Characterisation of the $\alpha_{2A}$-adrenoceptor antagonism by mirtazapine and its modifying effects on receptor signalling.

KENNETH KHOZA
(B.Pharm)

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

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This study is dedicated to my family’s most precious gift, my beloved brother & mentor

ERIC KHOZA
(22/10/71-26/07/03).

“Though your journey in life came to an early end, your selfless sacrifice will always be a part of each of my achievements. I continuously draw my inspiration from your life and I thank God for blessing me with a brother like you.

Etlela hi kurhula Mavhona Jakarantima!”
"Motivation is a fire from within. If someone else tries to light that fire under you, chances are it will burn very briefly."

*Stephen R. Covey*
Title: Characterisation of the $\alpha_2A$-adrenoceptor antagonism by mirtazapine and its modifying effects on receptor signalling

Mirtazapine is an atypical antidepressant employed clinically for the treatment of major depression. As a multipotent antagonist it acts at $\alpha_2A$-adrenergic receptors ($\alpha_2A$-ARs), serotonin type-2A receptors (5-HT$_2A$-Rs) and histamine type-1 receptors (H$_1$-Rs). Its actions at the $\alpha_2A$-AR have been proposed to play a role in its putative earlier onset of action. However, it is not known whether mirtazapine is a neutral antagonist or inverse agonist at $\alpha_2A$-ARs. The current study aimed to determine the mode of $\alpha_2A$-AR antagonism by mirtazapine, as well as to investigate in vitro the modulatory effects of mirtazapine pre-treatments on $\beta$-adrenergic receptor ($\beta$-AR), muscarinic acetylcholine receptor (mACHR) and $\alpha_2A$-AR functions.

Chinese hamster ovary (CHO-K1) cells expressing the porcine $\alpha_2A$-AR at high numbers ($\alpha_2A$-H), a constitutively active mutant $\alpha_2A$-AR ($\alpha_2A$-CAM), or mock-transfected control cells (neo) were radio-labelled with $[^3H]$-adenine and concentration-effect curves of mirtazapine, yohimbine, mianserin or idazoxan were constructed, measuring $[^3H]$-cAMP accumulation. In addition human neuroblastoma SH-SY5Y cells and CHO-K1 cells expressing the porcine $\alpha_2A$-AR at low numbers ($\alpha_2A$-L) were used to investigate the effect of mirtazapine pre-treatments on mACHRs and $\beta$-ARs or $\alpha_2A$-ARs respectively. After radio-labelling with myo-$[2-{^3H}]$-inositol or $[2-{^3H}]$-adenine, radio-label uptake was measured and receptor function was investigated by constructing concentration-effect curves, measuring $[^3H]$-IP$_x$ or $[^3H]$-cAMP accumulation respectively.

The results from the current study show that mirtazapine binds to the $\alpha_2A$-AR with an affinity value in the lower micromolar range ($K_i = 0.32 \mu M; \ pK_i = 6.50 \pm 0.07$). Mirtazapine is not a partial agonist at $\alpha_2A$-ARs as it does not affect $[^3H]$-cAMP accumulation in $\alpha_2A$-H cells. Preliminary results suggest that mirtazapine displays partial inverse agonism in $\alpha_2$-CAM cells, while mianserin displays neutral antagonism. Mirtazapine pre-treatment in SH-SY5Y cells does not alter muscarinic receptor function (different from fluoxetine and imipramine), but reduces $l$-isoproterenol-induced increase in $[^3H]$-cAMP accumulation in SH-SY5Y cells.
Abstract

(typically associated with chronic antidepressant activity). Although inconclusive, the data also suggests that mirtazapine may reduce $\alpha_{2A}$-AR function.

Key words: mirtazapine, antidepressant, onset of action, inverse agonism, neutral antagonism, $\alpha_{2A}$-adrenergic receptors, $\beta$-adrenergic receptors, muscarinic acetylcholine receptors.
**Abstrak**

**Titel:** Karakterisering van die α2A-adrenoseptor antagonisme deur mirtazapien en die modiferende effekte daarvan op reseptorseining

Mirtazapien is 'n atipiese antidepressant wat gebruik word vir die behandeling van major depressie. As multipotente antagonist werk dit op α2A-adrenergie se reseptore (α2A-AR), serotonien tipe-2A reseptore (5-HT2A-Re) en histamien tipe-1 reseptore (H1-Re). Die werking daarvan op die α2A-AR is voorgestel om 'n rol te speel in die moontlike vroeër aanvang van werking. Dit is egter nie bekend of mirtazapien 'n neutrale antagonist of inverse agonis op α2-AR is nie. Die huidige studie poog om die modus van α2A-AR antagonisme deur mirtazapien te bepaal, so wel as om die in vitro modulerende effekte van mirtazapien voorbehandelings op β-adrenergie reseptor- (β-AR), muskariniese asetielkolien reseptor- (mAChR) en α2A-AR-funksies te ondersoek.

Chinese hamster ovariale (CHO-K1) selle wat die vark α2A-AR in hoë getalle uitdruk (α2A-H), 'n konstitueel aktiewe mutant-α2A-AR (α2A-CAM), of fop-getransfekteerde kontroleselle (neo) was radio-aktief gemerk met [3H]-adenien en konsentrasie-effekskurves van mirtazapien, johimbien, mianserien of idazoksaaan is opgestel deur [3H]-cAMP-akkumulasie te meet. Addisioneel is menslike neuroblastoomselle (SH-SY5Y) en CHO-K1 selle wat die vark α2A-AR in lae getalle uitdruk (α2A-L) gebruik om ondersoek in te stel na die effekte van mirtazapien voorbehandelings op respektiewelik mAChR en β-AR of α2A-AR. Na radioaktiewe merking met mio-[2-3H]-inositol of [2-3H]-adenien, is die opname van die radioaktiewe merker gemes en reseptorfunksie is ondersoek deur konsentrasie-effekskurpes op te stel deur die meting van onderskeidelik [3H]-IP₃ of [3H]-cAMP akkumulasie.

Die resultate van die huidige studie toon aan dat mirtazapien aan die α2A-AR bind met 'n affiniteitswaarde in die hoër nanomolare orde (pKᵢ = 6.50 ± 0.07). Mirtazapien is nie 'n gedeeltelike agonis op α2A-ARE nie, aangesien dit nie [3H]-cAMP akkumulasie in α2A-H selle affekteer nie. Voorlopige resultate dui daarop dat mirtazapien gedeeltelike inverse agonisme vertoon in α2-CAM selle, terwyl mianserien neutrale antagonisme vertoon. Mirtazapien voorbehandeling in SH-SY5Y selle wysig nie muskariniese reseptor-funksie nie (anders as fluoksetien en imipramien), maar verlaag β-isoproterenol-geënduseerde verhoging in [3H]-cAMP akkumulasie in SH-SY5Y selle (tipies geassosieer met kroniese antidepressant-
aktiwiteit). Alhoewel onbeslis, dui die data daarop dat mirtazapien $\alpha_{2A}$-AR funksie mag verlaag.

Sleutelwoorde: mirtazapien, antidepressant, aanvang van werking, inverse agonisme, neutrale antagonisme, $\alpha_{2A}$-adrenergie se reseptore, $\beta$-adrenergie se reseptore, muskariniële asetielcholienreseptore.
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1.1 PROBLEM STATEMENT

Depression is one of the most common and serious psychiatric disorders world-wide. It has the potential to cause severe disability and places an economic burden on society. Although effective antidepressant therapies have been available for many years, major drawbacks associated with currently employed antidepressants include unwanted side-effects, resistance and delayed onset of action (with delayed remission rate). The introduction of selective serotonin re-uptake inhibitors (SSRIs) has improved the safety and tolerability of antidepressant therapy and they are the current drugs of choice for the treatment of patients with major depression. Newly developed agents with an effect on both the noradrenergic and serotonergic functions, e.g. mirtazapine and venlafaxine, have been reported to have an onset of action earlier than that of SSRIs (Olver et al., 2001). Several recent studies support the claim of early onset of antidepressant action by mirtazapine and venlafaxine (Guelfi et al., 2001; Szegedi et al., 2003; Ables and Baughman, 2003; Blier, 2003; Schutte & Van Oers, 2002). However, lack of clear understanding on the psychopharmacology of depression as well as the complexity of the mechanisms by which antidepressants act, are both thought to be responsible for the negative attributes in the development of therapeutically fast and well-tolerated antidepressants (Baldessarini, 2001).

Mirtazapine is a multipotent atypical antidepressant that blocks the α2A-adrenergic auto- and heteroreceptors (α2A-ARs), serotonergic type-2 receptors (5-HT2-Rs) and histamine type-1 receptors (H1-Rs). It has been suggested by De Boer (1996) that mirtazapine's principal action involves the blocking of α2-ARs, a feature thought to underlie much of its antidepressant activity.

As with many other antagonists at various G-protein-coupled receptors (GPCRs), several drugs that were classified as α2A-ARs antagonists have been shown to have inverse agonistic properties, e.g. rauwolscine and yohimbine, whereas idazoxan is essentially a neutral antagonist (Wade et al., 2000). However, it has not been established whether mirtazapine (or its older analogue mianserin) is a partial agonist, inverse agonist or neutral antagonist at the α2A-AR. In addition, the clinical importance of this property in
antidepressant action has not yet been established, e.g. how the inverse agonistic properties of yohimbine or the neutral antagonistic properties of idazoxan may influence antidepressant drug action. In general, due to lack of conclusive knowledge about inverse agonism to enable us to suggest its specific clinical relevance, existing suggestions are therefore speculative, although potentially important.

1.2 PROJECT AIMS

The main aims of the project were to:

- Characterise the $\alpha_2A$-ARs antagonism (and in particular possible inverse agonism) by mirtazapine; and
- investigate any in vitro modulatory effect of mirtazapine pre-treatment on muscarinic acetylcholine receptor (mACh-R) and beta adrenergic receptor ($\beta$-AR) function.

1.3 PROJECT LAYOUT

All the experiments in this study were conducted in the Laboratory for Applied Molecular Biology at the North-West University (Potchefstroom Campus), Republic of South Africa.

