies following chronic but not acute fluoxetine treatment (Vazquez-Palacios, et al., 2004). The data clearly simulates the delayed onset of antidepressant action observed in patients with depression, although species differences between rats and humans, as well as the differences between the FST and clinical depression, warrants cautious interpretation.

The observed parallel time-dependency in the fluoxetine-induced reduction of β -adrenoceptor density in the frontal cortex (Figure 13D), would indicate that the above behavioral changes may have an association with changes in β -adrenoceptor density, and which may represent a neurobiological marker for identifying early antidepressant activity. Indeed, fluoxetine and

desipramine together elicit an earlier antidepressant response in humans than either drug alone (Nelson, et al., 1991), while also demonstrating a more rapid downregulation of β -adrenoceptors (Baron, et al., 1988). Importantly, the data in Figure 13 suggest that any treatment that induces a measurable antidepressant-like response (behavioral and/or decreased β -adrenoceptor density) after 3 days of treatment, will have a more rapid onset of antidepressant-like response than fluoxetine.

Mirtazapine elicits earlier antidepressant-like responses and acts synergistically with fluoxetine in rats. Within the nominal 7 day treatment period, mirtazapine demonstrated antidepressant-like properties comparable to fluoxetine (Figure 14B), while a combination of fluoxetine with mirtazapine for 7 days was superior to mirtazapine alone, suggesting that fluoxetine augments the action of mirtazapine. A trend for superior response for this combination was also seen compared to fluoxetine alone.

Importantly, mirtazapine, as well as mirtazapine in combination with fluoxetine, significantly inhibited immobility after only 3 days (Figure 14A), which coincides with a putative earlier onset of antidepressant action for mirtazapine in humans (Wheatley, et al., 1998; Leinonen, et al., 1999; Benkert, et al., 2000; Benkert, et al., 2006). Mirtazapine is believed to antagonize α_2 -autoreceptors located on *I*-NE neurons in the locus coeruleus, leading to increased cell firing (de Boer, 1996) that in turn activates α_1 -heteroreceptors on 5-HT cell bodies in the raphe nuclei, leading to an increased 5-HT tone (Blier, 2003). Both these systems then project to critical mood regulating areas of the hippocampus. By blocking α_2 -autoreceptors and α_2 -heteroreceptors, mirtazapine prevents reactive dampening of *I*-NE and 5-HT release (Blier, 2003) resulting in the stimulation of postsynaptic 5-HT_{1A} receptors and α_1 -adrenoceptors located on pyramidal CA₃ neurons in the hippocampus. Increased swimming behavior, as seen for mirtazapine in Figure 14E & Figure 14F is most likely associated with such increased serotonergic activity (Lucki, 1997). Also, a significant increase in swimming after 3 days of treatment with mirtazapine compared to fluoxetine (Figure 14E) suggests an earlier postsynaptic serotonergic action (Lucki, 1997). for mirtazapine, supporting the hypothesis outlined above. Conversely, increased climbing behavior after 7 days of treatment with mirtazapine compared to fluoxetine (Figure 14D) correlates with the proposed noradrenergic actions (Lucki, 1997) of mirtazapine and the relative absence thereof with fluoxetine. The increased climbing behavior after 3 days of treatment with mirtazapine in combination with fluoxetine and a similar trend with mirtazapine alone (Figure 14C) suggest that fluoxetine may display synergism with mirtazapine. While the FST may have value in screening for time to onset of antidepressant action (depicted in Figure 13), caution should be advised when extrapolating these animal data to humans, since false positives and negatives have been reported for the FST (Porsolt, et al., 2000). Nevertheless,

these behavioral data support a quicker onset of action for mirtazapine. However, can this response be linked to α_{2A} -adrenoceptor antagonism?

