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

NICOLIEBENBERG
(B.Pharm)

Dissertation submitted for the degree Magister Scientiae
in
Pharmacology
at the
North-West University (Potchefstroom campus).

Study leader: Prof. C.B. Brink
Study co-leader: Prof. B.H. Harvey

2006
Potchefstroom
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.

NICOLE 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-HT$_2$ and 5-HT$_3$ receptor blocking properties, while its putative earlier onset of action is thought to be related to its $\alpha_2$-adrenoceptor lytic action (at $\alpha_2$-adrenergic autoreceptors and heteroreceptors), thereby modulating central serotonergic and noradrenergic neurotransmission.

The current study investigated the role of $\alpha_2$-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 $\beta$-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 $\alpha_2$-adrenoceptor antagonism for earlier onset of action, rats were treated for 3 and 7 days with fluoxetine, mirtazapine ($\alpha_2$-lytic mode unknown), yohimbine ($\alpha_2$-adrenoceptor inverse agonist), idazoxan ($\alpha_2$-adrenoceptor neutral antagonist), or combinations with fluoxetine, where after the FST was employed and cortical $\beta$-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 $\alpha_2$-lytic action in this regard. $\beta$-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 $\alpha_2$-adrenoceptor-lytic action may be important for the earlier onset of action by mirtazapine.
Abstrak

Tervyler '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 α₂-adrenoseptor litiese aksie (by α₂-autoreseptore en heteroreseptore), en hierdeur sentrale serotonergiese, asook noradrenergieuse neurotransmissie moduleer.

In die huidige studie is die rol van α₂-adrenoseptor antagonistisme 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. Daar is effekte wat na 3 dae waargeneem word in hierdie studie as vroë effekte beskou.

Rotte was behandel vir 3 en 7 dae met fluoksetien, mirtasepien (wyse van α₂-litiese werking onbekend), yohimbien (α₂-adrenoseptor inverse agonis), idazoxan (α₂-adrenoseptor neutrale antagonist), of kombinasies van hierdie middels met fluoksetien. Na behandeling is die geforseerde swem toets en meting van β-adrenoseptor en 5-HT₁A 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 α₂-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-HT₁A 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 α₂-litiese eienskappe nie, 'n belangrike rol speel in die versnelde aanvang van antidepressante werking van mirtasepien.
First and foremost, I thank God for giving me the opportunity and perseverance to overcome the obstacles during this study and to be able to do what I love. Without Him nothing is possible.

To my study leader and mentor, Prof. C.B. Brink, my greatest appreciation for your guidance and support during this study, and for sharing your valuable knowledge with me.

To my study co-leader, Prof. B.H. Harvey, for your valuable insights and proposals during this study.

To Prof. L. Brand for your support and advice.

To Sharlene Nieuwoudt, for your assistance inside and outside the laboratory.

To my colleague Hannes Clapton, for your friendship and patience with my occasional obsessive habits! It was a pleasure working with you.

To my family and girlfriend, Adele, for your endless love and encouragement.

To my friends and colleagues (Charise, Ilse, Benno, Leani, George, and Carl) for your friendship and for making the workplace such an enjoyable place to be.

To the National Research Foundation (NRF) for funding.
Table of contents

Abstract...............................................................................................................................i
Abstrak..............................................................................................................................ii
Acknowledgements............................................................................................................iii
Table of contents....................................................................................................................iv
List of figures........................................................................................................................x
List of tables........................................................................................................................xiv

CHAPTER 1: INTRODUCTION..............................................................................................1
  1.1 PROBLEM STATEMENT..............................................................................................1
  1.2 STUDY AIMS...............................................................................................................2
  1.3 STUDY LAYOUT..........................................................................................................2

CHAPTER 2: LITERATURE OVERVIEW..............................................................................4
  2.1 DEPRESSION...............................................................................................................4
    2.1.1 Diagnosis of depression ......................................................................................5
    2.1.2 Aetiology of depression ......................................................................................6
    2.1.3 Neuroanatomy implicated in depression ..............................................................7
    2.1.4 Neuropathological hypotheses of depression .....................................................10
      2.1.4.1 Monoamine hypothesis ................................................................................10
      2.1.4.2 Dysregulation of the hippocampus and hypothalamic-pituitary-adrenal axis .. 13
      2.1.4.3 Impairment of neurotrophic mechanisms .....................................................15
2.1.4.4 Impairment of brain reward pathways .......................................................... 17

2.2 DRUG TREATMENT OF DEPRESSION ............................................................... 19

2.2.1 Alteration of monoaminergic neurotransmission ............................................. 19

2.2.1.1 Tricyclic antidepressants ............................................................................. 22

2.2.1.2 Selective serotonin reuptake inhibitors ..................................................... 24

2.2.1.3 Atypical antidepressants .......................................................................... 26

2.2.1.4 Other α2-AR antagonists .......................................................................... 29

2.2.2 Alteration of neurogenesis ............................................................................ 30

2.3 ONSET OF ACTION OF ANTIDEPRESSANTS ................................................. 31

2.3.1 Delayed onset of antidepressant action ......................................................... 31

2.3.2 Pharmacological strategies for earlier onset of action .................................... 31

2.3.2.1 Mirtazapine ............................................................................................... 32

2.3.2.2 Venlafaxine ............................................................................................... 33

2.3.2.3 Nefazodone .............................................................................................. 33

2.3.2.4 SSRI augmentation with pindolol .............................................................. 33

2.3.3 Clinical evidence for early onset of antidepressants ...................................... 34

2.4 α2-ARs: Ligand actions and signalling mechanisms ........................................... 34

2.4.1 Background information on signal transduction systems ............................. 34

2.4.2 Distribution and function of α2-AR receptors .............................................. 35

2.4.3 Signal transduction of the α2-AR .................................................................. 36

2.4.4 Inverse agonism and the α2-AR receptor ..................................................... 36

2.4.4.1 Constitutive activity and inverse agonism at G-protein coupled receptors ... 36

2.4.4.2 Inverse agonism at α2-ARs in neuronal cells .......................................... 38
2.5 PRECLINICAL EVALUATION OF THE ACTION OF ANTIDEPRESSANT DRUGS .. 39

2.5.1 Animal models of depression ........................................................................................................... 39

2.5.1.1 Overview of animal models of depression ................................................................. 39
2.5.1.2 The Rat Forced Swim Test .................................................................................. 40
2.5.1.3 Learned helplessness .......................................................................................... 44
2.5.1.4 Genetic rat model of depression .......................................................................... 44

2.5.2 Alterations in $\beta$-AR concentration following antidepressant treatment .................. 44

2.6 SYNOPSIS ................................................................................................................................. 45

CHAPTER 3: MATERIALS AND METHODS .................................................................................. 47

3.1 OVERVIEW ............................................................................................................................... 47

3.2 ANIMALS AND MATERIALS USED ...................................................................................... 48

3.2.1 Animals ................................................................................................................................. 48
3.2.2 Drugs ....................................................................................................................................... 48
3.2.3 Radio-chemicals .................................................................................................................. 48
3.2.4 Other chemicals ................................................................................................................... 48
3.2.5 Instruments .......................................................................................................................... 49
3.2.6 Other materials .................................................................................................................... 49

3.3 PROJECT LAYOUT ................................................................................................................... 49

3.3.1 Pilot Study 1: Lab-validation of the rat forced swim test (FST) ........................................ 49
3.3.2 Pilot Study 2: Time-dependency of the antidepressant-like action of fluoxetine ......... 51
3.3.3 Experimental Study: $\alpha_2$-AR receptor antagonism and onset of antidepressant-like responses ................................................................. 53

3.4 DOSAGE CHOICES FOR THE DRUGS EMPLOYED ................................................................ 56

3.4.1 Principles of dosage selection in animal models .............................................................. 56
3.4.2 Fluoxetine ............................................................................... 57
   3.4.2.1 Doses in earlier studies ......................................................... 57
   3.4.2.2 Receptor binding profile ...................................................... 57
   3.4.2.3 Dose selection ...................................................................... 57
3.4.3 Mirtazapine ........................................................................... 58
   3.4.3.1 Doses in earlier studies ........................................................ 58
   3.4.3.2 Receptor binding profile ...................................................... 59
   3.4.3.3 Dose selection ...................................................................... 60
3.4.4 Idazoxan ................................................................................. 60
   3.4.4.1 Doses in earlier studies ........................................................ 60
   3.4.4.2 Receptor binding profile ...................................................... 60
   3.4.4.3 Dose selection ...................................................................... 61
3.4.5 Yohimbine ............................................................................... 61
   3.4.5.1 Doses in earlier studies ........................................................ 61
   3.4.5.2 Receptor binding profile ...................................................... 61
   3.4.5.3 Dose selection ...................................................................... 62
3.5 EXPERIMENTAL PROTOCOLS ......................................................... 62
   3.5.1 Drug administration ................................................................. 62
   3.5.2 The rat Forced Swim Test (FST) ............................................... 63
      3.5.2.1 Validation of the rat FST in our laboratory ......................... 63
      3.5.2.2 Detection of antidepressant-like responses ....................... 63
   3.5.3 Decapitation and dissection .................................................... 64
   3.5.4 Measurement of locomotor activity ................................. 64
3.5.5 Radio-ligand saturation binding studies ................................................................. 65

3.5.5.1 Preparation of membrane suspensions from brain tissue ................................. 65

3.5.5.2 Measurement of protein concentration: The Bradford method (Bradford, 1976) ................................................................. 66