To address the first main objective of the study (the characterisation of the $\alpha_2$-lytic action of mirtazapine), three Chinese hamster ovary (CHO-K1) cell lines were utilised, namely a cell line expressing the wild-type porcine $\alpha_2A$-AR at high numbers ($\alpha_2A$-H cells), a cell line expressing its constitutively active mutant receptor ($\alpha_2A$-CAM cells), or a mock-transfected control line (Neo cells). These cells were kindly provided by Dr. Richard Neubig (Department of Pharmacology, University of Michigan, Ann Arbor, USA). Competition-binding experiments were performed in $\alpha_2A$-H and the $K_i$ value of mirtazapine, UK-14,304, mianserin, or idazoxan were determined. The receptor concentrations ($B_{\text{max}}$ values) were determined for $\alpha_2A$-H, $\alpha_2A$-L (CHO cell line, expressing the wild-type porcine $\alpha_2A$-AR at lower numbers – also provided by Dr. Neubig) or $\alpha_2A$-CAM, by performing appropriate saturation-binding studies with cell membranes. Concentration-effect curves of mirtazapine were constructed, measuring [$^3$H]-cAMP accumulation and compared to that of yohimbine (inverse agonist at $\alpha_2A$-ARs), idazoxan (neutral antagonist at $\alpha_2A$-ARs) or mianserin (atypical antidepressant with $\alpha_2A$-AR lytic effects).

Human neuroblastoma cells (SH-SY5Y) were used to investigate the modulating effect of mirtazapine pre-treatment on mACh-R and $\beta$-AR functions. In addition $\alpha_2A$-L cells were used to investigate the modulatory effects of mirtazapine pre-treatment on the $\alpha_2A$-ARs. Cells
were pre-treated with mirtazapine for 24 hours, followed by appropriate functional and radio-
ligand-binding studies by constructing concentration-effect curves of metacholine, \( L \)-
isoproterenol or UK-14,304, measuring whole-cell inositol multiphosphate (\([^{3}\text{H}]\text{IP}_3\)) or \([^{3}\text{H}]\text{c-AMP} \) accumulation respectively.

The experimental layout described above allowed for characterisation of the mode of
antagonism by mirtazapine, as well as studying the modulatory effects of mirtazapine pre-
treatment on the functions of selected GPCRs.
2.1 INTRODUCTION

Delayed onset of action, poor tolerability and resistance to currently employed clinical antidepressants are critical challenges in antidepressant therapy to date. Effective antidepressant therapies have been available since the early 1950s, however they either have a potential for dangerous adverse effects or require at least 2-3 weeks (Leonard, 2003) following the commencement of therapy before initial antidepressant effect can be obtained. Full remission usually takes much longer, usually between 4 to 6 weeks following the initiation of therapy (Szegedi et al., 2003; Blier, 2003). As a result, there is prolonged patient suffering, even after the commencement of therapy, while the patient remains at greater risk of committing suicide (Blier, 2003). In addition, compliance may also be hampered by this delayed onset of symptom relief, since adverse effects are usually frequently higher during the initial phase of treatment. These challenges have stimulated the search for new antidepressants that are better tolerated, with an earlier onset of antidepressant action.

Selective serotonin uptake inhibitors (SSRIs) are currently the drugs of choice for the treatment of patients with major depression. However, mirtazapine, an atypical antidepressant, has been reported to relieve depressive symptoms more rapidly than SSRIs (Blier, 2003; Benkert et al., 2000; Leinonen et al., 1999; Wheatley et al., 1998; Van Oers et al., 2002). It has been speculated (De Boer, 1996) that the $\alpha_{2A}$-lytic effect of mirtazapine, thought to be a principal action, is responsible for much of its antidepressant activity, which in turn might be responsible for the faster relief of symptoms.

Detailed molecular and receptor studies on the $\alpha_{2A}$-adrenergic receptor ($\alpha_{2A}$-AR) actions of mirtazapine could not be found in the literature, which motivated the current study.

This chapter discusses the findings from a literature survey on mirtazapine. In addition, G-protein- coupled receptors (GPCRs) and their signal transduction mechanisms will be discussed, in particular as these relate to the action of mirtazapine in $\alpha_{2A}$-adrenergic receptors ($\alpha_{2A}$-ARs) and other GPCRs implicated in the pathophysiology and treatment of
depression. Lastly the pathophysiology, theories and treatment of depression will be discussed.

2.2 MIRTAZAPINE: AN ATYPICAL ANTIDEPRESSANT

2.2.1 Chemistry

Mirtazapine (1,2,3,4,10,14b-hexahydro-2-methyl-pyrazino[2,1-a]-pyrodo[2,3-c]benzazipine or (±)-Org 3770) is a tetracyclic antidepressant that is not functionally related to tricyclic antidepressants (TCAs) or SSRIs (Ables & Baughman, 2003). Mirtazapine is the 6-aza-analogue of mianserin, as illustrated in Figure 2-1 below.

![Mirtazapine and Mianserin](Mirtazapine-Mianserin.png)

Figure 2-1: Chemical structures of mirtazapine and mianserin

2.2.2 Mechanism of action

Mirtazapine is an atypical antidepressant referred to as a noradrenaline and specific serotonergic antidepressant (NaSSA) (Danilevicuite & Sveikata, 2002). Mirtazapine acts by antagonising central α2A-adrenergic auto and heteroreceptors, as well as by blocking serotonin (5-HT) type-2 (5-HT2) and type-3 (5-HT3) and H1 receptors (De Boer, 1996; Danilevicuite & Sveikata, 2002; Blier, 2003). It is believed that its actions at both α2A-ARs and 5-HT2 are responsible for much of its therapeutic action. The resulting outcomes are the simultaneous increase in the activity of both the noradrenergic and serotonergic systems. Mianserin on the other hand, is a second-generation antidepressant, which acts both as a moderate inhibitor of l-noradrenaline (l-NA) uptake and as an α2A-adrenergic auto receptor antagonist, which may combine to increase the availability of l-NA at the synapse (Baumann & Maitre, 1977).

Mirtazapine has a unique mechanism of action among currently available antidepressants. The reported blockade of presynaptic central α2-adrenergic autoreceptors leads to the
enhancement of noradrenergic neurotransmission through increased noradrenergic cell firing and 5-NA release (Danilevicute & Sveikata, 2002). It has been proposed by De Boer (1996) that mirtazapine's principal action is the blockade of α-TARs, and it is believed that this feature underlies much of its antidepressant activity.

As illustrated in Figure 2-2, there is an overlapping activity between the noradrenergic and serotonergic neuronal transmission. Noradrenergic cell bodies are concentrated mostly in locus coeruleus, whereby their axons project to various parts of the brain, including the dorsal raphe nuclei, thus regulating the firing of the serotonergic neurons. This synapse is regulated pre-synaptically by the α2A-adrenergic auto receptors on the noradrenergic terminals, which inhibit the release of 5-NA and hence the firing of 5-HT cells in the raphe. α-Lytic drugs, such as mirtazapine, uncouple this feedback mechanism, thus resulting in
increased I-NA in the synapse. This then results in the \( \alpha_1 \)-AR-mediated firing at the serotonergic neurons, with the \( \alpha_1 \)-ARs situated on the raphe cell bodies. The resulting overall effect of these pharmacological actions is the synergistically increased noradrenergic and serotonergic activity, which may possibly explain mirtazapine's putative earlier onset of therapeutic action (Blier, 2003) (see Figure 2-2). The serotonergic neurons of the dorsal raphe nuclei also project to the locus coeruleus, where they inhibit the noradrenergic activity via the excitatory postsynaptic 5-HT\( _{2A} \)R located on the GABA neuron (see Figure 2-2). Mirtazapine blocks the 5-HT\( _{2A} \)Rs, thus also uncoupling this feedback effect, resulting in increased activity at the noradrenergic nerve terminals (Blier, 2003). The combined actions of mirtazapine at both \( \alpha_{2A} \)-ARs and 5-HT\( _{2A} \)-Rs may explain its putative advantageous antidepressant actions over other antidepressants. The blocking of 5-HT\( _2 \) and \( H_1 \) by mirtazapine is believed to be more relevant to its favourable effect on sleep and appetite than to its antidepressant action (Blier, 2003).

Recent studies suggest that mirtazapine has an earlier onset of action than SSRIs, e.g. double-blind randomised studies where mirtazapine was found to have an earlier onset of action than paroxetine (Benkert et al., 2000), citalopram (Leinonen et al., 1999), fluoxetine (Wheatley et al., 1998) and sertraline (Van Oers et al., 2002).

### 2.2.3 Pharmacokinetics

Following oral administration of mirtazapine (Remeron®) tablets, it is rapidly and well absorbed from the gastro-intestinal tract, reaching peak plasma levels after about 2 hours. The bioavailability of mirtazapine is approximately 50%, with mean elimination half-life of 20-40 hours; (averaged 26 hours in males, 37 hours in females). It is approximately 85% bound to plasma proteins (South African Electronic Package Inserts, 2004).

The elimination half-life of mirtazapine of 20-40 hours is sufficient to justify a once-a-day dosing schedule. Mirtazapine is extensively metabolised in the liver, while elimination via urine and faeces occurs within a period of about four days (Sweetman, 2002). Major pathways of biotransformation are demethylation and oxidation, followed by conjugation of the parent drug (South African Electronic Package Inserts, 2004). However, the demethyl metabolite is pharmacologically active and appears to have the same pharmacokinetic profile as the parent compound, which also justifies once-a-day dosing to avoid mirtazapine toxicity. In vitro data from human liver microsomes indicates that cytochrome P450 enzymes such as CYP 2D6 and CYP 1A2 are involved in the formation of the 8-hydroxymetabolite of mirtazapine, whereas CYP 3A4 is considered to be responsible for the formation of the N-
demethyl and N-oxide metabolites (South African Electronic Package Inserts, 2004; Sweetman, 2002).

Mirtazapine is presented as a racemate, i.e. (±)-mirtazapine, and the two enantiomers are cleared through different metabolic processes. However, it is not known whether co-administering with food would affect the bioavailability of the two enantiomers (South African Electronic Package Inserts, 2004).

2.2.4 Clinical indications

Mirtazapine is currently indicated for the treatment of patients with major depression. However, mirtazapine should be used with caution in patients with epilepsy, hepatic or renal impairment, angina pectoris, myocardial infarction, hypotension, diabetes mellitus and in those patients with a history of bipolar depression (Sweetman, 2002). Mirtazapine is contraindicated in pregnancy, lactation and in children, as there is not sufficient scientific data available to demonstrate safety (South African Electronic Package Inserts, 2004). The use of mirtazapine is not recommended when the patient is currently on monoamine oxidase inhibitors (MAOls), or within 14 days of initiating or discontinuing therapy with MAOls. This is particularly important to avoid reported serious and sometimes fatal reactions, including nausea, vomiting, flushing, dizziness, tremor, myoclonus, rigidity, diaphoresis, hyperthermia, autonomic instability with rapid fluctuations of vital signs, seizures and mental status changes, ranging from agitation to coma (South African Electronic Package Inserts, 2004).