α_{2A} -adrenoceptor antagonism may not be important for earlier onset of antidepressant action. The α_2 -adrenoceptor inverse agonist yohimbine displays antidepressant-like properties comparable to fluoxetine and mirtazapine after 7 days treatment (Figure 14B). The effects of yohimbine and fluoxetine are, however, not additive. That yohimbine alone, or in combination with fluoxetine, did not yield an antidepressant-like response after 3 days supports the notion that the addition of α_2 -lytic properties to the SRI action of fluoxetine does not hasten onset of action. If α_2 -lytic properties do in fact contribute significantly to antidepressant action, an inverse agonist at α_2 -adrenoceptors, such as yohimbine, should theoretically be more potent than a neutral antagonist and hence more effectively amplify the behavioral effects of fluoxetine. Also, yohimbine blocks α_2 -adrenoceptors with higher affinity than mirtazapine (as discussed above). These findings therefore do not support the current hypotheses regarding the important role of α_2 -adrenoceptor antagonism in the earlier onset of antidepressant action of mirtazapine.

That the α_2 -adrenoceptor neutral antagonist idazoxan alone does not yield any antidepressant (or depressogenic) action confirms the above conclusion. Importantly, the combination of idazoxan with fluoxetine reversed the antidepressant-like effects induced by fluoxetine alone, congruent with idazoxan preventing the effect of desipramine in the FST (Cervo, et al., 1990). Other explanations involve idazoxan's effects in the FST response. Firstly, idazoxan has agonistic properties at 5-HT_{1A} autoreceptors on 5-HT cell bodies, leading to inhibition of 5-HT synthesis in vivo (Llado, et al., 1996), which would oppose the increase in postsynaptic availability of 5-HT induced by fluoxetine. Secondly, idazoxan displays a 250-fold greater potency at α_2 -auto than α_2 -heteroreceptors (Preziosi, et al., 1989), causing the idazoxan-induced *I*-NE outflow (de Boer, et al., 1996; Tao and Hjorth, 1992) to negatively modulate the release of 5-HT.

β -adrenoceptor downregulation in the rat frontal cortex mostly support behavioral data. Only the idazoxan-fluoxetine combination downregulated β -adrenoceptors after 3 days (Figure 15A), corresponding with the corresponding general lack of inhibition of immobility (Figure 14A), except for mirtazapine alone and in combination with fluoxetine (displaying inhibited immobility but no parallel β -adrenoceptor downregulation) and the idazoxan-fluoxetine combination (displaying β -adrenoceptor but no parallel inhibition of immobility). In contrast, all treatments produced significant downregulation of β -adrenoceptors after 7 days of treatment (Figure 15B). Others (McGrath, et al., 1998) found a trend towards a mirtazapine-induced reduction in β -adrenoceptor density in rat cortex. Thus, although antidepressant treatment leads to a

decrease in cortical β -adrenoceptor density, such changes do not necessarily correlate with early behavioral changes in the FST.

Changes in 5-HT_{1A} density in the rat hippocampus do not correlate with antidepressant-like behavioral responses. None of the treatments produced down-regulation of 5-HT_{1A} receptors after 3 days of treatment (Figure 15C). Other studies have found that mirtazapine does not exert direct effects on 5-HT_{1A} receptors besides a reduction in 5-HT₂ receptor density (McGrath, et al., 1998). After 7 days of treatment, however, all combinations, but no single drug treatment, elicited significant down-regulation of 5-HT_{1A} receptors in the hippocampus (Figure 15D), suggesting that hippocampal 5-HT_{1A} receptor density is not indicative of antidepressant-like action.

Conclusions. The present study confirms an earlier onset of antidepressant-like action for mirtazapine in the FST. Furthermore, it confirms the α_2 -lytic action of mirtazapine (as neutral antagonist or inverse agonist), but suggests that these receptor actions are not important for its putative earlier onset of action, which may more likely result from complex, multifactorial pharmacological properties. These findings warrant further investigation, employing animal models of chronic depression.

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Footnotes

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Figure Legends

Figure 11 Competition-binding curves of brimonidine (A), idazoxan (B), mirtazapine (C) and mianserin (D) in α_{2A} -H cells against 5 nM [methyl- ^3H]-yohimbine. Results were calculated as a percentage of control and are expressed as mean $\pm$ SEM. The reported K_i values were calculated from the IC_{50} values, applying the Cheng-Prusoff correction.