3.5.5.3 Measurement of β-AR density .......................................................................... 67

3.5.5.4 Measurement of 5-HT₁A receptor density ......................................................... 68

3.5.5.5 Calculations ..................................................................................................... 69

3.6 DATA ANALYSIS .................................................................................................... 70

CHAPTER 4: RESULTS AND DISCUSSION ........................................................................ 71

4.1 PILOT STUDY 1: VALIDATION OF THE RAT FORCED SWIM TEST (FST) ........ 72

4.1.1 Development of behavioural despair .................................................................... 72

4.2 PILOT STUDY 2: Time-dependency of the antidepressant-like action of fluoxetine .. 74

4.2.1 The Forced Swim Test ......................................................................................... 74

4.2.2 β-AR concentration ............................................................................................ 75

4.3 EXPERIMENTAL STUDY: α₂-AR receptor antagonism and onset of antidepressant-like responses ................................................................. 76

4.3.1 The Forced Swim Test (FST) ............................................................................. 76

4.3.1.1 Interactions of α₂-AR antagonists with fluoxetine in the FST ......................... 81

4.3.2 Changes in β-AR and serotonin 5-HT₁A receptor concentration ....................... 85

4.3.2.1 β-AR concentration ....................................................................................... 85

4.3.2.2 Serotonin 5-HT₁A-receptor concentration .................................................... 86

CHAPTER 5: CONCLUSION ............................................................................................. 87

5.1 SUMMARY OF RESULTS ....................................................................................... 87

5.2 CONCLUSIONS .................................................................................................... 89
List of figures

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).................................................................................................................. 9

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). ............................................................................................................. 14

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). .............................................. 20

Figure 2-4: The chemical structure of some tricyclic antidepressants ........................................................................................................................................................................... 23

Figure 2-5: The chemical structure of some selective serotonin inhibitors ................................................................................................................................. 25

Figure 2-6: The chemical structure of some atypical antidepressants ................................................................................................................................. 26

Figure 2-7: The spectrum of efficacy of drugs acting at GPCRs. (R̅) 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 R̅, thereby decreasing the basal activity of the receptor system. .................. 37

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) .................................................................................................. 38
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) .......................................................... 41

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 utilised 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. .......................................................... 50

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. .......................................................... 51

Figure 3-3: Schematic illustration of the treatment timescale for Pilot Study 2. .................. 53

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. .......................................................... 54

Figure 3-5: Schematic illustration of the treatment timescale for the Experimental Study. ........ 56

Figure 3-6: Example of results obtained with a single saturation binding study of [3H]-8-OHDPAT to 5-HT₁A receptors. The (A) binding curve depicts total binding, non-specific binding (defined with 10 μM serotonin), and specific binding (calculated by subtracting non-specific binding from total binding). The raw values of Bₘₐₓ and Kᵦ are indicated on the graph. The (B) standard curve was obtained by measuring the total radioactivity of 10 μl of each radio-ligand concentration.......................................................... 69

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 ± standard error of the mean, where *** represents p < 0.001 .......................................................... 72

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 ± standard error of the mean, where *** represents p < 0.001. .......................................................... 73
List of figures xii

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 ± standard error of the mean, where * represents p < 0.05 and *** represents p < 0.001.

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 (Bmax) is expressed in terms of fmol receptors per mg protein, and as the mean ± standard error of the mean where ** represents p < 0.01.

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 ± standard error of the mean.

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 ± standard error of the mean, where * represents p < 0.05, and *** represents p < 0.001 (Tukey, see Figure 4-5).

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 and (B) 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 ± standard error of the mean, where * represents p < 0.05, and *** represents p< 0.001 (Tukey, see Figure 4-5).
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 ± standard error of the mean, where * represents p < 0.05, and *** represents p < 0.001 (Tukey, see Figure 4-5).

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 (Bmax) is expressed in terms of fmol receptors per mg protein, and as mean ± standard error of the mean, where * represents p < 0.05 and ** represents p < 0.01.

Figure 4-10: Effects on serotonin 5-HT1A-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-HT1A-receptor density (Bmax) is expressed in terms of fmol receptors per mg protein, and as mean ± standard error of the mean, where * represents p < 0.05 and ** represents p < 0.01.
List of tables

Table 2-1: Diagnostic criteria for major depression ................................................................. 5
Table 2-2: Examples of proposed subtypes of depression ......................................................... 6
Table 2-3: Potencies of antidepressants at human transporters for monoamines ............... 22
Table 2-4: Receptor binding profiles of atypical antidepressants compared to serotonin reuptake inhibitors .................................................................................................................... 27
Table 2-5: Receptor binding profile mirtazapine .................................................................. 29
Table 3-1: Treatment regimes in Pilot Study 2 .................................................................... 52
Table 3-2: Treatment regimes for the Experimental Study .................................................. 55
Table 3-3: Receptor binding profile of fluoxetine ................................................................. 58
Table 3-4: Receptor binding profile of mirtazapine .............................................................. 59
Table 3-5: Receptor binding profile of idazoxan ................................................................. 61
Table 3-6: Receptor binding profile of yohimbine ............................................................... 62
Table 3-7: Buffers used for radio-ligand saturation binding assays .................................. 65
Table 3-8: Preparation of protein standards ......................................................................... 66
Table 5-1: Effects of antidepressants in the rat FST after 3 and 7 days of treatment ........ 87
Table 5-2: Interactions of α2-lytic drugs with fluoxetine in the rat FST .............................. 88
Table 5-3: Effects on cortical β-AR density ......................................................................... 88
Table 5-4: Effects on 5-HT1A receptor density .................................................................... 89
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 (Blier, 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 α2-adrenoceptor (α2-AR) blocking property is also believed to be a major mechanism by which it elicits an antidepressant response (de Boer, 1996). In addition, the α2-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 $\alpha_2$-lytic properties of mirtazapine in its putative earlier onset of antidepressant-like action; and

- Explore whether an inverse agonist at $\alpha_2$-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 $\beta$-adrenoceptor ($\beta$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-AR inverse agonist yohimbine (3 mg/kg/day), and the $\alpha_2$-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 $\alpha_2$-AR antagonism in eliciting an earlier onset of antidepressant-like action.
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 α2-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>
<thead>
<tr>
<th>Diagnostic criteria for major depression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depressed mood</td>
</tr>
<tr>
<td>Irritability</td>
</tr>
<tr>
<td>Low self-esteem</td>
</tr>
<tr>
<td>Feelings of hopelessness, worthlessness, and guilt</td>
</tr>
<tr>
<td>Decreased ability to concentrate and think</td>
</tr>
<tr>
<td>Decreased or increased appetite</td>
</tr>
<tr>
<td>Weight loss or weight gain</td>
</tr>
<tr>
<td>Insomnia or hypersomnia</td>
</tr>
<tr>
<td>Low energy levels, fatigue, or increased agitation</td>
</tr>
<tr>
<td>Decreased interest in pleasurable stimuli (e.g., sex, food, social interactions)</td>
</tr>
<tr>
<td>Recurrent thoughts of death and suicide</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Depression subtype</th>
<th>Main features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melancholic depression¹</td>
<td>Severe symptoms; prominent neurovegetative abnormalities</td>
</tr>
<tr>
<td>Reactive depression²</td>
<td>Moderate symptoms apparently in response to external factors</td>
</tr>
<tr>
<td>Psychotic depression</td>
<td>Severe symptoms; associated with psychosis</td>
</tr>
<tr>
<td>Atypical depression</td>
<td>Associated with labile mood, hypersomnia, increased appetite, and weight gain</td>
</tr>
<tr>
<td>Dysthymia</td>
<td>Milder symptoms, but with a more protracted course</td>
</tr>
</tbody>
</table>

¹Melancholic depression is similar to a syndrome classified as "endogenous depression".  
²Reactive 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 no 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 I-NE (e.g. imipramine and amitriptyline), while others are more selective in blocking the reuptake of 5-HT (e.g. clomipramine) or I-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 α2-ARs. However, the adverse effects of this antidepressant, namely postural hypotension and sedation, have been attributed to its antagonistic action on α1-ARs and histaminergic H1 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 (SSRls), e.g. fluoxetine and paroxetine were introduced in the early 1990s (Fuller, 1995). During the same period, the highly potent α2-AR antagonist mirtazapine (Smith et al., 1990) was discovered. The last antidepressant introduced by the end of the last century was the highly selective I-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, SSRls and NRls) 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 α2 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 α1-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 α2-AR sensitivity after chronic therapy with antidepressants have also been reported. The inhibitory α2-heteroreceptors are believed to control 5-HT release from the serotonergic nerve terminal (Limberger et al., 1986), while α2-autoreceptors control the release of l-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 l-NE and 5-HT at the synapse.

In summary, the chronic administration of antidepressants desensitises α2-ARs and increases the sensitivity of stimulatory α1-ARs, thus increasing the release of both 5-HT and l-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-HT2A receptors are not very consistent. Several studies in rodents have shown a decrease in the number or function of 5-HT2A 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.
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).

![Diagram of the hypothalamic-pituitary-adrenal (HPA) axis.](image)

**Figure 2-2: The hypothalamic-pituitary-adrenal (HPA) axis.** Abbreviations: CRH = corticotropin-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; Sklair tavoron & 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-\(\alpha\)) (Tuglu et al., 2003), interleukin-1 beta (IL-1\(\beta\)) (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-\(\alpha\)) 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-\(\alpha\)-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 1-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 (l-DA) release in the NAc and onset of antidepressant action (Dremencov et al., 2004; Dremencov et al., 2005). The study proposes that 5-HT2c receptors might be hyperfunctional in depressed individuals, where these receptors play an inhibitory role in serotonin-induced l-DA release in the NAc. It is suggested that the onset of an antidepressant-like response is dependent on the reversal of 5-HT2c receptor hyperfunctionality, which would allow 5-HT to effectively increase l-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 [1-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 \( \alpha_2 \) 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 1-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.