The most commonly reported adverse effects of mirtazapine include fatigue, dizziness, transient sedation, increased appetite and weight gain, asthenia, flu syndrome, increased sweating, abnormal dreams, paresthesia, tremor, vertigo, dry mouth, constipation and nausea (Ables & Baughman, 2003; South African Electronic Package Inserts, 2004). However, in contrast to SSRIs, mirtazepine is not associated with sexual dysfunction and is reported to be devoid of anticholinergic, adrenergic, and 5-HT related side effects (Hirschfield, 1999).

Drug interactions associated with mirtazapine have not been extensively studied, but mirtazapine is not expected to interact with the metabolism of other drugs, since it does not inhibit cytochrome P450 enzyme systems, as reported by Boer and Westenburg (Danilevicuite & Sveikata, 2002). It has been reported that the use of mirtazapine with alcohol, anxiolytics, or hypnotics may potentiate sedative effects (Sweetman, 2002).
2.3 EARLY ONSET OF ANTIDEPRESSANT ACTION

The delay in the onset of therapeutic activity of antidepressants appears to be common amongst different chemical classes, although it is believed that this feature is not a characteristic of the disease, since sleep deprivation and electroconvulsive shock therapy (ECT) have been reported to have a faster onset of action (Gillin, 1993; Dalay et al., 2001; Blier, 2003). There have been several reported claims of early onset of action for third-generation antidepressants, such as mirtazapine, venlafaxine, reboxetine and nefazodoneone. These claims have encouraged the development of new therapeutic strategic approaches, which in addition to preserving the overall therapeutic actions of the existing treatments also hasten their onset of action (Blier, 2003). Another obstacle in the development of therapeutically fast and well-tolerated antidepressants is the lack of a clear understanding of the psychopharmacology of depression and the complex mechanisms by which antidepressants act (Baldessarini, 2001).

Drugs with an effect on both the noradrenergic and serotonergic functions (e.g. mirtazapine and venlafaxine) have been reported to have an onset of action earlier than that of SSRIs (Olver et al., 2001). Venlafaxine, like the TCAs, blocks the NA and 5-HT reuptake pathways, but lacks the anticholinergic, antihistaminic and α1-AR-lytic properties that might explain its better tolerability profile compare to TCAs (Blier, 2003). As already mentioned (see § 2.2.2), mirtazapine has also been reported to have an earlier onset of action than the SSRIs. In the study done by Guelfi et al. (2001) in hospitalised, severely depressed patients with melancholic features, both venlafaxine and mirtazapine were effective in improving patients’ overall depressive symptoms and their quality of life. Although not statistically significant, data showed a trend for an onset of action of mirtazapine earlier than that of venlafaxine. Again in the later study, mirtazapine was reported to have a better tolerability profile than venlafaxine (Guelfi et al., 2001). However, it is believed that mirtazapine’s unique mechanism of action, especially the antagonism of α2-AR, is responsible for its advantage over other antidepressants (Blier, 2003).

Due to the reported earlier onset of action with mirtazapine, some of the focus has shifted to the noradrenergic pathway, in particular at the adrenergic α2A-auto and heteroreceptors. Most recently, Sanacora et al. (2004) reported that co-administering of yohimbine, an α2-AR inverse agonist (Wade et al., 2000) with fluoxetine reduces the delay in the onset of therapeutic actions (Sanacora et al., 2004). Although inconclusive, there is reported data suggesting that idazoxan (an α2-AR neutral antagonist (Wade et al., 2000)) is as effective as bupropion in the treatment of patients with major depression. As a result, it could be
speculated that $\alpha_2$-ARs plays a vital role in antidepressant strategies and might also be implicated in the putative earlier onset of actions seen with mirtazapine.

However, there is no reported data on the mode by which mirtazapine acts as antagonist at $\alpha_2$-ARs (i.e. whether it acts as partial agonist, neutral antagonist or inverse agonist). Neither did comprehensive literature searches reveal any data on the binding affinity values of mirtazapine at $\alpha_2$-ARs.

2.4 G-PROTEIN-COUPLED RECEPTORS AND SIGNAL TRANSDUCTION MECHANISMS

Mirtazapine is a multipotent antagonist believed to exert its antidepressant actions by acting on the $\alpha_2A$-ARs and 5-HT$_2A$ receptors. Both these receptors are coupled with guanosine triphosphate (GTP)-binding proteins, commonly referred to as G-proteins. G-proteins are intracellular membrane-associated heterotrimeric proteins. Receptors that interact with G-proteins are referred to as G-protein-coupled receptors (GPCRs). Some of the longest known and best-described GPCRs include the muscarinic acetylcholine receptors (mACh-Rs), serotonergic receptors (5-HT-Rs), dopaminergic receptors (DA-Rs) and adrenergic receptors (ARs). Antidepressants affect one or more of these receptors, either directly or indirectly. G- Proteins form a large super-family of trimeric proteins, composed of three major subunits, namely alpha ($\alpha$), beta ($\beta$) and gamma ($\gamma$) subunits. G-proteins are further classified according to their $\alpha$-subunits into three families, namely $G_{\text{ia}}$ (inhibit adenylyl cyclase), $G_s$ (activates adenylyl cyclase), and $G_{q/11}$ (activates phospholipase C). The subunits are further subdivided into several subtypes each, so that more than 30 subfamilies of G-proteins exists (Parnot et al., 2002; Ulloa-Aguirre et al., 1999).

GPCRs play a vital role in signal transduction mechanisms in various parts of the body. They are the main targets for a large number of currently employed drugs, including antidepressants, and in the development of new therapeutic drugs. GPCRs form a large family of integral membrane proteins that are classified into several classes (A-E) according to sequence homologies. They are characterised by their seven transmembrane (TM I-VII) alpha helices, joined together by three intracellular (i$_{1,3}$) and three extracellular (e$_{1,3}$) peptide loops with an extracellular N-terminal and cytosolic carboxy-terminal (Parnot et al., 2002) (see Figure 2-3).
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Figure 2-3: Counter-clockwise orientation of GPCR from transmembrane domains I-VII. The closed-loop structure is representative of receptors for small ligands such as biogenic amines and nucleosides. In this arrangement, the core is comprised mainly of TM domains II, III, V, and VI, whereas TM domains I and IV are peripherally sequestered (Ulloa-Aguirre et al., 1999).

GPCRs are specifically activated by a ligand, including cations, monoamines, neurotransmitters, lipids, odorant molecules, and various peptides. As discussed by Parnot et al., (2002), mammalian GPCRs are found in class A-C and other GPCRs, including those of fungal phenotype receptors in class D and class E (see Table 2-1).

GPCRs convey extracellular instructions from ligands to the heterotrimeric G-protein, which in turn stimulate membrane-bound enzyme systems (e.g. adenylyl cyclase), referred to as G-protein effectors. Activation of these effectors typically leads to the release of active second messengers, resulting in diverse cellular responses (Linder & Gilman, 1992). Two major effects are exerted by the G-protein, namely the regulation of ion channels or second-messenger systems (Hyman & Nestler, 1996).
Table 2-1 Classes of GPCRs

<table>
<thead>
<tr>
<th>Class</th>
<th>Source</th>
<th>Ligand (receptor)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mammalian</td>
<td>Endocrine&lt;br&gt;Oxytocin, gonadotropin, prostaglandin, lutenizing hormone, melanocortin, thyrotropin, adrenomedullin, melatonin, gonadotropin-releasing hormone, thyrotropin-releasing hormone, follicle-stimulating hormone, somatostatin.&lt;br&gt;Neurotransmitters&lt;br&gt;Acetylcholine (e.g. muscarinic), neuropeptide Y, neotensin, serotonin, opioid, I-NA (α- and β-adrenergic), dopamine&lt;br&gt;Cardiovascular&lt;br&gt;Angiotensine, bradykinin, endothelin, tachykinin, vasopressin, thrombin&lt;br&gt;Others&lt;br&gt;Histamine, chemokine, interleukin, olfactory receptors, rhodopsin (light-sensing receptors), chemoattactant C5a, purine receptors</td>
</tr>
<tr>
<td></td>
<td>Fungal</td>
<td>Yeast α-factor receptor, yeast a-factor</td>
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<tr>
<td></td>
<td></td>
<td>Dictyostellium chemoattractant receptors</td>
</tr>
</tbody>
</table>
2.4.1 Mechanisms of signal transduction

Upon activation of a GPCR by an agonist (i.e. a shift of the equilibrium towards active receptor states – see § 2.4.3) it couples with the membrane-bound G-protein, thereby initiating the release of GDP from the $\alpha$-subunit and the subsequent binding of GTP. This is followed by the dissociation of the G-protein trimeric protein into its $\alpha$- and $\beta\gamma$-subunits. Both the $G_{\alpha(GTP)}$ and the $\beta\gamma$ subunits interact with their respective effectors, which in turn could regulate the activities of diverse effector proteins inside the cell. Signal transduction is terminated by the hydrolysis of GTP to GDP, as catalysed by GTPase (intrinsic to the $\alpha$-subunit), resulting in the re-association of the $\alpha$ and $\beta\gamma$ subunit, thus inactivating the G-protein (Ross, 2001; Linder & Gilman, 1992). As a result, G-protein acts as a switch that controls the signal transduction pathways (Linder & Gilman, 1992). The basic signalling mechanism of GPCRs is illustrated in Figure 2-4 below.

![Figure 2-4: Schematic illustration of the G-protein activation/inactivation cycle](obtained from Addison Wesley Longman, (2003))
2.4.1.1 GPCRs associated with phospholipase C

Phospholipase C (PLC) is an intracellular membrane-bound enzyme that mediates the formation of second messengers. Several isoforms of PLC exist, including the membrane-bound PLC-β, cytosolic PLC-γ, and PLC-δ. GPCRs such as muscarinic (M₁ & M₂) acetylcholine receptors, adrenergic (α₁A, 1B & 1D) receptors, and serotonergic (5-HT₂A, 2B, & 2C) receptors regulate the activity of PLC through interaction with all four members of the G₉ subfamily (Gα₅, Gαq/11, Gαq/14 & Gαq/16) and Gβγ subfamily (Sternweis & Smrcka, 1992; Berridge, 1993).