Figure 12 Only brimonidine yields a dose-response curve in α_{2A} -H cells. Dose-response curves of brimonidine (A), yohimbine (B), idazoxan (C), mirtazapine (D) and mianserin (E) in α_{2A} -H cells and control neo cells, measuring cAMP production. Results were calculated as a percentage of control (no drug) and are expressed as mean $\pm$ SEM.

Figure 13 Fluoxetine produces antidepressant-like responses in the FST after 7 and 11 days, but not 3 days of treatment. Behavioral parameters were distinguished as immobility (A), climbing (B) and swimming (C), while a neuroreceptor response was measured as changes in β -adrenoceptor density (D). Results from the FST were calculated as a percentage of the respective vehicle treated control and are expressed as mean $\pm$ SEM. Receptor densities are calculated in terms of fmol/mg protein and are expressed as mean $\pm$ SEM. Statistics: All data were analyzed using a one-way ANOVA followed by a Dunnett post-test where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Figure 14 Behavioral effects in the FST are produced in distinct patterns and to a variable extent by fluoxetine (Flx), yohimbine (Yoh), mirtazapine (Mirt), idazoxan (Idaz), and combinations of fluoxetine with α_2 -adrenoceptor antagonists (Flx+Yoh; Flx+Mirt; Flx+Idaz). Behavioral parameters were distinguished after separate treatment periods: immobility after 3 (A) and 7 days (B); climbing after 3 (C) and 7 (D) days; and swimming after 3 (E) and 7 (F) days. Results are calculated as a percentage of the respective vehicle-treated control and are expressed as mean $\pm$ SEM. Statistics: One-way ANOVA followed by a Dunnett post-test (i.e. versus vehicle control) where $*p < 0.05$ and $**p < 0.01$, or a Tukey-Kramer post-test where $*p < 0.05$ and $***p < 0.001$.

Figure 15 Changes in cortical β -adrenoceptor concentration after 3 (A) and 7 days (B), and changes in hippocampal serotonin 5-HT $_1A$ -receptor concentration after 3 (C) and 7 days (D) of treatment with fluoxetine (Flx), yohimbine (Yoh), mirtazapine (Mirt), idazoxan (Idaz), and combinations of α_2 -adrenoceptor antagonists with fluoxetine (Flx+Yoh; Flx+Mirt; Flx+Idaz). Receptor densities are calculated in terms of fmol/mg protein and are expressed as mean $\pm$ SEM. Statistics: All data were analyzed using a one-way ANOVA followed by a Dunnett post-test (i.e. versus vehicle control) where $*p < 0.05$ and $**p < 0.01$.