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 1-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 α2-AR and 5-HT1A 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 α1-heteroreceptors on the raphe cell bodies. These synapses are also regulated by α2-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-HT1A and α1-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-HT2A 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

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

Note: Potency is expressed as inhibition constant (Ki) 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 l-NE transport, while most tertiary-amine tricyclic compounds inhibit 5-HT reuptake in addition to l-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).
Chapter 2: Literature overview

The tricyclic antidepressants do not block L-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 D_2-receptors (autoreceptors), which could lead to enhanced forebrain L-DA neurotransmission, and this, together with the inhibition of transport of L-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 L-NE receptors. Most tricyclic antidepressants have at least moderate affinity for α₁-ARs, much less for α₂-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 L-NE and/or 5-HT levels (Frazer, 1997b). These presumably include indirect activation of postsynaptic α₁-ARs and 5-HT₁A receptors located on pyramidal neurons of the hippocampus (Figure 2-3).

An acute increase in postsynaptic L-NE availability would lead to enhanced stimulation of α₂-ARs on L-NE cell bodies and nerve terminals. These α₂-ARs would include presynaptic autoreceptors which inhibit L-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 l-NE through the rate-limiting step at tyrosine hydroxylase, presumably through \(\alpha_2\)-AR attenuation of cAMP-mediated phosphorylation (Baldessarini, 2001).

The \(\alpha_2\)-AR negative feedback response is rapidly activated upon administration of tricyclic antidepressants, however, with repeated drug exposure, \(\alpha_2\)-AR responses are eventually reduced, which may be a result of desensitisation secondary to increased exposure to l-NE (Baldessarini, 2001). This adaptation allows the presynaptic production and release of l-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 l-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 l-NE or l-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-HT\(_3\) 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).
A feature of both 5-HT and l-NE neurons is that negative feedback mechanisms rapidly emerge to restore homeostasis (Blier, 2003). In the 5-HT system, 5-HT₁A and 5-HT₁B 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-HT₁B 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-HT₂A receptors that may contribute to antidepressant effects, and indirect enhancement of l-NE transmission by a reduction of tonic inhibitory effects of 5-HT₂A 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.

![Chemical structures of Mirtazapine, Venlafaxine, Reboxetine, and Nefazodone](image)

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 (\(\alpha_1\)- and \(\alpha_2\)-ARs, histaminergic 1 (H\(_1\)) receptors, and muscarinic acetylcholine (mAch) receptors), compared to those of some SSRIs. The affinities for l-NE and 5-HT transporters of these drugs are displayed in Table 2-3.
Chapter 2: Literature overview

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Receptor affinity $K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$a_1$</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Nefazodone</td>
<td>42</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>372</td>
</tr>
<tr>
<td>Reboxetine</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>5900</td>
</tr>
<tr>
<td>Sertraline</td>
<td>300</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Citalopram</td>
<td>4500</td>
</tr>
</tbody>
</table>

(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 $l$-NE reuptake properties of many tricyclic antidepressants (Table 2-3), it lacks anticholinergic, antihistaminic, and $\alpha_1$-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 $l$-NE reuptake (Table 2-3), but is a potent antagonist at 5-HT$_2$ 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 $\alpha_1$-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 $l$-NE or 5-HT, or inhibit monoamine oxidase, mirtazapine potentiates both $l$-NE and 5-HT neurotransmission at selected receptor subtypes. Its principal mechanism of antidepressant action is thought to be the
antagonism of α₂-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

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Tissue</th>
<th>Affinity (pA₂ or pKᵢ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-NE interactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha₂ )-autoreceptor</td>
<td>Synaptosomes</td>
<td>7.7</td>
</tr>
<tr>
<td>( \alpha₂ )-heteroreceptor</td>
<td>Synaptosomes</td>
<td>8</td>
</tr>
<tr>
<td>( \alpha₂ )-postsynaptic</td>
<td>Rat cortex</td>
<td>7.3</td>
</tr>
<tr>
<td>( \alpha₂ )-presynaptic</td>
<td>Rat vas deferens</td>
<td>6.8</td>
</tr>
<tr>
<td>( \alpha₁ )-adrenoceptor</td>
<td>Rat cortex</td>
<td>6.3</td>
</tr>
<tr>
<td>( \beta₁ )-adrenoceptor</td>
<td>Rat vas deferens</td>
<td>6.5</td>
</tr>
<tr>
<td>( \beta₂ )-adrenoceptor</td>
<td>Rat atrium</td>
<td>&lt; 4.5</td>
</tr>
<tr>
<td>I-NE -transporter</td>
<td>Synaptosomes</td>
<td>5.8</td>
</tr>
<tr>
<td>5-HT interactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT₁A receptor</td>
<td>Rat hippocampus</td>
<td>5.3</td>
</tr>
<tr>
<td>5-HT₁B receptor</td>
<td>Rat striatum</td>
<td>4.9</td>
</tr>
<tr>
<td>5-HT₂A receptor</td>
<td>Pig striatum</td>
<td>5.3</td>
</tr>
<tr>
<td>5-HT₂B receptor</td>
<td>Rat cortex</td>
<td>8.2</td>
</tr>
<tr>
<td>5-HT₂C receptor</td>
<td>Rat fundus</td>
<td>6.7</td>
</tr>
<tr>
<td>5-HT₃ receptor</td>
<td>Pig choroid plexus</td>
<td>7.9</td>
</tr>
<tr>
<td>5-HT₃ receptor</td>
<td>Mouse</td>
<td>8.1</td>
</tr>
<tr>
<td>5-HT₄ receptor</td>
<td>neuroblastoma</td>
<td></td>
</tr>
<tr>
<td>5-HT₅ receptor</td>
<td>Mouse</td>
<td>7.1</td>
</tr>
<tr>
<td>5-HT transporter</td>
<td>Rat esophagus</td>
<td>&lt; 4.5</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>Synaptosomes</td>
<td>&lt; 4.5</td>
</tr>
</tbody>
</table>

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

2.2.1.4 Other \( \alpha₂ \)-AR antagonists

The \( \alpha₂ \)-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 \( \alpha₂ \)-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 \( \alpha₂ \)-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 $\text{i}2$-imadozoline receptors (Miralles et al., 1993), and acts agonistically at $5\text{-HT}_{1\text{A}}$ receptors (Kawai et al., 1994).

It has been demonstrated that idazoxan increases the synthesis of $l$-NE in the cerebral cortex and hippocampus of rats following the powerful blockade of $\alpha_2$-autoreceptors but, in contrast, decreases $5\text{-HT}$ synthesis in these brain regions via stimulation of presynaptic $5\text{-HT}_{1\text{A}}$ 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 $l$-NE and $5\text{-HT}$ 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 $\alpha_2$-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 $\alpha$-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 $5\text{-HT}_{1\text{A}}$ 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 I-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 I-NE allowed to be effectively increased to alleviate depressive symptoms. Putative
Chapter 2: Literature overview

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

2.3.2.1 Mirtazapine

Mirtazapine enhances 5-HT and l-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 l-NE neurotransmission

As described in § 2.2.1 and as depicted in Figure 2-3, l-NE transmission is under negative feedback control via $\alpha_2$-autoreceptors, which, when stimulated inhibit the release of l-NE. Mirtazapine blocks the cell body autoreceptors and thereby uncouples the feedback inhibition on l-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 $\alpha_2$-autoreceptors which reduce the amount of l-NE released. However, these receptors are also blocked by mirtazapine, and this permits the increase in l-NE neurotransmission (Figure 2-3). This increase in l-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 l-NE neurotransmission in terminal projection areas. These include projections to the dorsal raphe where it stimulates 5-HT neurotransmission via indirect activation of $\alpha_2$-heteroreceptors on 5-HT cell bodies secondary to an increased release of l-NE (Figure 2-3). This enhancing effect would normally be dampened by the activation of $\alpha_2$-heteroreceptors on 5-HT nerve terminals (Figure 2-3) resulting from the enhanced release of l-NE by mirtazapine. However, these receptors are also blocked by mirtazapine (de Boer, 1996).