Following the stimulation of these receptor types, the G-protein is then activated and Gαq(GTP) binds to PLC-β (PLC isoform commonly modulated by GPCRs), thus catalysing the hydrolysis of intracellular-associated phosphatidylinositol-4,5-bisphosphate (PIP₂) to form diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃). Gβγ-GTP subunit also binds to certain PLC-β isoforms, resulting in activation of the enzyme and formation of IP₃ and DAG (Rhee & Choi, 1992).

IP₃ is a soluble molecule that diffuses through the cytosol and binds to IP₃ receptors on the endoplasmic reticulum, causing the release of calcium ions (Ca²⁺) into the cytosol. The resulting rise in intracellular calcium triggers a cellular response. DAG remains in the inner layer of the plasma membrane, which then activates protein-kinase C (PKC), a calcium-dependent kinase that phosphorylates many other proteins to bring changes in the cellular function (Hurley & Meyer, 2001).

2.4.1.2 GPCRs associated with adenylyl cyclase

Adenylyl cyclase (AC) is an integral membrane protein consisting of two bundles of six transmembrane segments, with two catalytic domains (forskolin-binding sites) extending as loops into the cytoplasm. This is depicted in Figure 2-5 below (Houslay & Milligan, 1997). The two large cytoplasmic loops (C1 and C2) appear to have similar homologous sequence between the family members, while the two groups of putative transmembrane helices are quite different (Houslay & Milligan, 1997). However, in contrast to G-proteins, the transmembrane helices are highly similar between family members (see Figure 2-5).
Several GPCRs, including cholinergic (M₂) receptors, serotonergic (5-HT₁) receptors and adrenergic (α₂) receptors couple to Gi proteins. Go interacts with either of the two soluble active sites of the adenyl cyclase, thus inhibiting the formation of cAMP from ATP. This is followed by activation of K⁺-dependent channels and suppression of the activity of the voltage-gated Ca²⁺ channels in the cells, resulting in a cellular response (e.g. the inhibition of monoamine release from the stores) (Houslay & Milligan, 1997).

However, activation of all β-adrenergic receptor (β-AR) subtypes (β₁,2,3,5) results in coupling of the receptor to the Gα₅, thus catalysing the conversion of ATP to cAMP, which in turn leads to an increase in intracellular levels of cAMP. cAMP binds and activates the regulatory subunit of cAMP-dependent protein kinase (PKA). PKA is responsible for the phosphorylation of specific protein substrates to modify their activity, resulting in a cellular response (e.g. activation of glycogen phosphorylase in the liver, or promotion of smooth muscle relaxation) (Hoffman, 1998).

### 2.4.2 Constitutively-active mutants of G-protein-coupled receptors

The constitutive activity of pharmacological receptors could be defined as ligand-independent activity, resulting in the production of a second messenger, even in the absence of an agonist (also referred to as “gain-of-function” phenotype or “basal” activity (Parnot et al., 2002). In the wild-type (naturally occurring, non-mutated) receptor, constitutive activity was
first reported for delta (δ) opioid receptors (Costa & Herz, 1989). The concept of genetic manipulation of wild-type receptors to constitutively active mutant (CAM) receptors was first reported for the α₁-adrenergic receptors (Cotecchia et al., 1990). This led to the introduction of the “extended ternary complex (ETC) model” (Samama et al., 1993) and the “cubic ternary complex model” (Weiss et al., 1996). These models suggest that the receptor exists in equilibrium between the inactive state(s) (R) and the active state(s) (R*) in the absence of an agonist. However, this equilibrium varies with each receptor type and in the wild-type receptors, R predominates and as a result there is minimal receptor activity in the absence of an agonist.

Due to low constitutive activity in the wild-type receptors, various genetic manipulations to increase this activity have been explored for several GPCR types. Observations indicated that the basal activity of wild-type GPCRs might vary from totally inactive to fully active, depending on the nature of the GPCR. This complicates the definition of a CAM receptor, except that it is characterised by an increase in basal activity relative to its wild-type counterpart (Parnot et al., 2002).

2.4.3 Inverse agonism at constitutively-active mutant of G-protein-coupled receptors

According to the theory of multiple receptor activation states (extended ternary complex model), a GPCR exists in equilibrium between two or more conformational states, of which some are active (R* coupling with G-proteins) and some are inactive (R not coupling with G-proteins). Consequently, the binding of a ligand to a specific receptor results in a shift of the equilibrium between the receptor conformations (states) according to the selectivity of the ligand for the active or inactive conformation(s) (see Figure 2-6).
Figure 2.6: Extended ternary complex model. Simple version of the extended ternary complex model in which the receptor exists in two states, an inactive R and an active R* state. $K_A$ and $K_{A*}$ and $K_1$ represent the affinities of the ligand A for R, R* and R*G, respectively, whereas $L$ and $L_{(A)}$ represent the initial equilibrium between the inactive and active conformations of the receptor and $K_G$ and $K_{G(A)}$ represent equilibrium constants describing R*-G and AR*-G-coupling (adapted from Brink, 2002).

An agonist is defined as a drug with higher affinity for R*, whereby its binding to the receptor shifts the equilibrium towards R* to promote coupling to the G-protein. A neutral antagonist binds to the R and R* with equal affinity (thereby not disturbing the existing equilibrium between R and R*), competing with other ligands for binding to the receptor. An inverse agonist binds with higher affinity to R, thereby shifting the equilibrium towards R and thereby reducing R*G complex formation. If a significant proportion of the receptors are in the R* state (significant constitutive activity), basal activity is reduced (inverse effect) by the inverse agonist (Barker et al., 1994; Chidiac et al., 1994; Bond et al., 1995).

Inverse agonism was first described for GABA$_A$ receptors (Ehlert et al., 1983), but has recently been extensively described for GPCRs, including the $\alpha_{1A*}$, $\alpha_{1B*}$, $\alpha_{2A*}$- and $\beta_2$-ARs, 5-HT$_{1A}$-Rs and H$_2$- and H$_3$-histaminic receptors and other GPCR types (Strange, 2002).

Since the discovery of inverse agonism, several drugs previously classified as neutral antagonists have been found to have inverse agonist activity (Chidiac et al., 1994; Wade et al., 2000). The therapeutic utility of the inverse agonists have not yet been clearly
characterised, although several reports suggest an important role. These include reports that 90 hours prolonged treatment with β₂-AR inverse agonists ICI-118,551 and propranolol in transgenic mice (mutants with cardiac overexpression of human β₂-ARs) enhanced baseline atrial contractility (Nagajara et al., 1999). Also, the prolonged treatment of about 24 hours with inverse agonists in human 5-HT₂C receptor (expressed stably in CHO-K1 cells with no receptor reserve), selectively enhance 5-HT₂C-mediated inositol phosphate accumulation (Berg et al., 1999). As a result, it is crucial to understand and differentiate agonists, partial agonists, inverse agonists and neutral antagonists in order to broaden our understanding of drug mechanisms.

2.4.4 The role of G-proteins in antidepressant action

Clinically effective treatments for depression have been available over the past four decades. Several distinct pharmacological compounds show therapeutic efficacy. On the basis of their time of discovery, antidepressants are divided into three generations. The first-generation antidepressants include monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCAs), after which followed second-generation drugs such as SSRIs, mianserin and moclobemide, with lastly the so-called third-generation antidepressants, including venlafaxine, mirtazapine, reboxetine and nefazodone (Ovler et al., 2001).

In order to elicit an antidepressant action, antidepressants may have one or more primary targets. At cellular level, those targets may be near or part of the membrane and their stimulation alter intracellular signalling. Antidepressant treatment may either act by altering neurotransmitter function indirectly through the presynaptic regulation of intracellular signalling, or postsynaptically though the signal-transducing G-protein, which plays a vital role in the amplification and integration of signals in the central nervous system (CNS).
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Figure 2-7: Schematic representation of possible targets of antidepressant action (Donati & Rasenick, 2003).

There are several possible ways by which an antidepressant could modulate the activity of G-proteins, i.e. (1) the number or affinity of receptors could be altered; (2) the coupling between receptor and G-protein could be changed; (3) the number of G-proteins could be changed, or the intrinsic properties of a G-protein could be modified; (4) the coupling between G-proteins and their effectors could be altered; or (5) the effectors themselves could be increased in the number or intrinsic activity (see Figure 2-7) (Donati & Rasenick, 2003).

2.5 DEPRESSION

2.5.1 Introduction

Depression can be defined as a mental illness characterised by sadness, general apathy, a loss of self-esteem, feelings of guilt, and, at times, suicidal tendencies. It is one of the most common and serious psychiatric disorders world-wide. Murray et al. (1997) predicted that bipolar major depression would be the second most prevalent cause of world-wide illness-induced disability by 2020. Lifetime prevalence of 4.4-19.6% for major depression and 3.1-3.9% for dysthymia (minor depression) has been reported (Angst, 1992). The lifetime risk for major depression is estimated to be 7-12% for men and 20-25% for women (Akisal et al., 2000). The symptoms of depression are mostly difficult to notice and are usually
unrecognised by both the patient and the physician. The diagnosis and classification of depression are done according to the current Diagnosis and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1994).

Clinically effective antidepressant treatments currently available attempt to alleviate the symptoms of the disorder, thus decreasing possible functional disability in the affected individuals and enhance their general well-being, quality of life and overall functions. The antidepressant strategies available to date include psychotherapy, pharmacological, electroconvulsive and magnetic therapies (Nestler, 1998; Stahl, 1998; Ressler & Nemeroff, 1999).

2.5.2 Aetiology and neurobiology of depression

Depression is a broad heterogenous psychiatric disorder affecting people at all ages, from early childhood to late adulthood with varying severity and duration. As a result it is most likely that there are several causes of depression, which may include factors such as genetic predisposition, the influence of childhood experiences, psychosocial adversity, drug-induced and biological and physiological effects of other diseases and environmental factors (Shah, 2002). Depression could be either reactive (neurotic) or endogenous. Reactive depression is the mild form of depression thought to be relative to the particular external stressors for the individual and can be relieved by the removal of the external situations. Endogenous depression is classified as the most severe, is unrelated to life stressors, but more likely to be alleviated by drug treatment.

Recent data suggests that depression may be associated with structural and functional alteration of certain areas of the brain. The following brain structures are reported to be implicated in depressive disorders; pre-frontal cortex, the limbic system and subcortical regions, including the hippocampus, amygdala, posterior cingulate, striatum and thalamus (see Figure 2-8).
Structural changes have been reported following neuropathologic studies in humans where a
decrease in glial cell number and density and reduced neuronal density were reported in the
prefrontal cortex (Rajkowska et al., 2001). Reduced glial cell number and density in the
subgenual anterior cingulate gyrus (Ongur et al., 1998; Rajkowska, 2002) and in the
hippocampus (Benes et al., 1998) was also reported.