FIGURES

Figure 1

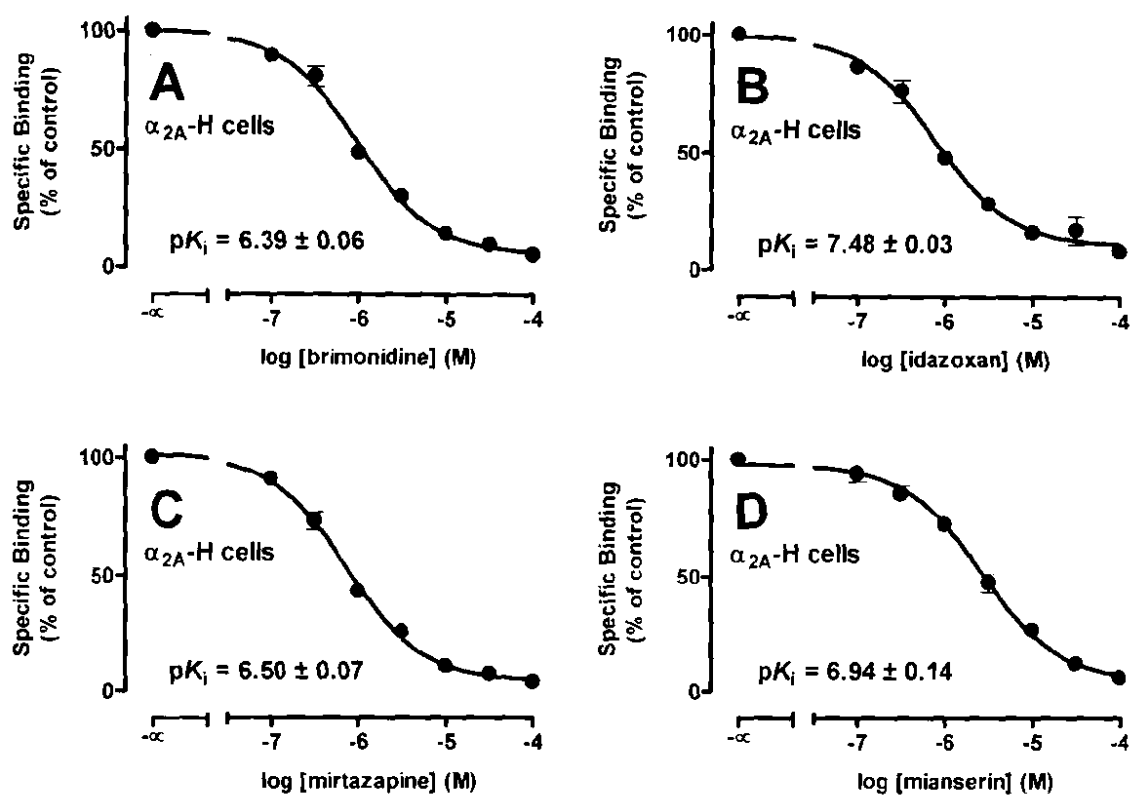


Figure 2