This modulation of 5-HT and l-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 l-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-HT₁A and 5-HT₁B receptors (Barnes & Sharp, 1999). One study has suggested that pindolol may block, specifically, the inhibitory 5-HT₁A autoreceptors on the cell bodies of 5-HT neurons, and less effectively, the postsynaptic 5-HT₁A receptors in the hippocampus (Romero & Artigas, 1997). This is an important property since postsynaptic 5-HT₁A 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-HT₁A autoreceptors) normally occurring with SSRIs (see § 2.2.1.2). Therefore, by giving pindolol, desensitisation of 5-HT₁A 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-HT₁A receptor agonistic
property. It was indeed shown that a 21 day treatment can desensitise the rat 5-HT\textsubscript{1A}
autoreceptor in the dorsal raphe (Haddjeri & Blier, 2000). Therefore, while preventing an
excessive activation of 5-HT\textsubscript{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 (Dremerov 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\textsubscript{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 adenylate 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 (Gq proteins, which activate adenylate cyclase), pertussis toxin (Gi and Gq proteins, which inhibit adenylyl cyclase and Ca2+ channels, as well as promote K+ channel function) or neither (Gq proteins, which stimulate phospholipase C activity) (Simon et al., 1991). G₁₂/₁₃ 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 l-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 α₂-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 α₂-AR receptors

The α₂-ARs couples to G1 proteins (GPCRs). The amino sequences for three distinct subtypes of α₂-ARs (i.e. α₂A, α₂B, and α₂C) have been deduced (Bylund, 1992). These receptors are located in the periphery on pancreatic island cells (β-cells) where they inhibit insulin secretion and on platelets where they promote aggregation, on nerve terminals where they decrease the release of l-NE, and on vascular smooth muscle where they mediate contraction (Hoffman & Taylor, 2001). Furthermore, α₂-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 α₂A-AR subtype has an important role in the regulation of the central sympathetic nervous system. As mentioned above, activation of α₂-ARs inhibits the release of l-NE from nerve endings. Therefore, activation of α₂A-ARs in the central nervous system inhibits sympathetic
nervous system activity, while blockade of $\alpha_{2A}$-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 $\alpha_2$-ARs in this dissertation in the context of presynaptically located receptors on nerve cells, is indeed of the $\alpha_{2A}$-AR subtype.

### 2.4.3 Signal transduction of the $\alpha_2$-AR

$\alpha_2$-ARs couple to a variety of effectors (Bylund, 1992; Aantaet 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). $\alpha_2$-ARs are also known to inhibit voltage-gated $Ca^{2+}$ channels, mediated via $G_0$ proteins.

### 2.4.4 Inverse agonism and the $\alpha_2$-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 commonly accepted that GPCRs exist 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.](image)

(R0) 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 R0, 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 R0, thereby shifting the equilibrium towards R0, stabilising this conformation and reducing Gi protein coupling. Neutral antagonists have equal affinity for both states, and merely block the effect of the endogenous ligands. They do no alter spontaneous (basal) G protein coupling.
Chapter 2: Literature overview

Figure 2.1-2: 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; a and b = 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 \(\alpha_2\)-ARs in neuronal cells

The same theory described in the previous section (§ 2.4.4.1) can be applied to \(\alpha_2\)-ARs located in the central nervous system. Under normal neurophysiological conditions, a large fraction of \(\alpha_2\)-ARs in the CNS can be expected to exist in the inactive state (\(\alpha_2\)-AR\(^b\)), while a small fraction will be in the active state (\(\alpha_2\)-AR\(^a\)). Upon stimulation of endogenous I-NE the equilibrium is shifted towards \(\alpha_2\)-AR\(^a\), leading to increased coupling to G\(_i\) proteins and hence, either an inhibition of adenylate cyclase with reduced cAMP production or activation of K\(^{\text{r}}\)-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 \(\alpha_2\)-ARs, such as idazoxan (see below), will compete with I-NE for binding to \(\alpha_2\)-ARs. At relatively high concentrations of the neutral antagonist, when all of the I-NE is displaced, the equilibrium between \(\alpha_2\)-AR\(^b\) and \(\alpha_2\)-AR\(^a\) 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 \(\alpha_2\)-ARs. However, at relatively high concentrations of the inverse agonist, when all of the I-NE is displaced, the equilibrium will shift towards \(\alpha_2\)-AR\(^b\) thereby preventing any spontaneous coupling to G\(_i\) proteins (see Figure 2-7 and Figure 2-8). If there is any constitutive activity of \(\alpha_2\)-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 $\alpha_2$-ARs and remains speculative, although data for other GPCRs suggest that this may be possible.

Relevant to the current study, the $\alpha_2$-ARs are located presynaptically on 1-NE neuronal cell bodies and terminals ($\alpha_2$-autoreceptors) and on 5-HT neuronal cell bodies and terminals ($\alpha_2$-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 1-NE and 5-HT neurotransmission mediated by $\alpha_2$-ARs, whereas neutral antagonists will not.

Recent studies report that drugs acting at $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-ARs (Wade et al., 2001). The differences between the modes of antagonism of these drugs at $\alpha_2$-ARs could account for in vivo pharmacological differences between them.

Since the $\alpha_2$-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 $\alpha_2$-ARs (Khoza, 2004). Since $\alpha_2$-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.,
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.

![Behavioural parameters in the modified forced swim test](image)

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 L-NE levels. For example, agents that enhance central L-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; Reneric et al., 2002a). The modified FST has also been used with much success, indicating the reliability of the modified version of this model (Reneric et al., 2001; Reneric et al., 2002a; Reneric 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-HT_3 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-HT_3 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 D_2 and D_3, but not D_4 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 CRF_1,
receptors (Chaki et al., 2004), NMDA receptors (Padovan & Guimaraes, 2004), adenosine A$_{2A}$ receptors (El et al., 2001; El et al., 2003), imidazoline I$_2$ 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 $\alpha_2$-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 $\alpha_2$-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 $\beta$-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 $\beta$-ARs downregulation in the alleviation of depressive symptoms is unclear.
Materials and methods

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 α₂-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-HT₁A 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 \textit{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

$[^3]H$-Propranolol hydrochloride (20.0 Ci/mmol) was obtained from GE Healthcare (formerly Amersham Biosciences, Buckinghamshire, England). $[^3]H$-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 N$_2$ was obtained from Afrox (Johannesburg, South Africa), Propranolol hydrochloride, Bradford reagent, EDTA, Ascorbic acid, Sigmacote$^\textregistered$ and fractioned BSA were obtained from Sigma-Aldrich (St. Louis, USA), Tris HCl and MgCl$_2$ 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.
Chapter 3: Materials and methods

Pilot Study 1:
Lab-validation of FST

7 days

Group C4:
Vehicle

Group T1:
Fluoxetine

Group C1:
Vehicle

Pre-exposure
(15 minutes)

Swim trial
(15 minutes)

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 SSR1 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 α2-lytic drugs. Figure 3-2 illustrates schematically the experimental layout, as conducted in this phase of the study.

![Pilot Study 2: Determination of time to onset of action of fluoxetine](image)

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

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

In total, 15 rats were used in the FST and 6 rats for assessment of locomotor activity for each treatment group.
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: \( \alpha_2 \)-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 α₂-lytic drug, including mirtazapine (α₂-lytic mode unknown), yohimbine (inverse agonist) and idazoxan (neutral antagonist), or with a combination of fluoxetine and each of the respective aforementioned α₂-lytic drugs (see Table 3-2). This was done in order to enable us to investigate whether α₂-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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug(s)</th>
<th>Days</th>
<th>Rats / group</th>
<th>Groups</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5A</td>
<td>vehicle</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T4A</td>
<td>fluoxetine</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T5A</td>
<td>mirtazapine</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T6A</td>
<td>yohimbine</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T7A</td>
<td>idazoxan</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>C6A</td>
<td>vehicle</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T8A</td>
<td>fluoxetine + mirtazapine</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T9A</td>
<td>fluoxetine + yohimbine</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T10A</td>
<td>fluoxetine + idazoxan</td>
<td>3 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>C5B</td>
<td>vehicle</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T4B</td>
<td>fluoxetine</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T5B</td>
<td>mirtazapine</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T6B</td>
<td>yohimbine</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T7B</td>
<td>idazoxan</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>C6B</td>
<td>vehicle</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T8B</td>
<td>fluoxetine + mirtazapine</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T9B</td>
<td>fluoxetine + yohimbine</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>T10B</td>
<td>fluoxetine + idazoxan</td>
<td>7 days</td>
<td>7</td>
<td>3</td>
<td>21</td>
</tr>
</tbody>
</table>
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 n-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

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_i$-value (nM)</th>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT transporter</td>
<td>6.8</td>
<td>Rat brain</td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>10.8</td>
<td>Rat cortex</td>
<td>(Shank et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>Rat cortex</td>
<td>(Owens et al., 1997)</td>
</tr>
<tr>
<td>I-DA transporter</td>
<td>3160</td>
<td>Rat striatum</td>
<td>(Shank et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>Rat striatum</td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td>I-NE transporter</td>
<td>794.12</td>
<td>Rat frontal cortex</td>
<td>(Cordi et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>Rat brain</td>
<td>(Beique et al., 1998)</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-HT$_{1A}$</td>
<td>Rat cortex</td>
<td>(Wong et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>11481</td>
<td></td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>5-HT$_{2A}$</td>
<td>Rat cortex</td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>710</td>
<td></td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>5-HT$_{2C}$</td>
<td>Rat cloned cells</td>
<td>(Rothman et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-AR</td>
<td>$\alpha_1$</td>
<td>Rat cortex</td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>14000</td>
<td></td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>$\alpha_2$</td>
<td>Rat cortex</td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>2800</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_1$</td>
<td>Rat brain</td>
<td>(Sanchez &amp; Hyttel, 1999)</td>
</tr>
<tr>
<td></td>
<td>3200</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 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>
<thead>
<tr>
<th>Receptor</th>
<th>$K_a$-value (nM)</th>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>&gt; 18 000</td>
<td>Human</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>2.0</td>
<td>Rat cortex</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
<tr>
<td>5-HT2C</td>
<td>5.5</td>
<td>Rat tissue</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
<tr>
<td>5-HT3</td>
<td>6.31</td>
<td>Mouse neuroblastoma</td>
<td>(de Boer, 1996)</td>
</tr>
<tr>
<td>α1</td>
<td>372</td>
<td>Rat frontal cortex</td>
<td>(de Boer et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>1 050</td>
<td>Rat whole brain</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
<tr>
<td>α2</td>
<td>112</td>
<td>Rat frontal cortex</td>
<td>(de Boer et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>Rat cortex</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
<tr>
<td>mAch</td>
<td>794</td>
<td>Rat whole brain</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
<tr>
<td>H1</td>
<td>0.5</td>
<td>Rat frontal cortex</td>
<td>(de Boer et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>5.1</td>
<td>Guinea pig lung</td>
<td>(Wikstrom et al., 2002)</td>
</tr>
</tbody>
</table>

Since the role of α2-AR antagonism in antidepressant action is the focus of the current study, the affinity of mirtazapine for the α2-AR, relative to other receptors, is important for considering an appropriate dose.