2.5.3 Neurochemistry of depression

Since the acceptance of the monoamine theory in the mid-1960s, depressive illness was
recognised as a biochemical phenomenon and became a widely accepted theory
(Schildkraut, 1965). This theory states that mental depression is due to the deficiency of
brain monoaminergic activity and that it is treated by drugs that increase this activity
(Schildkraut, 1965). Speculations that biogenic monoamines play a role in the aetiology of
depression initially came from the following three main lines of evidence: (1) the fact that
drugs such as reserpine that causes depletion of central monoamines could induce
symptoms of depression; (2) some depressed patients have reduced levels of
monoaminergic metabolites in some body fluids, usually in the cerebrospinal fluid; and (3)
drugs that relieve depression seem to immediately attenuate the mechanism by which
serotonin and noradrenaline are inactivated (Blier, 2003). There are several different
mechanisms by which current antidepressants increase brain monoamine availability,
including (1) inhibition of the reuptake of monoamines from the synapse; (2) inhibition of the
intraneuronal metabolism of monoamines, or (3) by blocking the presynaptic inhibitory auto-
or heteroreceptors.
However, the monoamine hypothesis has several drawbacks, such as that (1) it does not explain why drugs such as cocaine and amphetamine that also increase the brain monoamine activity, are clinically ineffective as antidepressants; and (2) it fails to explain the delayed onset of antidepressant action, where the changes in monoamine levels at the synapse occur within hours after administering the antidepressants (Baldessarini, 1989).

This lead to the development of the modified monoamine theory (Pineyro & Blier, 1999), suggesting that the acute increase in monoamines at the synapse may be an early step in the complex set of events that ultimately result in antidepressant therapy. The resulting increase in synaptic monoamines has been found to induce desensitisation of the inhibitory auto and heteroreceptors and some post-synaptic receptors located in certain brain regions.

Monoamine neurotransmitters share many properties, but have different brain distribution patterns, with varying mechanisms of action at different receptor types. Neurotransmitters implicated in the aetiology of depression include L-NA, 5-HT, dopamine, γ-aminobutyric acid (GABA), acetylcholine (ACh) and glutamate. However L-NA will be discussed in more details in this chapter since it is highly implicated in mirtazapine’s antidepressant properties.

2.5.3.1 Noradrenaline

L-NA (also known as norepinephrine) is one of the neurotransmitters belonging to a class of compounds referred to as catecholamines (containing a characteristic catechol nucleus with an amine substituent). L-NA is found in most brain regions and is also released from the adrenal gland together with adrenaline (also known as epinephrine) (Leonard, 2003). According to Cameron (quoted by Keltnar et al., 2001), the adrenergic nervous system is implicated in mechanisms involving cortical activation, learning, memory, and attention due to the extensive connection made by the locus ceruleus to the hippocampus and the cortex.

2.5.3.1.1 Noradrenaline cycle

Synthesis: In the brain L-NA is synthesised from dietary amino acid precursor L-tyrosine (Cooper et al., 1996). L-tyrosine is transported by the blood stream and taken up by the brain and other sympathetically innervated tissues through an active transport mechanism.

While in the cytosol of the neuronal cell body and in its nerve terminals L-tyrosine is transformed to L-DOPA by the rate-limiting enzyme tyrosine hydroxylase (TH) (Ressler & Nemeroff, 1999). In order to display maximal effect, TH requires dihydropteridine reductase, Fe²⁺ and oxygen (Cooper et al., 1996). DOPA-decarboxylase rapidly converts L-DOPA to dopamine, the immediate precursor of L-NA. Competitive inhibitors of TH, such as α-methyl-
tyrosine (AMPT - the analogue of tyrosine), have been shown to precipitate depressive episodes in some individuals (Delgado et al., 1993).

Storage and release: In L-NA-specific neurons, dopamine is transported into storage vesicles via amine-specific transporters. While in the vesicles, dopamine is rapidly transformed by dopamine-β-hydroxylase contained in these vesicles to L-NA via hydroxylation of the β-carbon (Ressler & Nemeroff, 1999). This enzyme requires ascorbic acid, Cu²⁺, and oxygen as cofactors (Leonard, 2003). Increase L-NA synthesis has a negative feedback effect on the rate-limiting enzyme TH, thus reducing further synthesis of the transmitter (Leonard, 2003) (see Figure 2-9).

![Figure 2-9: Biosynthesis and transmission at the adrenergic nerve terminal (University of Guelph, 2004).](image)

(DOPA = 3,4-dihydroxyphenylalanine; MAO = monoamine oxidase inhibitor; NE = norepinephrine; Tyr = tyrosine)

Reserpine, an antihypertensive agent, causes a rapid (sometimes gradual) onset of depression by blocking the vesicular monoamine transporter, thus preventing neuronal storage of neurotransmitters, leading to rapid cytosolic metabolism (Freis, 1954; Muller et al., 1955; Schildkraut, 1965). The original catecholamine hypothesis was based largely on this observation (Schildkraut, 1965).
Following its biosynthesis, I-NA is stored in these highly specialised subcellular vesicles in the CNS, sympathetic nerve endings and the chromaffin cells, where it is bound and stored. This leads to a reduction in its diffusion from the neurone, thus protecting it from being metabolised by monoamine oxidases (Rang et al., 1999). Once stored, the transmitter may be released from the vesicle upon physiological stimuli (Rang et al., 1999).

The intravesicular I-NA is rapidly released into the synaptic cleft through exocytosis following nerve stimulation. The mechanism by which I-NA is released is dependent on the Ca²⁺ influx that follows nerve stimulation (Cooper et al., 1996), although this mechanism can be blocked by drugs such as guanethidine or bretylium (Mycek et al., 2000). I-NA at the synaptic cleft regulates its own release by interacting with presynaptic α₂-adrenergic autoreceptors, thus inhibiting further release via (1) inhibition of the voltage-sensitive Ca²⁺ channels, (2) a blockade of the spread of the action potential along the terminal varicosity, (3) opening of K⁺ channels, leading to hyperpolarisation of the neuron terminals and (4) inhibition of adenylyl cyclase, resulting from the decrease in intracellular c-AMP and Ca²⁺ levels (Cooper et al., 1996). For example, mirtazapine is an atypical antidepressant believed to exert part of its antidepressant actions by antagonising the α₂A-adrenergic autoreceptors, thus increasing I-NA neurotransmission.

Removal: Synaptic I-NA may either (1) diffuse from the synaptic cleft, entering the general circulation, (2) being metabolised to O-methylated derivatives by post-synaptic cell membrane associated catechol O-methyltransferase (COMT) in the synaptic space, (3) being recaptured into the presynaptic neuron (Uptake 1), or (4) being taken up by the postsynaptic neuron (Uptake 2) (Mycek et al., 2000). Uptake 1 involves a NA⁺-K⁺-activated ATPase, which can be inhibited by TCAs such as imipramine (Mycek et al., 2000). Cocaine, a local anaesthetic, also blocks NA⁺-K⁺-activated ATPase, but unlike TCAs it does not display antidepressant properties, but instead causes euphoria.

2.5.3.1.2 Noradrenergic receptors and their role in depression

Noradrenergic receptors are classified into alpha- (α-) and beta- (β-) adrenergic receptors (Bylund et al., 1994). Three families of noradrenergic receptors, namely β, α₁ and α₂, have been reported to be present in the CNS (Keltner et al., 2001). These receptors are further subdivided into several subtypes designated α₁A, α₁B, α₂A, α₂B, for α-AR class, while β-ARs are divided into β₁, β₂, & β₃. All known adrenergic receptors are coupled to G-proteins and modulate either AC (β₁, β₂, β₃ & α₂-ARs), thereby influencing the formation of second messenger c-AMP, or PLC (α₁-ARs), leading to the formation of second messenger IP₃ and DAG (Taussig & Gilman, 1995; Lingett, 1996).
\(\alpha_1\)-ARs are generally postsynaptic and excitatory, while \(\alpha_2\)-ARs are inhibitory in nature (Elhwuegi, 2004). However, \(\alpha_2\)-ARs could either function as presynaptic autoreceptors and following their activation, there is a decrease in the release of l-NA, or they could be heteroreceptors controlling the release of other neurotransmitters, e.g. 5-HT. Most antidepressant drugs act by increasing l-NA-availability at the synapse, so that their long-term use results in altered receptor function and expression (Cooper et al., 1996; Keltner et al., 2001).

Chronic administering of most antidepressants have been reported to increase the number and function of the postsynaptic \(\alpha_1\) adrenergic receptors in the frontal cortex of rats (Maj et al., 1985). Similar results have also been reported following chronic treatment with mirtazapine (Rogoz et al., 2002), reboxetine (Rogoz & Kolasiewicz, 2001), milnacipran (Maj et al., 2000) and trimipramine (Maj et al., 1998).

An increase of 31-40% in the number of \(\alpha_2\)-ARs has been reported in the prefrontal cortex of suicide victims and in antidepressant-free depressed suicide victims, which supports the theory of the existence of supersensitive \(\alpha_2\)-ARs in major depression (Garcia-Sevilla et al., 1999). Desipramine, a selective l-NA reuptake inhibitor, has been reported to induce \(\alpha_2\)-ARs desensitisation after chronic treatment in rats in the following brain areas: hypothalamus and corpus striatum, brainstem, cerebral cortex, and hippocampus (Barturen & Garcia-Sevilla, 1992). Similarly, chronic therapy with reboxetine has also been reported to induce desensitisation of \(\alpha_2\)-ARs in the dorsal raphe nuclei (Szabo & Blier, 2001). Amitryptyline has also been reported to induce \(\alpha_2\)-ARs autoreceptor desensitisation (Charney et al., 1983). However, chronic treatment of depressed patients with clinically effective antidepressant mianserin did not produce significant changes in the sensitivity of \(\alpha_2\)-ARs, suggesting that desensitisation of \(\alpha_2\)-ARs autoreceptors might not be a prerequisite for all effective antidepressant treatment (Charney et al., 1984).

The finding that several antidepressants cause down-regulation of post-synaptic \(\beta\)-ARs is often regarded as an indication of antidepressant potential for new agents (Leonard, 2003). Consistent decrease in the \(\beta\)-AR number and function has been reported in rat cortex after chronic treatment for 14 days with desipramine, electroconvulsive therapy or reboxetine (Heal et al., 1987; Heal et al., 1989; Harkin et al., 2000). It has been shown that \(\beta\)-AR levels and function are restored following antidepressant therapy (Leonard, 2003).