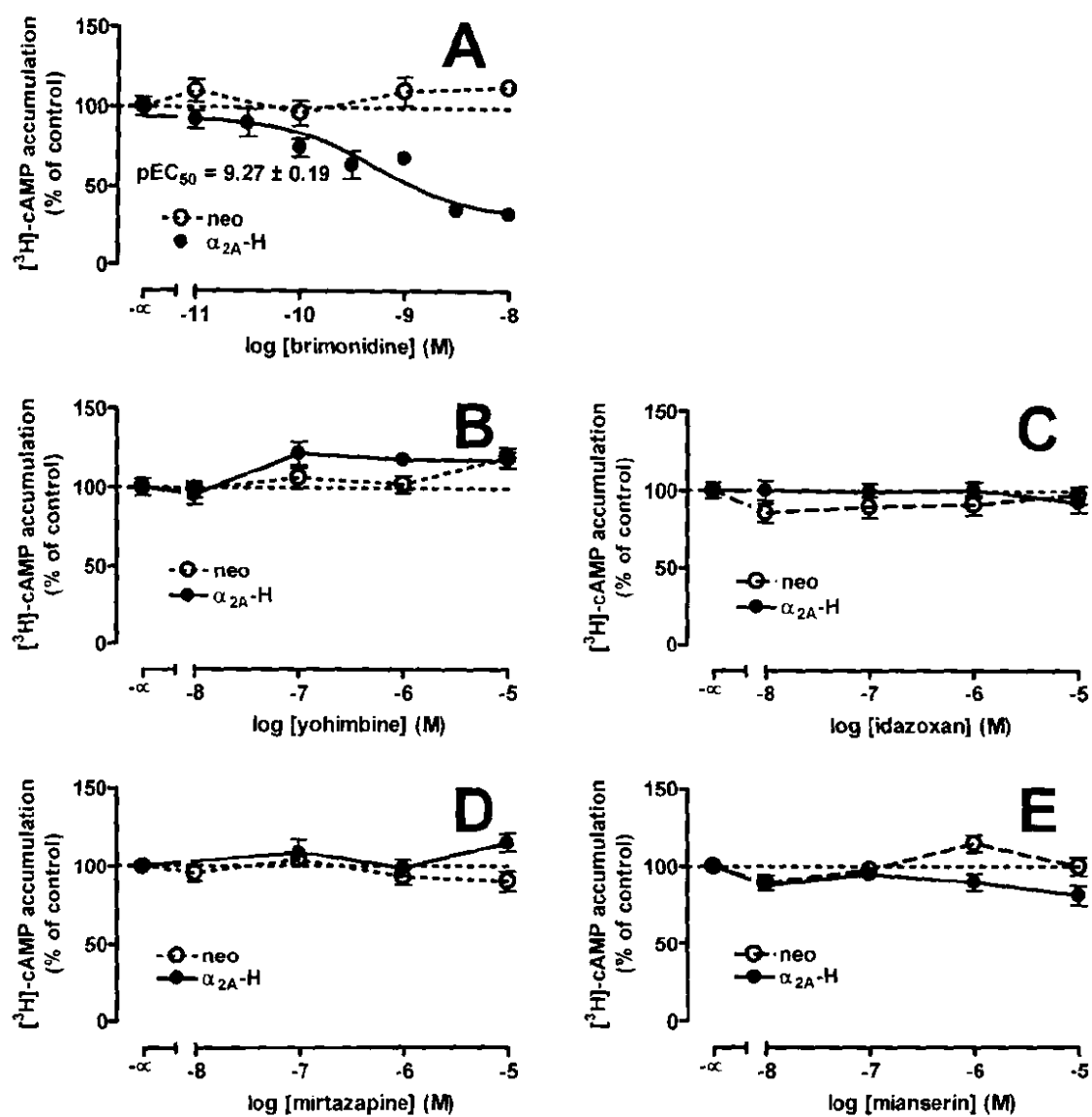


Figure 3

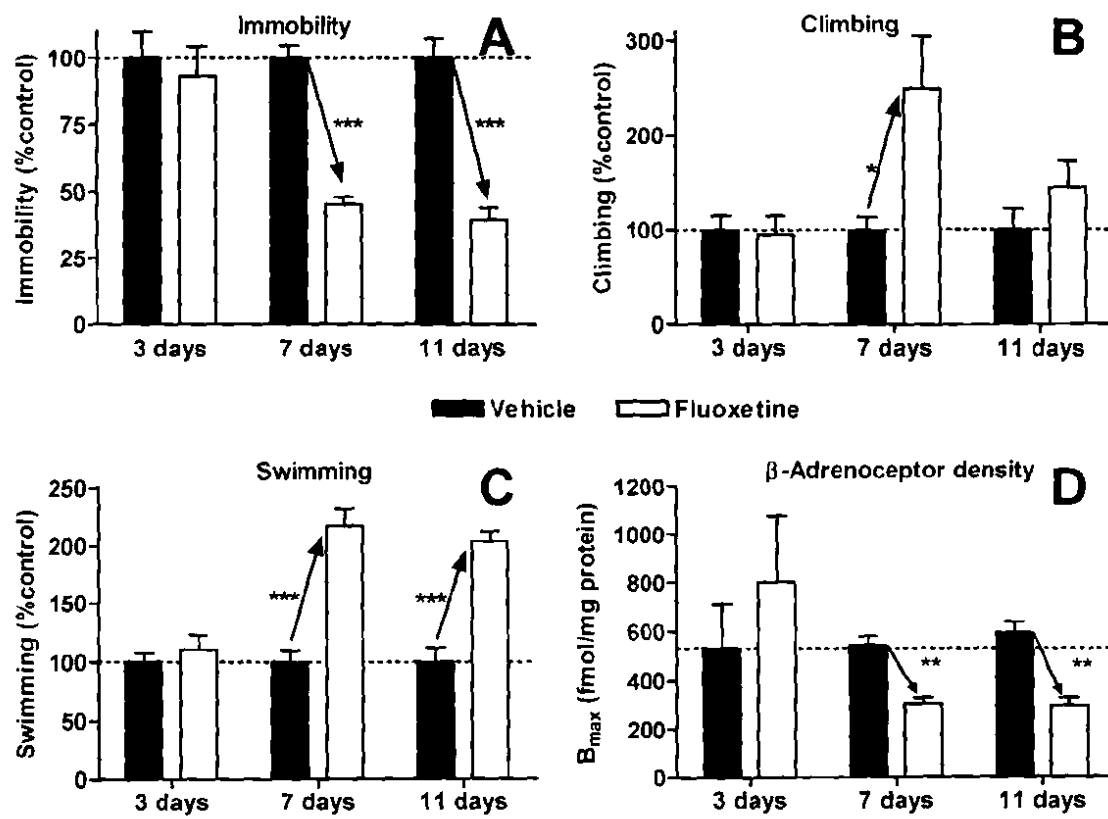