The affinity of mirtazapine at α2-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-HT$_{3}$ 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 α1-ARs is lower than at α2-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 H1-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 α2-lytic effects of mirtazapine would become more significant, leading to increased I-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 I-NE neurons (Szabo & Blier, 2001). At a dose of 1.5 mg/kg, idazoxan increased the levels of I-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 α2-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 α2-ARs, some affinity for 5-HT1A-receptors and relatively low affinity for α1-ARs.
3.4.4.3 Dose selection

A moderate dosage should provide effects which are primarily α₂-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.
Chapter 3: Materials and methods

Table 3-6: Receptor binding profile of yohimbine

<table>
<thead>
<tr>
<th>Receptor</th>
<th>K_i-value (nM)</th>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT <em>HT1</em></td>
<td>125.89</td>
<td>Rat hippocampus</td>
<td>(Millan et al., 2000)</td>
</tr>
<tr>
<td>α_1</td>
<td>251.18</td>
<td>Rat frontal cortex</td>
<td>(Millan et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>230.00</td>
<td>Rat brain</td>
<td>(Doxey et al., 1984)</td>
</tr>
<tr>
<td>α_2</td>
<td>2.67</td>
<td>CHO-K1 cells</td>
<td>(Bodenstein et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>8.67</td>
<td>CHO-K1 cells</td>
<td>(Brink et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>14.3</td>
<td>Rat brain</td>
<td>(Boyajian &amp; Leslie, 1987)</td>
</tr>
<tr>
<td></td>
<td>31.62</td>
<td>Rat cortex</td>
<td>(Millan et al., 2000)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>501.18</td>
<td>Rat striatum</td>
<td>(Millan et al., 2000)</td>
</tr>
</tbody>
</table>

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 administrated 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 significantly 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 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 µl dilutions as indicated in Table 3-8:

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Volume of 2 mg/ml BSA</th>
<th>Volume of 50 mM Tris buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/ml</td>
<td>0 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>5 µl</td>
<td>95 µl</td>
</tr>
<tr>
<td>0.4 mg/ml</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>0.7 mg/ml</td>
<td>35 µl</td>
<td>65 µl</td>
</tr>
<tr>
<td>1.0 mg/ml</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>1.4 mg/ml</td>
<td>70 µl</td>
<td>30 µl</td>
</tr>
</tbody>
</table>

2 x 5 µl of each protein standard dilution, as well as 2 x 5 µl of the membrane suspension were added to separate wells of a 96-well plate, i.e. all in duplicate.

250 µl 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$_{\text{max}}$) 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 [3H]-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$_{\text{max}}$.

- Sigmaclone® 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 [3H]-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 [${}^3$H]-8-OH-DPAT was used to define total binding, while the same series, but also containing 20 µM 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 [${}^3$H]-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 µl of membrane suspension was added, as well as 50 µl of assay buffer, and to the 8 tubes for measurement of non-specific binding 400 µl of membrane suspension was added, as well as 50 µl of a 200 µM serotonin solution (containing 0.02% ascorbic acid). The final assay volume in the binding study was 500 µl. Therefore, each tube contained a total of 250 µg protein.

- 50 µl 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.
Chapter 3: Materials and methods

- 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 \( B_{\text{max}} \) and \( K_d \) values determined using GraphPad Prism® (Version 4.02). The raw \( B_{\text{max}} \) values derived from the plotted graphs are in terms of cpm/250 µg protein (since each tube contained 500 µl of a protein concentration of 500 µg/ml). The standard curve was also plotted for each experiment (10 µl for \([\text{3H}]-8-\text{OH-DPAT} \) binding, and 20 µl for \([\text{3H}]-\text{propranolol} \) 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 \( B_{\text{max}} \) value to units of mol receptors/mg protein.

**Example:**

This is an example of the calculations for a single saturation binding study of \([\text{3H}]-8-\text{OH-DPAT} \) 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 \([\text{3H}]-8-\text{OH-DPAT} \) to 5-HT\(_{1A}\) receptors. The (A) binding curve depicts total binding, non-specific binding (defined with 10 µM serotonin), and specific binding (calculated by subtracting non-specific binding from total binding). The raw values of \( B_{\text{max}} \) and \( K_d \) are indicated on the graph. The (B) standard curve was obtained by measuring the total radioactivity of 10 µl of each radio-ligand concentration.](image-url)
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, $B_{\text{max}}$ is calculated as $8326 \pm 261.9 \text{ cpm}/250 \mu\text{g protein}$ (Figure 3-6A) and the $K_D$ value as $1.9 \pm 0.17 \text{ nM}$. The $K_D$ value compares well with published affinity values of $[^3\text{H}]$-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 $1359 \pm 13.6 \text{ cpm}/\text{nM}$ (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 $\mu$l). As a result, the value of the slope becomes $1.359 \times 10^{17} \text{ cpm/mol}$. The raw $B_{\text{max}}$ (in terms of cpm/250 $\mu\text{g protein}$) is divided by the slope of the standard curve to yield a value of $6.127 \times 10^{-14} \text{ mol}/250 \mu\text{g protein}$. This value is then converted to represent a final value in terms of mol/mg protein. This is done by dividing the $B_{\text{max}}$ value by the total amount of protein in the final assay volume (0.25 mg, since a total assay volume of 500 $\mu$l was used with a protein concentration of 500 $\mu$g/ml). The 5-HT$_{1A}$ receptor concentration ($B_{\text{max}}$), for this experiment, was subsequently calculated to be $2.451 \times 10^{-13} \text{ mol/mg protein}$ (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) 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.
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 $\alpha_2$-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 $\beta$-AR density in the frontal cortex (since $\beta$-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 $\alpha_2$-AR antagonists (i.e. mirtazapine, yohimbine and idazoxan) or a combination of fluoxetine with each of the $\alpha_2$-AR antagonists for both treatment periods. The $\alpha_2$-AR antagonists were also specifically chosen to include an inverse agonist (yohimbine) and a neutral antagonist (idazoxan) at $\alpha_2$-ARs. This was to investigate whether an $\alpha_2$-AR antagonist can hasten the onset of action of a conventional antidepressant (such as fluoxetine), and also to investigate whether an inverse agonist at $\alpha_2$-
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-HT1A 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 ± standard error of the mean, where *** represents p < 0.001.](image-url)
Chapter 4: Results and discussion

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 ± 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.

![Graphs A, B, C, D](image)

**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 ± 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 ± 4.8% versus 45 ± 2.9% with p < 0.001) and 11 days (100 ± 7.4 versus 39 ± 4.4% with p < 0.0001) but not after 3 days (100 ± 9.7% versus 93 ± 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 ± 9.5% versus 216 ± 14.5% with p < 0.0001) and 11 days (100 ± 11.8% versus 203 ± 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 ± 14.0% versus 249 ± 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.
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: α₂-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 α₂-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 α₂-lytic properties. This would be the case if α₂-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 α₂-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 α₂-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 ± standard error of the mean.
Consistent with data from Pilot Study 2, fluoxetine alone produces a significant reduction in immobility (100 ± 4.1% versus 60 ± 9.8% with \( p < 0.01 \)) and significantly increases swimming behaviour (100 ± 14.8% versus 284 ± 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 \( \alpha_2 \)-AR inverse agonist yohimbine significantly reduces immobility (100 ± 4.1% versus 69 ± 6.4% with \( p < 0.01 \)) and increases swimming behaviour (100 ± 14.8% versus 258 ± 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 ± 2.7% versus 83 ± 4.1% with \( p > 0.05 \)). It does however significantly increase swimming behaviour after 3 days (100 ± 13.3% versus 206 ± 22.14% with \( p < 0.05 \)) compared to control. The \( \alpha_2 \)-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 ± 2.7% versus 74 ± 7.0% with \( p < 0.01 \)) compared to control. It also significantly increases swimming behaviour after this short treatment period (100 ± 13.3% versus 226 ± 37.6% with \( p < 0.01 \)) compared to control. After 7 days immobility is still significantly reduced (100 ± 4.1% versus 75 ± 4.8% with \( p < 0.05 \)), but swimming behaviour is no longer significantly elevated (100 ± 14.8% versus 165 ± 22.4% with \( p > 0.05 \)) compared to control. This is caused by a significant increase in climbing behaviour (100 ± 15.1% versus 190 ± 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 ± 4.1% versus 69 ± 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 ± 14.8% versus 232 ± 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 ± 4.1% versus 41 ± 4.9% with \( p < 0.01 \)), significantly increases climbing behaviour after 3 (100 ± 21.7% versus 233 ± 48.9% with \( p < 0.01 \)) and 7 days (100 ± 15.1% versus 253 ± 28.7% with \( p < 0.01 \)), and significantly increases swimming behaviour after 3 days (100 ± 13.3% versus 255 ± 39.7% with \( p < 0.01 \)) and 7 days (100 ± 14.8% versus 285 ± 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 \( \alpha_2 \)-autoreceptors and reportedly increase synaptic availability of I-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 $\alpha_2$-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 L-NE transmission, most probably caused by antagonism of $\alpha_2$-autoreceptors on L-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 L-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 L-NE neurotransmission. A possible explanation for the ability of the FST to distinguish between mirtazapine and the other $\alpha_2$-lytic drugs employed here regarding climbing behaviour, is the reported capability of mirtazapine to bind to, and stimulate postsynaptic $\alpha_2$-ARs (de Boer, 1996), which would simulate an increased L-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 $\alpha_1$-heteroreceptors on serotonergic cell bodies in the dorsal raphe, secondary to an increased availability of L-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 $\alpha_2$-autoreceptors located on the cell bodies of L-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 \( \alpha_1 \)-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 \( \alpha_2 \)-heteroreceptors on serotonergic terminals caused by the increased release of 5-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 \( \alpha_1 \)-ARs located on pyramidal CA3 neurons in the hippocampus (see § 2.2.1.3.3).