However, the \(\beta\)-AR hypothesis has several drawbacks. Firstly, there is evidence suggesting that the down-regulation of \(\beta\)-ARs is dependent on the 5-HT system (Manier et al., 1987).
Secondly the clinical effectiveness of SSRIs, which do not down-regulate the β-ARs, also does not support the β-ARs down-regulation hypothesis (Goodnough & Baker, 1994). Lastly, the fact that the β-AR antagonist propranolol, which crosses the blood-brain barrier (BBB), does not have any antidepressant properties, makes it even more difficult to be reconciled with the hypothesis. This is especially true, since one would have expected that the blocking of the receptor and receptor desensitisation/down-regulation would produce the same effect on neuronal function, which does not seem to occur.

It is clear though, that the l-NA system plays an important role in psychiatry. Its dysregulation in the brain could have serious repercussions, especially in disorders affecting mood control (Keltner et al., 2001).

### 3.5.3.2 Serotonin

Serotonin (5-HT) is believed to be involved in the pathophysiology of several stress-related disorders such as post-traumatic stress disorder (PTSD), anxiety and depression. In addition, the serotonergic system appears to play a crucial role in coping with, and to ward off, the feeling of fear and helplessness (Vogt, 1982).

At least 14 distinct mammalian 5-HT receptor subtypes have been identified (Martin & Humphrey, 1994). Currently, seven classes of 5-HT receptor (5-HT₁, 5-HT₂ and 5-HT₃ receptor classes) have been defined well (Martin & Humphrey, 1994).

The desensitisation of 5-HT₁A auto-receptor has been reported for chronic citalopram treatment in rats (Invernizzi & Samanin, 1994). Hervas et al, (2001) reported similar results in dorsal raphe nuclei (DRN), following two weeks of ongoing treatment with fluoxetine. However, reports regarding the changes in sensitivity and density of 5-HT₂A are not consistent.

### 2.5.3.3 Dopamine

Like l-NA, dopamine also belongs to a class of chemical compounds known as catecholamines. Two types of dopaminergic receptors have been identified in the human brain, namely dopamine type-1 (D₁) and type-2 (D₂) receptors (Leonard, 2003).

Dopamine is one of the important transmitters in the brain, where it plays a vital role in the control of movement, behaviour and some endocrine functions (Elhujuegi, 2004). However, dopamine is more specifically implicated in disorders such as Parkinsonism and schizophrenia than in affective disorders. Nevertheless, dopaminergic systems have been
implicated in the aetiology of depression. There are several reports that chronic treatment with several antidepressant drugs e.g. desipramine, mianserin, and fluvoxamine, has resulted in postsynaptic dopamine receptor (D$_2$/D$_3$) supersensitivity in the nucleus accumbens, a terminal area of the mesolimbic dopaminergic system (Durlach-Misteli & Van Ree, 1992). D'Aquila et al. (2003) reported similar results following chronic treatment of male Sprague-Dawley rats with imipramine, where there was an enhanced dopaminergic neurotransmission in the mesolimbic dopamine system. In the later study (D'Aguila et al., 2003) it was observed that there was an increase in motor activity of the rats following quinpirole (D$_2$ receptor agonist) treatment 24 hours after imipramine discontinuation, with no effect after 12 and 40 days after discontinuation thereof. The above results suggest that there is a reversal of the imipramine-induced dopaminergic supersensitivity after 40 days of withdrawal.

2.5.3.4 Acetylcholine

Acetylcholine (ACh) was identified as a neurotransmitter at the skeletal neuromuscular junction (Feldman & Quenzer, 1984). Cholinergic neurons are widely distributed in both the periphery and the brain and highly implicated in parasympathetic functions. ACh is widely distributed in the brain, occurring in all parts of the forebrain, midbrain and brain stem, with some occurrence in the cerebellum (Rang et al., 1999). ACh is the predominant excitatory neurotransmitter in the brain.

Two classes of cholinergic receptors have been identified, namely the muscarinic receptors (mACh-Rs) and nicotinic receptors (Cooper et al., 1996). Five mACh-R subtypes have been identified (M$_1$-M$_5$), all of which are coupled to G-proteins and either act directly on ion channels or modulate a variety of second-messenger systems (Cooper et al., 1996). The nicotinic receptors, both ligand-gated ion channels, are divided into two classes, namely the muscle and neuronal types. Muscle receptors are present in the skeletal neuromuscular junction (NMJ), while nicotinic receptors are present in the autonomic ganglia and the brain (Rang et al., 1999).

Depression is usually associated with mACh-R supersensitivity, and is normalised by chronic antidepressant treatment (Leonard, 2003). Thus, the anticholinergic properties of some antidepressants, e.g. TCAs, might contribute to their efficacy. However, this activity is usually associated with their unacceptable peripheral side effects, a property that most new-generation antidepressants lack, thus adding to their therapeutic popularity over TCAs.

ACh has also been implicated in mania. There is evidence showing that cholinomimetic drugs and anticholinesterases have antimanic properties, although their effects appear to be
short-lived (Leonard, 2003). Fryer and Lucas (1999) reported that sertraline, paroxetine, nefazodone and venlafaxine non-competely inhibit nicotinic Ach-receptor (nAChR) functions. This study suggests that nAChR may play a vital role in clinical depression.

2.5.3.5 GABA

In mammals, γ-aminobutyric acid (GABA) is primarily a central amino acid neurotransmitter, with only trace amounts in other tissues, e.g. the retina (Rang et al., 1999). GABA is the major inhibitory neurotransmitter in the brain (Godfraind et al., 1970), a function that is vital to the brain, analogous to the brake in the operation of an automobile (Keltner et al., 2001). GABA pathways in the brain occur mainly in the nigrostriatal system, with low occurrence throughout the grey matter. In the CNS, reduction in the GABAergic activity is associated with convulsions and seizures, and most anticonvulsants alter GABAergic transmission either directly or indirectly (Leonard, 2003).

Three types of GABA receptors have been identified to date, namely GABA_A, GABA_B and GABA_C (Leonard, 2003). There is emerging clinical data implicating GABAergic-dysfunction in the pathophysiology of mood disorders (Krystal et al., 2002; Brambilla et al., 2003). It has been speculated in earlier studies that GABA levels in plasma and cerebrospinal fluid are reduced in depressed patients (Petty, 1995) and in depressed alcohol-abusing patients (Roy et al., 1991). In addition recent studies suggest that major depressive disorder is associated with a reduction in cortical GABAergic transmission (Krystal et al., 2002). A reduced number of cortical GABAergic neurons have also been reported to be associated with mood disorders, as suggested by post-mortem studies (Rajkowska et al., 1999). This deficit in cortical GABA levels appears to be reversed by antidepressant treatment, including electroconvulsive therapy (Lloyd et al., 1989).

Several studies in rat brain regions such as the cortex, hippocampus and hypothalamus have shown that there is a reduction in the levels of GABA_A receptors following chronic administration of antidepressants, such as imipramine, desipramine, trimipramine, maprotiline, nomifensine and citalopram (Suzdak & Gianutsos, 1985; Suranyi-Caudotte et al., 1984; Barbaccia et al., 1986; Pilc & Lloyd, 1984).

In contrast, GABA_B receptors have been reported to increase in the cortex and hippocampus in rats following chronic treatment with several antidepressants and electroconvulsive treatment (Brambilla et al., 2003).
2.5.3.6 Glutamate

Glutamate belongs to a class of neurotransmitters referred to as excitatory amino acids (EAAs) (Rang et al., 1999). Glutamate is the primary excitatory neurotransmitter in the brain (Godfraind et al., 1970). In addition to its important role as a neurotransmitter, glutamate also plays a vital metabolic role in the brain for the synthesis of GABA, where it acts as a precursor (Leonard, 2003).

Four main types of glutamate receptors have been identified to date, namely the ionotropic receptors (N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole, α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) and kinase types) and a group of metabotropic receptors (Leonard, 2003). The ionotropic receptors are ligand-gated ion channels and are named on the basis of their specific agonists, while the metabotropic receptors modulate G-protein function (Rang et al., 1999). The AMPA and kinase receptors modulate fast excitatory transmission, while the NMDA receptors mediate slower excitatory responses and are implicated in mediating synaptic plasticity (Leonard, 2003).

There is experimental evidence showing that TCAs inhibit the binding of dizolcipine to the ion channels of NMDA receptors (Leonard, 2003). Recently, it has been shown that both typical and atypical antidepressants reduce the binding of dizolcipine to NMDA receptors, although it is uncertain whether antidepressants exert their effects directly on the ion channels or indirectly through other mechanisms (Leonard, 2003). It has also been reported that repeated treatment with imipramine may induce subsensitivity of metabotropic glutamate receptors in the hippocampus (Pilc et al., 1998).

In a study by Layer et al. (1995) it was indicated that the functional NMDA-receptor antagonist eliprodil possesses antidepressant-like actions. The mechanism by which the NMDA antagonists exert this antidepressant effect is not clearly understood, although, like other antidepressants, these antagonists down-regulate β-ARs (Wedzony et al., 1995) and also enhance serotonergic function (Lejeune et al., 1994).

2.5.4 Clinical classification and diagnosis of depression

The current Diagnosis and Statistical Manual of Mental Disorders (DSM-IV) categorises mental disorders according to their clusters of clinical signs, symptoms and their time course. However, it is difficult to make a diagnosis for a specific psychiatric disorder, because of factors such as a lack of an objective biological 'gold standard' by the DSM, co-morbidity of psychiatric disorders, and the complexity of the disorders.
Even though depression is one of the psychiatric disorders that is hard to diagnose, it can be subdivided into several mood episodes, namely major depressive episodes, manic episodes, mixed episodes and hypomanic episodes.

2.5.4.1 Major depressive episode

According to DSM-IV diagnostic criteria, major depression is defined as a chronic or prolonged state of patient suffering (for 2 or more weeks) from at least one of the following core symptoms and at least four of the secondary symptoms (American Psychiatric Association, 1994).

The core symptoms include the following:

- A depressed mood most of the day, i.e. lack of motivation and loss of interest in practically everything; and
- inability to experience pleasure in anything (anhedonia).

The secondary symptoms are as follows:

- Significant weight loss or weight gain, or increased or decreased appetite;
- sleep disturbances (i.e. insomnia or hypersomnia);
- motor retardation or agitation;
- continuous fatigue (loss of energy);
- feelings of worthlessness and inappropriate guilt;
- diminished ability to think or concentrate; and
- recurrent thoughts of death.