Figure 4

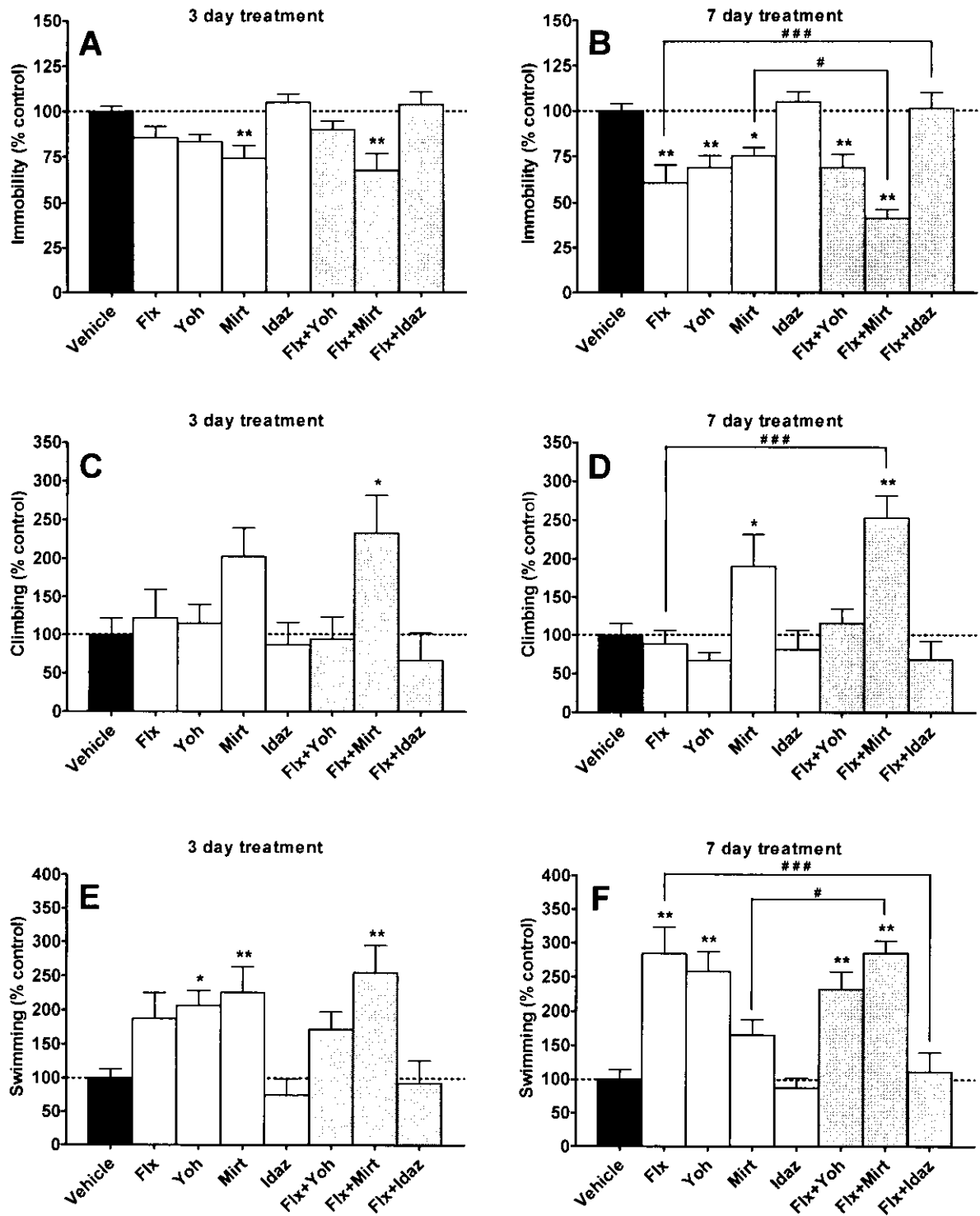


Figure 5