Since yohimbine and idazoxan also antagonise \( \alpha_2 \)-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 \( \alpha_2 \)-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-HT\(_2 \) and 5-HT\(_3 \) 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-HT\(_2 \) 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-HT\(_2 \) 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-HT\(_3 \) 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 \( \alpha_2 \)-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 \( \alpha_2 \)-lytic drugs with fluoxetine on behaviour in the FST. More specifically, this was to investigate more closely the mechanisms of interaction of the three \( \alpha_2 \)-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 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 ± 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 \( (75 ± 4.8\% \text{ versus } 41 ± 4.9\% \text{ with } p < 0.05) \) and increases swimming behaviour \( (165 ± 22.4\% \text{ versus } 285 ± 18.3\% \text{ with } p < 0.05) \) 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 \( (61 ± 9.8\% \text{ versus } 41 ± 4.9\% \text{ with } p > 0.05) \) and swimming \( (284 ± 39.4 \text{ versus } 285 ± 18.3\% \text{ with } p > 0.05) \). Also, although a mirtazapine-fluoxetine combination increased climbing significantly compared to fluoxetine alone \( (89 ± 17.3\% \text{ versus } 253 ± 28.7\% \text{ with } p < 0.001) \), the effect of the combination treatment did not differ significantly from the effect produced by mirtazapine alone \( (190 ± 41.7\% \text{ versus } 253 ± 28.7\% \text{ with } p > 0.05) \).

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 ± 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 \( (61 ± 9.8\% \text{ versus } 101 ± 8.5\% \text{ with } p < 0.001) \) and decreases swimming behaviour \( (284 ± 39.4\% \text{ versus } 111 ± 28.4\% \text{ with } p < 0.001) \) 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 \( \alpha_2 \)-autoreceptors thereby increasing noradrenergic tone (Tao & Hjorth, 1992; de Boer et al., 1996) and should indirectly (via \( \alpha_1 \)-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 \( \alpha_2 \)-autoreceptors than for \( \alpha_2 \)-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 ± standard error of the mean.

As depicted in Figure 4-8, there are no significant alterations in immobility (61 ± 9.8% versus 69 ± 6.9% with \( p > 0.05 \)), climbing (89 ± 17.3% versus 115 ± 19.6% with \( p > 0.05 \)), or swimming (284 ± 39.4% versus 232 ± 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 \( \alpha_2 \)-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-HT1A 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.

![Graph A: 3 day treatment](image)

![Graph B: 7 day treatment](image)

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 (Bmax) is expressed in terms of fmol receptors per mg protein, and as mean ± 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-HT₁A-receptor concentration

The hippocampus regions of the animals used in the FST were removed and used in receptor binding experiments for measurement of 5-HT₁A receptor density.

Figure 4-10: Effects on serotonin 5-HT₁A-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-HT₁A-receptor density (B_max) is expressed in terms of fmol receptors per mg protein, and as mean ± 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-HT₁A 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-HT₁A receptors in the hippocampus.

The values for 5-HT₁A receptor density that were obtained in this experiment include both presynaptic and postsynaptic 5-HT₁A receptors, while the presynaptic 5-HT₁A 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-HT₁A receptors is therefore impaired. Nonetheless, it seems that combinations of fluoxetine with α₂-AR antagonists in general are more effective in decreasing 5-HT₁A 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 ± 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).
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 α₂-lytic drugs, whereafter the rats were exposed to the FST and cortical α₂-AR and hippocampal 5-HT₁A receptor concentration. With this study design the aim was to investigate the role of the α₂-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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect elicited in the FST</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-day treatment</td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>↔</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>↓↓</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>↔</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>↔</td>
</tr>
<tr>
<td>Fluoxetine + Mirtazapine</td>
<td>↓↓</td>
</tr>
<tr>
<td>Fluoxetine + Yohimbine</td>
<td>↔</td>
</tr>
<tr>
<td>Fluoxetine + Idazoxan</td>
<td>↔</td>
</tr>
</tbody>
</table>

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 α₂-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 α₂-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 \(\alpha_2\)-AR antagonists used in the current study in combination with fluoxetine are depicted in Table 5-2.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Interactions with fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mirtazapine</td>
<td>Immobility: Possible additive effect; Climbing: Possible additive effect; Swimming: Possible additive effect</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>Immobility: No interaction; Climbing: No interaction; Swimming: No interaction</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>Immobility: Antagonism; Climbing: No interaction; Swimming: Antagonism</td>
</tr>
</tbody>
</table>

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 \(\beta\)-AR density in the frontal cortex of the rats are summarised in Table 5-3:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect on (\beta)-AR density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>←→</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>←→</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>←→</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>←→</td>
</tr>
<tr>
<td>Fluoxetine + Mirtazapine</td>
<td>←→</td>
</tr>
<tr>
<td>Fluoxetine + Yohimbine</td>
<td>←→</td>
</tr>
<tr>
<td>Fluoxetine + Idazoxan</td>
<td>↓</td>
</tr>
</tbody>
</table>

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

\(\beta\)-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 \(\beta\)-AR concentration in the rats.
The effects of the drugs employed in the current study on 5-HT₁₆ receptor density in the hippocampus of the rats are summarised in Table 5-4:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect on 5-HT₁₆ density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>←</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>←</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>←</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>←</td>
</tr>
<tr>
<td>Fluoxetine + Mirtazapine</td>
<td>←</td>
</tr>
<tr>
<td>Fluoxetine + Yohimbine</td>
<td>←</td>
</tr>
<tr>
<td>Fluoxetine + Idazoxan</td>
<td>←</td>
</tr>
</tbody>
</table>

Note: In the table, a horizontal arrow denotes no effect, while a downward arrow indicates a reduction in 5-HT₁₆ density. Two arrows indicate a more significant effect than one arrow.

5-HT₁₆ 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-HT₁₆ receptors.

5.2 CONCLUSIONS

The current study has provided useful and novel information regarding the role of the α₂-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 $\alpha_2$-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 $\alpha_2$-AR antagonism plays an important role in the early onset of action of mirtazapine, we found that neither the $\alpha_2$-AR inverse agonist yohimbine, nor the $\alpha_2$-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 $\alpha_2$-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 $\alpha_{2\theta}$-AR, an orthologue of the human $\alpha_{2A}$-AR (Lanier et al., 1991; Harrison et al., 1991). While the pharmacological profiles of the $\alpha_{2A}$- and $\alpha_{2\theta}$-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 $\alpha_{2\theta}$-AR will match the profiles in humans and closely resemble that for the rat $\alpha_{2\theta}$-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 $\alpha_2$-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-HT$_2$ and 5-HT$_3$ 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-HT\textsubscript{2} receptors (specifically the 5-HT\textsubscript{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 l-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\textsubscript{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 l-DA in the NAC to basal state. This ability of nefazodone has been attributed to its 5-HT\textsubscript{2c} receptor antagonistic property, since this receptor plays an inhibitory role in the release of central l-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\textsubscript{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\textsubscript{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\textsubscript{1A} receptors, and thereby produce a more rapid onset of antidepressant action. Such a comparison of 5-HT\textsubscript{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\textsubscript{1A} receptors has been shown to have neuroprotective effects (Gould, 1999). Therefore, mirtazapine may have a neuroprotective action via indirect enhancement of 5-HT\textsubscript{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 5HT₁A 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-HT₁A 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.

  o 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
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 α1-AR and 5-HT1A receptor agonistic properties (de Boer, 1996). The importance of the roles of such diverse receptor activities may also be further explored.


References


CONTI, A.C., CRYAN, J.F., DALVI, A., LUCKI, I., & BLENDY, J.A. 2002. cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. *Journal of Neuroscience, 22*(8):3262-3268.


References


HENSLER, J.G. 2003. Regulation of 5-HT1A receptor function in brain following agonist or antidepressant administration. Life Sciences, 72(15):1665-1682.


KHOZA, K. 2004. The characterisation of the a2a-adrenoceptor antagonism by mirtazapine and its modifying effects on receptor signalling. Potchefstroom: North-West University (PUK). (Dissertation - M.Sc.).


References


References


References


References


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
- Gastrointestinal, Hepatic, Pulmonary, and Renal
- Inflammation, Immunopharmacology, and Asthma
- Metabolism, Transport, and Pharmacogenomics
- Neuropharmacology
- Toxicology
- Other

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:


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

- Unnumbered footnote providing the source of financial support, thesis information, citation of meeting abstracts where the work was previously presented, etc.
- The name and full address (with street address or P.O. box, and postal code) and e-mail address of person to receive reprint requests.
- Numbered footnotes, using superscript numbers, beginning with those (if any) to authors’ names and listed in order of appearance.