However, these symptoms must occur nearly every day and for most of the day. Another symptom associated with major depressive disorder (MDD) is chronic pain (Kramlinger et al., 1983; Geisser et al., 1997, Geisser et al., 2000). The main factor contributing to the diagnostic problem in MDD is the fact that chronic pain is itself associated with many somatic symptoms.
2.5.4.2 Manic episode

As reported in the DSM-IV, a manic episode is characterised by a time period of an elevated, expansive or notably irritable mood, lasting for at least one week. A manic episode is not a disorder in itself, but is a part of other disorders, usually bipolar disorder. According to the DSM-IV, during this period of manic episode, three or more of the following symptoms would be experienced:

- Inflated self-esteem or grandiosity;
- Decreased need for sleep, e.g. feels rested after only 3 hours of sleep;
- More talkative than usual or pressure to keep talking;
- Flight of ideas or subjective experience that thoughts are racing;
- Distractibility, i.e. attention is easily drawn to unimportant or irrelevant items;
- Increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation;
- Excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g., engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments).

As a result of this disorder in its severe state, difficulty in occupational, social and educational or other important functions may occur. As such, hospitalisation may be necessary to prevent harm to others and self (American Psychiatric Association, 1994).

2.5.4.3 Mixed episode

A mixed episode is characterised by meeting the criteria of both a manic episode as well as a major depressive episode nearly every day for about a week. Like some other psychiatric disorders, hospitalisation may be required due to impairment in occupational functioning and normal social relationships (American Psychiatric Association, 1994).

2.5.4.4 Hypomanic episode

During a hypomanic episode, a distinct period of persistently elevated, expansive, or irritable mood, lasting throughout at least 4 days, that is clearly different from the usual non-depressed mood, is experienced. During this period, three or more of the following symptoms are present (American Psychiatric Association, 1994):
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- Inflated self-esteem or grandiosity;
- Decreased need for sleep, e.g. feels rested after only 3 hours of sleep;
- More talkative than usual or pressure to keep talking;
- Flight of ideas or subjective experience that thoughts are racing;
- Distractibility, i.e. attention is easily drawn to unimportant or irrelevant items;
- Increase in goal-directed activity (either socially, at work or school, or sexually) or psychomotor agitation;
- Excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g. engaging in unrestrained buying sprees, sexual indiscretions, or foolish business investments).

The episode is associated with unequivocal change (mood disturbances that can clearly be observed by others), that is not characteristic of a person when not symptomatic. Hospitalisation may not be necessary in this case, because unlike manic and mixed episodes, it is not sufficiently severe to cause impairment in social or occupational functioning (American Psychiatric Association, 1994).

2.5.5 Antidepressant treatments

2.5.5.1 Monoamine oxidase inhibitors

Monoamine oxidase inhibitors (MAOIs) are the first clinically successful antidepressants introduced clinically in the mid-1950s. Their discovery came after the realisation that the antituberculosis agent iproniazid (the isopropyl derivative of isoniazid) had mood-elevating effects in tuberculosis patients. In 1952 it was found that iproniazid, in contrast to isoniazid, inhibits the enzyme MAO (Baldessarini, 2001). However, despite these advances, MAOIs appeared to be limited in efficacy at therapeutic doses and presented both toxic risks and potential dangerous drug interactions, thus limiting their acceptance in favour of other antidepressants such as TCAs (Baldessarini, 2001).

MAO is an enzyme produced by the mitochondria and is responsible for inactivating biogenic amines such as 5-NA, DA, 5-HT and melatonin (Leonard, 2003). MAOIs act by inhibiting this enzyme, thus increasing the availability of these neurotransmitters to interact with their respective receptors. As discussed by Kamil (1996), there are two subtypes of MAO enzymes, namely MAO-A and MAO-B. MAO-A is responsible for the degradation of 5-NA,
dopamine and 5-HT, while MAO-B metabolises dopamine and exogenously occurring monoamines such as tyramine. MAOIs are classified as either selective or non-selective and either as reversible and irreversible. The selective MAOIs are capable of inhibiting either MAO-A or MAO-B, while the non-selective MAOIs antagonise both enzymes. The irreversible MAOIs form a bond with the MAO enzyme to inactivate it in such a way that additional enzyme would need to be synthesised for the biological activity to be re-established. This results in a sustained effect over an extended period, with no correlation with the plasma levels of the drug (Kamil, 1996).

2.5.5.1.1 Irreversible monoamine oxidase inhibitors

Irreversible MAOIs include isocarboxazid, phenelzine, tranylcypromine and pargyline. In addition to othostatic hypotension, the most common side effect of these agents induce severe hypertension due to increased sympathomimetic or serotonergic activity (Baldessarini, 2001). During treatment, including a two-week period after discontinuation of therapy, food containing tyramine such as most cheeses, yeast extract, stewed fruits, broad beans, etc, as well as medication that have sympathomimetic or serotonergic activity, should be avoided (Kamil, 1996).

The irreversible MAOIs could precipitate serotonin syndrome when co-administered with 5-HT enhancing medications. Other side effects may include tremor, tinnitus, sexual dysfunction as well as peripheral oedema (Kamil, 1996).

2.5.5.1.2 Reversible monoamine oxidase inhibitors

2.5.5.1.2.1 Reversible monoamine oxidase-A inhibitors

Reversible MAOIs include moclobemide, tolazatone and brafaromine. These agents have a selective and short-acting inhibitory effect on MAO-A enzyme. They are considered safe when co-administered with sympathomimetic amines. The diet of the patient also does not have to be altered (Kamil, 1996). Headaches and insomnia are the most common side effects, while overdosing could result in tremor, anxiety and light-headaches. Nausea, dry mouth and sweating could also occur, although are not commonly reported.

2.5.5.1.2.2 Reversible monoamine oxidase-B inhibitors

An example of a MAO-B inhibitor is selegiline. The enzyme MAO-B is preferentially concentrated in the basal ganglia where it is responsible for the degradation of DA. Penny and Young (1998) indicated that the suppression of the enzyme MAO-B has clinical importance in the treatment of Parkinson's disease. It has also been indicated that, like
reversible MAO-A inhibitors, selegiline does not interact with tyramine-containing food. Adverse effects of these agents include hypotension, nausea, confusion, agitation, increased dyskinesia and hallucinations (Reynolds, 1989).

2.5.5.2 Tricyclic antidepressants

The discovery of TCAs came after Haflinger and Schindler in the late 1940s had synthesised a series of more than 40 iminodibenzyl derivatives for possible use as antihistamines, sedatives, analgesics and anti-Parkinson drugs. Imipramine, a dibenzazepine compound, was one of the compounds synthesised. It differs from phenothiazines by the replacement of the sulphur with an ethylene bridge producing a seven-membered central ring analogue. It was fortuitously found by Kuhn (1958) following the clinical investigations of these putative phenothiazine analogues that imipramine was relatively ineffective as an antipsychotic, but that had remarkable effects in depressed patients (Baldessarini, 2001; Hollister, 1978).

In the early 1960s, imipramine, amitriptyline, their N-demethyl derivatives, and other related compounds were the first successful antidepressants and have since been used for the treatment of major depression (Baldessarini, 2001). TCAs act by inhibiting the neuronal transport (reuptake) of monoamine neurotransmitters, e.g. 5-NA, 5-HT and sometimes DA, enhancing the availability of these neurotransmitters. Imipramine-like TCAs inhibit both the 5-NA and 5-HT and include imipramine, amitriptyline and clomipramine. Studies conducted by Amsterdam (1998) suggest that TCAs, in particular clomipramine, are superior to SSRIs in treating melancholic depression. TCAs with a secondary amine side chain or the N-demethylated, such as amoxapine, desipramine, maprotiline, norclomipramine, nordoxepin and nortriptyline, are relatively selective for 5-NA transport (Baldessarini, 2001).

In addition to the blockade of neurotransmitter uptake, TCAs also have an effect on several heterogenous receptors, including the H₁, α₁-adrenoceptors and the mACH-Rs (Kamil, 1996). It has been reported that tertiary amines are potent antagonists of these receptors and that this antagonism is the major cause of multiple side effects and toxicity of the TCAs. TCAs have also been reported to have cardiovascular side effects related to those of class I antiarrhythmic drugs (Kamil, 1996). Side effects such as dry mouth, blurred vision, urinary retention, tachycardia and impaired orgasmic ability, are thought to be associated with anticholinergic effects, while postural hypotension results from α-AR antagonism. Antihistaminergic side effects include sedation and weight gain (Baldessarini, 2001).
2.5.5.3 Selective serotonin reuptake inhibitors

Selective serotonin reuptake inhibitors (SSRIs) have currently replaced TCAs as the drugs of choice in the treatment of depressive disorders, mainly because of their better tolerated profile and safety when taken in an overdose (Ables & Baughman, 2003). They are also effective in the treatment of obsessive-compulsive disorder (OCD), panic disorder, and social phobia. New indications for selective serotonin reuptake inhibitors include post-traumatic stress disorder (PTSD), premenstrual dysphoric disorder, and generalised anxiety disorder (Ables & Baughman, 2003).

SSRls were first developed in the early 1970s. Their discovery came after the realisation that antihistamines such as chlorpheniramine and diphenhydramine inhibit the transport of 5-HT or I-NA (Carlsson & Wong, 1997). Zimelidine, fluoxetine and fluvoxamine were the first to be introduced clinically, while zimelidine was withdrawn due to its association with febrile illnesses and Guillain-Barre ascending paralysis (Baldessarini, 2001; Carlsson & Wong, 1997). SSRls that are currently on the market include fluoxetine, sertraline, paroxetine, citalopram and fluvoxamine (Ables & Baughman, 2003).

SSRls are a structurally heterogeneous group of compounds and have less antihistaminic, alpha-adrenergic and anticholinergic effects compared with TCAs (Kamil, 1996; Harvey, 1997). They selectively block the neuronal transport of serotonin, leading to complex secondary responses (Baldessarini, 2001). Although this is the general mechanism of action of this class of compounds, they each have a slightly different pharmacological profile, leading to varying clinical activity, side effects and drug interactions (Stahl, 1998). The resulting increase in synaptic availability of serotonin activates a variety of postsynaptic 5-HT receptor types (Azmitia & Whitaker-Azmitia, 1995). Stimulation of 5-HT3 receptors is suspected of being responsible for the common adverse effects characteristic in this class of drugs, including gastrointestinal (nausea and vomiting) and sexual effects (delayed or impaired orgasm). Stimulation of 5-THz receptors is suggested to contribute to the risk of agitation or restlessness, usually induced by SSRls (Baldessarini, 2001). Their repeated treatment leads to secondary changes, such as gradual down-regulation of postsynaptic 5-HT2A receptors that may contribute to antidepressant effects.