11. Legends for figures. Figures are numbered consecutively with Arabic numerals and listed in order rather than one per page. Legends must provide sufficient explanation for the reader to understand the figure independent of the text.

12. Tables. Each table must be double-spaced and begin on a separate page, each page numbered continuous with the rest of the manuscript. Tables are numbered consecutively with Arabic numerals. A brief descriptive title is provided at the top of each table. General statements about the table follow the title in paragraph form. Footnotes to tables are referenced by italicized, lower case, superscript letters and defined beneath the table. Acceptable formats for tables are Word and WordPerfect.

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.
The \( \alpha_2 \)-lytic properties of mirtazapine may not be critical for a putative earlier onset of antidepressant action. Evidence from rat behavioral and neuro-receptor studies.

**Names of authors**

Christiaan B Brink, Nico Liebenberg, Kenneth Khoza and Brian H Harvey

**Primary laboratory of origin**

Division of Pharmacology, North-West University (PUK), Potchefstroom, South Africa (CBB, NL, KK, BHH)
RUNNING TITLE PAGE

Running title

α2-Adrenoceptor antagonism and mirtazapine onset of action

Corresponding author

Christiaan B Brink, Internal Box 16, Division of Pharmacology, North-West University (PUK), Potchefstroom, 2520, South Africa, Tel. +27 18 299 2226/34; Fax. +27 18 299 2225; Email: Tiaan.Brink@nwu.ac.za

Document statistics

Number of text pages: 39
Number of tables: 0
Number of figures: 5
Number of references: 61
Number of words in the Abstract: 250 (250 max)
Number of words in the Introduction: 723 (750 max)
Number of words in the Discussion: 1469 (1500 max)

List of non-standard abbreviations: 5-HT, 5-hydroxytryptamine; CHO-K1, Chinese hamster ovary cell line; α2A-H, Chinese hamster ovary cell line expressing the porcine α2A-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 α2-adrenoceptor lytic action (at α2-adrenergic autoreceptors and heteroreceptors), thereby modulating central serotonergic and noradrenergic neurotransmission. The current study investigated the role of α2-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 α2-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 α2-adrenoceptors. Rats were treated for 3 and 7 days with fluoxetine, mirtazapine, yohimbine (α2-adrenoceptor strong inverse agonist), idazoxan (α2-adrenoceptor neutral antagonist), or combinations with fluoxetine, whereafter the forced swim test (FST) were employed and cortical β-adrenoceptor and hippocampal 5-HT1A receptor densities were measured. Results with the FST support an earlier onset of action by mirtazapine, but do not support an important role for α2-lytic action in this regard. β-Adrenoceptor density generally decreased with antidepressant action, but is not a good marker of hastened onset. 5-HT1A receptor density is not a good marker for antidepressant action. It was concluded that a property different from its α2-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 α2-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 α2-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 α2-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 1-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; Reneric, 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 α2-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 α2-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-HT1A 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 α2-adrenoceptor inverse agonist (yohimbine), an α2-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-1H-imidazol-2-yl)-6-quinoxalineamine) 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 α₂₅-adrenoceptor at high numbers (cell line denoted α₂₅-H) and the other mock-transfected (control cell line containing the selection plasmid but no α₂₅-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 α₂₅-H cell line have been previously characterized, where the determined α₂₅-adrenoceptor concentration was reported as 19 ± 2 pmol/mg membrane protein (Brink, et al., 2000). We confirmed the high receptor expression for α₂₅-H in our laboratory, determined as 46 ± 5 pmol/mg membrane protein (Bodenstein, et al.,...
2005). These cell lines were used to investigate the $\alpha_2$-adrenoceptor binding and signaling properties of the test drugs.

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

**Preparing membranes from $\alpha_{2A}$-H cells.** Membranes were prepared from $\alpha_{2A}$-H cells essentially as described before (Bodenstein, et al., 2005). After the appropriate pre-treatment of whole $\alpha_{2A}$-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 $\alpha_{2A}$-adrenoceptors from radioligand saturation-binding experiments in whole $\alpha_{2A}$-H cells, defining non-specific binding with 10 μM 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 μM 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 pKᵢ value of the α₂ₐ-adrenoceptor ligands at α₂ₐ-adrenoceptors in α₂ₐ-H cells we performed competition binding assays in whole α₂ₐ-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 α₂ₐ-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 μCi 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 μM 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 ± 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 × 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 µg/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 MgCl2 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 $\beta$-adrenoceptor density. Eight concentrations of [3$^3$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 μM 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$^3$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 MgCl$_2$, 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 μM 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 MgCl$_2$ 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 ± 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 $K_i$ 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 \( \alpha_{2A}-\)adrenoceptors. The \( K_i \) value of the inverse agonist \([\text{methyl-}^3\text{H}]\)-yohimbine at \( \alpha_{2A} \)-adrenoceptors in the \( \alpha_{2A}-\)H cells (expressing the wild type \( \alpha_{2A} \)-adrenoceptor at relatively high numbers) was calculated from saturation binding curves as 6.72 ± 2.05 nM and the \( B_{\text{max}} \) 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 \( \alpha_{2A} \)-adrenoceptor ligands brimonidine (agonist; \( pK_i = 6.39 \pm 0.06 \), i.e. \( K_i = 407 \) nM), idazoxan (neutral antagonist; \( pK_i = 7.48 \pm 0.03 \), i.e. \( K_i = 33 \) nM), mirtazapine (atypical antidepressant; \( pK_i = 6.50 \pm 0.07 \), i.e. \( K_i = 316 \) nM) and mianserin (atypical antidepressant; \( pK_i = 6.94 \pm 0.14 \), i.e. \( K_i = 115 \) nM), against 5 nM \([\text{methyl-}^3\text{H}]\)-yohimbine in \( \alpha_{2A}-\)H cells. The affinities of these ligands for \( \alpha_{2A} \)-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 \( \alpha_{2A} \)-adrenoceptors.

Dose-response curves of \( \alpha_{2A} \)-adrenoceptor ligands. Figure 12 depicts dose-response-relationships of the \( \alpha_{2A} \)-adrenoceptor ligands brimonidine, yohimbine, idazoxan, mirtazapine and mianserin in neo cells (negative control, mock-transfected) and in \( \alpha_{2A}-\)H cells (transfected to express the porcine \( \alpha_{2A} \)-adrenoceptor at relatively high numbers). It is important to note that only brimonidine displayed a dose-response curve in \( \alpha_{2A}-\)H cells (\( pEC_{50} = 9.27 \pm 0.19 \); \( E_{\text{max}} = 69.0 \pm 5.8\% \) inhibition of \([\text{H}]\)-cAMP accumulation), while all other drugs did not elicit any significant \( \alpha_{2A} \)-adrenoceptor-mediated inhibition of cAMP production in \( \alpha_{2A}-\)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 ± 8.2% to 143 ± 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 ± 4.6% versus 143 ± 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 \( \alpha_2 \)-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 ± 4.8% versus 45 ± 2.9%, \(p < 0.001\)) and 11 days (100 ± 7.4 versus 39 ± 4.4%, \(p < 0.0001\)), but not after 3 days (100 ± 9.7% versus 93 ± 11.3%, \(p > 0.05\)). Conversely, as shown in Figure 13C, swimming behavior was significantly increased compared to control after 7 days (100 ± 9.5% versus 216 ± 14.5%, \(p < 0.0001\)) and 11 days (100 ± 11.8% versus 203 ± 8.5%, \(p < 0.0001\)), but not after 3 days (100 ± 8.1% versus 110 ± 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 ± 14.0% versus 249 ± 55.2%, \(p = 0.00136\)), while no significant changes were observed after 3 days (100 ± 15.5% versus 94 ± 21.2%, \(p > 0.05\)) or 11 days (100 ± 23.2% versus 145 ± 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).

**\(\beta\)-Adrenoceptor density:** As depicted in Figure 13D \(\beta\)-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 \(\beta\)-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.

**\(\alpha\)-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 ± 2.7% versus 74 ± 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 ± 4.1% versus 61 ± 9.8%, \(p < 0.01\)), yohimbine (100 ± 4.1% versus 69 ± 6.4%, \(p < 0.01\)), mirtazapine (100 ± 4.1%
versus 75 ± 4.8%, p < 0.05), a combination of yohimbine with fluoxetine (100 ± 4.1% versus 69 ± 6.9%, p < 0.01), and a combination of mirtazapine with fluoxetine (100 ± 4.1% versus 41 ± 4.9%, p < 0.01). Importantly, a combination of mirtazapine with fluoxetine was superior to mirtazapine alone (75 ± 4.8% versus 41 ± 19.0%, p < 0.05) but not statistically significantly to fluoxetine alone (61 ± 9.8% versus 41 ± 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 ± 6.4% versus 69 ± 6.9%, p > 0.05) or fluoxetine (61 ± 9.8% versus 69 ± 6.9%, p > 0.05) alone. Interestingly, the combination of mirtazapine with fluoxetine was superior to fluoxetine alone (61 ± 9.8% versus 101 ± 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 ± 21.7% versus 233 ± 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 ± 15.1% versus 190 ± 41.7, p < 0.05) and mirtazapine in combination with fluoxetine (100 ± 15.1% versus 253 ± 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 ± 17.3% versus 253 ± 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 ± 13.3% versus 226 ± 37.6%, p < 0.01), mirtazapine in combination with fluoxetine (100 ± 13.3% versus 255 ± 39.7%, p < 0.01), as well as yohimbine (100 ± 13.3% versus 207 ± 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 ± 14.8% versus 284 ± 39.4%, p < 0.01), yohimbine (100 ± 14.8% versus 258 ± 28.8%, p < 0.01), yohimbine in combination with fluoxetine (100 ± 14.8% versus 232 ± 25.7%, p < 0.01), and mirtazapine in combination with fluoxetine (100 ± 14.8% versus 285 ± 18.2%, p < 0.01). In addition, the combination of mirtazapine with fluoxetine was superior to mirtazapine alone (165 ± 22.4% versus 285 ± 18.3%, p < 0.05), but not to fluoxetine alone (284 ± 39.4% versus 285 ± 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 ± 39.4% versus 111 ± 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-HT₁A receptor density: As depicted in Figure 15C, none of the treatments produced a significant down-regulation of 5-HT₁A 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-HT₁A receptors in the hippocampus, while mono treatments still did not induce any significant changes in 5-HT₁A 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 $\alpha_2$-adrenoceptors with an affinity in the high nanomolar range. This measured affinity ($K_i$ value) of mirtazapine at $\alpha_2\alpha$-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 $<\text{idazoxan}<\text{yohimbine}$). 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 $\alpha_2$-adrenergic autoreceptor selectivity. However, these reported $pA_2$ values do not necessarily reflect drug-receptor dissociation constants ($K_i$), which requires specific kinetic conditions to be met (Neubig, et al., 2003).

Mirtazapine is a neutral antagonist or inverse agonist at $\alpha_2$-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 $\alpha_2$-H cells, only the agonist UK-14-304 (brimonidine) elicits a full dose-response curve. $\alpha_2\alpha$-H cells express a high concentration of $\alpha_2\alpha$-adrenoceptors (46.18 $\pm\text{5.23 pmol/mg protein}$) 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 $\alpha_2\alpha$-adrenoceptors, but rather are neutral antagonists or inverse agonists. It has been shown before in CHO-K1 cells expressing constitutively activated mutant $\alpha_2\alpha$-adrenoceptors, that yohimbine is a strong inverse agonist and idazoxan a neutral antagonist at $\alpha_2\alpha$-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 $\beta$-adrenoceptor density in the frontal cortex (Figure 13D), would indicate that the above behavioral changes may have an association with changes in $\beta$-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 α2-autoreceptors located on I-NE neurons in the locus coeruleus, leading to increased cell firing (de Boer, 1996) that in turn activates α2-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 α2-autoreceptors and α2-heteroreceptors, mirtazapine prevents reactive dampening of I-NE and 5-HT release (Blier, 2003) resulting in the stimulation of postsynaptic 5-HT1A receptors and α1-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 $\alpha_{2A}$-adrenoceptor antagonism?

$\alpha_{2A}$-adrenoceptor antagonism may not be important for earlier onset of antidepressant action. The $\alpha_2$-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 $\alpha_2$-lytic properties to the SRI action of fluoxetine does not hasten onset of action. If $\alpha_2$-lytic properties do in fact contribute significantly to antidepressant action, an inverse agonist at $\alpha_2$-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 $\alpha_2$-adrenoceptors with higher affinity than mirtazapine (as discussed above). These findings therefore do not support the current hypotheses regarding the important role of $\alpha_2$-adrenoceptor antagonism in the earlier onset of antidepressant action of mirtazapine.

That the $\alpha_2$-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 $\alpha_2$-auto than $\alpha_2$-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.

$\beta$-adrenoceptor downregulation in the rat frontal cortex mostly support behavioral data. Only the idazoxan-fluoxetine combination downregulated $\beta$-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 $\beta$-adrenoceptor downregulation) and the idazoxan-fluoxetine combination (displaying $\beta$-adrenoceptor but no parallel inhibition of immobility). In contrast, all treatments produced significant downregulation of $\beta$-adrenoceptors after 7 days of treatment (Figure 15B). Others (McGrath, et al., 1998) found a trend towards a mirtazapine-induced reduction in $\beta$-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-HT₁A density in the rat hippocampus do not correlate with antidepressant-like behavioral responses.** None of the treatments produced down-regulation of 5-HT₁A receptors after 3 days of treatment (Figure 15C). Other studies have found that mirtazapine does not exert direct effects on 5-HT₁A 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-HT₁A receptors in the hippocampus (Figure 15D), suggesting that hippocampal 5-HT₁A 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 α₂-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.

**Acknowledgements**

Mirtazapine was kindly provided by Organon (Netherlands) and fluoxetine was kindly provided by Aspen (South Africa). The authors wish to express our gratitude towards Mr. Cor Bester for assistance with logistic matters in the Centre for Laboratory Animals at the North-West University, and Mrs. Maureen Steyn and Mrs. Sharleen Nieuwoudt for general laboratory assistance the handling of logistic matters related to the project.
References


Appendix


Reneric JP, Bouvard M and Stinus L (2002a) In the rat forced swimming test, chronic but not subacute administration of dual 5-HT/NA antidepressant treatments may produce greater effects than selective drugs. *Behav Brain Res* **136**:521-532.
Appendix

Reneric JP, Bouvard M and Stinus L (2002b) In the rat forced swimming test, NA-system mediated interactions may prevent the 5-HT properties of some subacute antidepressant treatments being expressed. *Eur Neuropsychopharmacol* 12:159-171.


Footnotes

Present address: Department of Pharmacology, University of Kwazulu Natal, Durban, South Africa (KK)

The project was supported financially by a grant from the South African National Research Foundation.
Figure Legends

Figure 11 Competition-binding curves of brimonidine (A), idazoxan (B), mirtazapine (C) and mianserin (D) in α2A-H cells against 5 nM [methyl-3H]-yohimbine. Results were calculated as a percentage of control and are expressed as mean ± SEM. The reported Ki values were calculated from the IC50 values, applying the Cheng-Prusoff correction.

Figure 12 Only brimonidine yields a dose-response curve in α2A-H cells. Dose-response curves of brimonidine (A), yohimbine (B), idazoxan (C), mirtazapine (D) and mianserin (E) in α2A-H cells and control neo cells, measuring cAMP production. Results were calculated as a percentage of control (no drug) and are expressed as mean ± 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 ± SEM. Receptor densities are calculated in terms of fmol/mg protein and are expressed as mean ± 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 α2-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 ± 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-HT1A-receptor concentration after 3 (C) and 7 days (D) of treatment with fluoxetine (Flx), yohimbine (Yoh), mirtazapine (Mirt), idazoxan (Idaz), and combinations of α2-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 ± 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.
Figure 1

**A**

\[ \alpha_2A-H \text{ cells} \]

\[ pK_i = 6.39 \pm 0.06 \]

**B**

\[ \alpha_2A-H \text{ cells} \]

\[ pK_i = 7.48 \pm 0.03 \]

**C**

\[ \alpha_2A-H \text{ cells} \]

\[ pK_i = 6.50 \pm 0.07 \]

**D**

\[ \alpha_2A-H \text{ cells} \]

\[ pK_i = 6.94 \pm 0.14 \]
Figure 2

A

\[ \frac{[\text{Hi}-cAMP accumulation}}{\text{% of control}} \]

log [brimonidine] (M)

\( pEC_{50} = 9.27 \pm 0.19 \)

- neo
- \( \alpha_{2A}-\text{H} \)

B

\[ \frac{[\text{Hi}-cAMP accumulation}}{\text{% of control}} \]

log [yohimbine] (M)

- neo
- \( \alpha_{2A}-\text{H} \)

C

\[ \frac{[\text{Hi}-cAMP accumulation}}{\text{% of control}} \]

log [idazoxan] (M)

- neo
- \( \alpha_{2A}-\text{H} \)

D

\[ \frac{[\text{Hi}-cAMP accumulation}}{\text{% of control}} \]

log [mirtazapine] (M)

- neo
- \( \alpha_{2A}-\text{H} \)

E

\[ \frac{[\text{Hi}-cAMP accumulation}}{\text{% of control}} \]

log [mianserin] (M)

- neo
- \( \alpha_{2A}-\text{H} \)
Figure 3

A: Immobility

B: Climbing

C: Swimming

D: β-Adrenoceptor density

Vehicle  Fluoxetine
Figure 4

A | 3 day treatment
---|---
Vehicle | Fix | Yoh | Mirt | Idaz | Fix+Yoh | Fix+Mirt | Fix+Idaz
Immobility (% control)

B | 7 day treatment
---|---
Vehicle | Fix | Yoh | Mirt | Idaz | Fix+Yoh | Fix+Mirt | Fix+Idaz
Immobility (% control)

C | 3 day treatment
---|---
Vehicle | Fix | Yoh | Mirt | Idaz | Fix+Yoh | Fix+Mirt | Fix+Idaz
Climbing (% control)

D | 7 day treatment
---|---
Vehicle | Fix | Yoh | Mirt | Idaz | Fix+Yoh | Fix+Mirt | Fix+Idaz
Climbing (% control)

E | 3 day treatment
---|---
Vehicle | Fix | Yoh | Mirt | Idaz | Fix+Yoh | Fix+Mirt | Fix+Idaz
Swimming (% control)

F | 7 day treatment
---|---
Vehicle | Fix | Yoh | Mirt | Idaz | Fix+Yoh | Fix+Mirt | Fix+Idaz
Swimming (% control)
Figure 5

β-Adrenoceptor density

3 days treatment

7 days treatment

5-HT1A-receptor density

3 days treatment

7 days treatment