In addition to nausea, vomiting and sexual effects, other side effects associated with SSRIs include insomnia and headaches (Harvey, 1997). The change in sleeping patterns seen with SSRls is thought to be due to stimulation of 5-HT2 receptors (Thase, 1999). Concomitant, treatment with low doses of trazodone (a 5-HT2 receptor antagonist) helps to alleviate insomnia (Thase, 1999). Other reported side effects include movement disorders, acute
dystonia, anorgasmia and reduced libido, yawning, bruxism and hyponatraemia (Harvey, 1997).

"Serotonin syndrome" is another side effect associated with SSRIs. It is diagnosed by using Sternbach’s criteria (1991), as a condition characterised by the presence of at least three of these symptoms: agitation, diaphoresis, diarrhoea, fever, hyperreflexia, inco-ordination, mental status change (confusion, hypomania), myoclonus, shivering and tremor.

2.5.5.4 Atypical antidepressants

Atypical antidepressants, including mirtazapine and mianserin (see § 2.2.), are those compounds that do not have structural or functional similarities to TCAs, SSRI and MAOIs. They can be classified according to their mechanism of actions as follows:

2.5.5.4.1 Noradrenaline and dopamine reuptake inhibitors (NDRI)

Bupropion is an example of this class of antidepressants. It is a weak inhibitor of dopamine reuptake, with slight effects on the L-NA reuptake and is devoid of effects on 5-HT reuptake (Danileviciute & Sveikata, 2002). Bupropion is metabolised into three metabolites (hydroxybupropion, threohydroxydibupropropiol and erythrohydrobupropion), which are all active and responsible for the inhibition of dopamine reuptake (Danileviciute & Sveikata, 2002). It appears to be devoid of adverse effects on sexual function. Seizures are the main safety risk associated with bupropion therapy.

Both the inhibition of dopamine and L-NA reuptake transporters are responsible for bupropion’s antidepressant effects. However, its dopaminergic effects may cause psychomotor activation and precipitation or aggravation of psychosis (Danileviciute & Sveikata, 2002). These psychotic effects include hallucinations and delusions, over-stimulation, agitation and nausea.

2.5.5.4.2 Selective noradrenaline reuptake inhibitors (NARI)

Reboxetine is the first antidepressant drug to be specifically selective for the L-NA reuptake without affecting 5-HT and dopamine reuptake. TCAs such as desipramine or nortryptiline are relatively more potent as L-NA-uptake blockers than as 5-HT-uptake inhibitors, however, they cannot be regarded as selective L-NA-reuptake blockers, since they block other pathways as well (Danileviciute & Sveikata, 2002). Reboxetine was introduced in 1997 as the first drug of new antidepressants class (Kadhe et al., 2003).

Although there is insufficient published data on reboxetine, it has been reported to have greater efficacy than placebo (Montgomery, 1997) and similar efficacy to the SSRI fluoxetine
(Massana et al., 1999) and the TCAs desipramine and imipramine (Ban et al., 1998; Berzewski et al., 1997). It has also been reported that reboxetine may prevent a relapse in patients that have responded to short-term antidepressant therapy for about 6 weeks, although these studies need to be replicated (Holm & Spencer, 1999). The onset of its antidepressant effect is about 2-3 weeks, which is comparable to other antidepressants (Danileviciute & Sveikata, 2002).

Adverse events mostly reported in the study of reboxetine versus placebo included dry mouth, constipation, headaches, increased sweating, tachycardia, vertigo, urinary hesitation and/or retention (Mucci, 1997; Doster et al., 1997; Olver et al., 2001). From in vitro studies, reboxetine appears to be metabolised by the CYP3A4 isoenzyme, making it prone to drug interactions.

2.5.5.4.4 Serotonin and noradrenaline reuptake inhibitors (SNRI)

Two examples in this class include venlafaxine and milnacipran.

Venlafaxine is a dual-acting 5-HT and 1-NA reuptake inhibitor that has been reported to be more effective than placebo (Guelfi et al., 1995) and fluoxetine (Clerc et al., 1994) in the treatment of hospitalised patients with melancholic depression. The superior effectiveness of venlafaxine is suggested to be due to its dual-acting antidepressant properties. According to Gabbard (quoted by Danileviciute & Sveikata, 2002), venlafaxine blocks 5-HT-reuptake potently (at low doses of less than 150 mg/day), while it blocks 1-NA-reuptake only weakly, making its function at low doses basically similar to that of the SSRIs. Danileviciute and Sveikata (2002) proposed that the faster desensitisation of β-ARs seen with higher doses of venlafaxin may also contribute to its rapid onset of action.

Commonly reported side effects of venlafaxine are nausea, insomnia, somnolence, dizziness, dry mouth, headaches, constipation, asthenia, nervousness, increased perspiration and sexual dysfunction (Danileviciute & Sveikata, 2002).

Milnacipran, like venlafaxine, is a dual 5-HT and 1-NA-reuptake inhibitor. It has recently been reported to be more effective than fluvoxamine and paroxetine in the treatment of depression in patients aged 50 or older (Morishita, 2004). In a comparative study with imipramine, milnacipran was shown to have similar efficacy in reducing depressive symptoms (Lopez-Ibor et al., 2004). In the latter study, it was also indicated that the frequency of most adverse events in the milnacipran-treated patients was lower than that observed in the imipramine group, particularly those related to anticholinergic symptoms.
The results of this study support others, which demonstrated that milnacipran has equivalent efficacy, but superior tolerability to a TCA such as imipramine (Lopez-Ibor et al., 2004).

Milnacipran, unlike TCAs, is essentially devoid of antagonistic activity at mAChRs, histaminergic receptors and adrenergic receptors, contributing to its superior tolerability profile. Dysuria and shivering, however, were more common side effects associated with milnacipran (Lopez-Ibor et al., 2004).

2.5.5.4.4 Serotonin (5-HT$_{2A}$) receptor blockade with serotonin reuptake inhibition

Nefazodone is an example of antidepressants that block 5-HT$_{2A}$-Rs and inhibit 5HT reuptake. It was developed from structural modifications of trazodone, an earlier antidepressant that has been withdrawn because it is more sedating and causes postural hypotension (Kent, 2000). It is metabolised by CYP303/4 to form its active metabolite m-chlorophenylpiperazine (mCPP), which is a potent 5-HT$_{2C}$ receptor agonist. However, nefazodone has a tendency to cause paradoxical effects, including anxiety and stimulation, instead of anxiety reduction and sedation (Danileviciute & Sveikata, 2002).

Adverse effects associated with nefazodone treatment include drowsiness, nausea, dizziness and hypotension (Olver et al., 2001). However, the use of nefazodone as an antidepressant has recently been also discontinued because of its association with fulminant liver failure in previously well patients. These signs of liver damage seems to emerge between 14 and 28 weeks following chronic nefazodone treatment (Olver et al., 2001).

2.5.5.5 Electroconvulsive shock treatment

Electroconvulsive shock treatment (ECT) is an effective treatment for a wide range of psychiatric disease, ranging from severe suicidal depression and mania to some forms of schizophrenia. It is generally indicated for the treatment of depression that is resistant to treatment (Rang et al., 1999). ECT is reported to be at least as effective as antidepressant drugs, with response rates ranging between 60% and 80%. Most recently, in the study by Van den Broek et al., (2004), it was shown that medication resistance does not influence short-term response to subsequent ECT and it could still be of considerable efficacy. However, despite these advances, there are dangers associated with this method of treatment, the main being confusion and memory loss lasting for days or weeks (Rang et al., 1999).

The rise in the seizure threshold during the course of ECT treatment and the corresponding change in blood flow may reflect profound changes in cerebral metabolism (Leonard, 2003).
Experimental studies in models of depression indicated that there is decreased β-AR responsiveness in both ECT and chronic antidepressant therapy (Rang et al., 1999). In other studies conducted in rodents, it was found that there are similar chronic effects with both ECT and antidepressant drug treatment. These effects include increased l-NA and 5-HT levels in the brain, decreased α2-AR levels and their functional activity and decreased functional activity of dopamine auto receptors (Leonard, 2003). Glutamate-receptor functions are also altered by both chronic ECT and antidepressant drug treatments. Decreased NMDA-receptor binding was reported for traditional antidepressants, including SSRIs, TCAs and ECT (Paul et al., 1993), while an increase in the number of metabotropic glutamate receptors in the hippocampus was observed following ECT and chronic imipramine (Bajkowska et al., 1999).

Since the central cholinergic system is implicated in the pathogenesis of affective disorders and memory function, the memory deficit elicited by chronic ECT in both patients and animals may be associated with the decreased density and function of central mAChRs (Leonard, 2003).

Brain GABA is closely associated with the induction of seizures. There is evidence suggesting that there is decreased GABA synthesis and an increase in GABA\(_B\) receptor density in the limbic region following chronic ECT and antidepressant drug treatment (Leonard, 2003).

### 2.6 SUMMARY AND CONCLUSIONS

The mechanism by which mirtazapine (atypical antidepressant with putative earlier onset of action than older antidepressants) attains its antidepressant effects is unique among currently employed antidepressants. Thus understanding this mechanism may facilitate the development of antidepressants with a faster onset of action and better tolerability profile.
3.1 PROJECT DESIGN

3.1.1 Introduction

This chapter will discuss the experimental layout, cell lines, materials, and assays used in the study. This includes functional studies (measuring agonist-induced, receptor-mediated second-messenger formation), radio-ligand-receptor binding studies and myo-[2-3H]-inositol and [2-3H]-adenine cellular uptake studies. The study can be divided into two main sections: part A, entailing the characterisation of the mode of action of mirtazapine at \( \alpha_2 \)-adrenergic receptors (\( \alpha_2 \)-ARs) (see Figure 3-1), and part B, entailing investigations into the modulatory effect of mirtazapine pre-treatments on the muscarinic acetylcholine receptor (mACH-R) and the \( \beta \)-adrenergic receptor (\( \beta \)-AR) function (see Figure 3-2).
Figure 3-1 Schematic layout of the experiments conducted for part A of the study.
($D_{max} =$ receptor concentration; CAM = constitutively active mutant).
Chapter 3: Materials and methods

Part B
investigating the modulatory effects of mirtazapine pre-treatments

SH-SY5Y cells
(human neuroblastoma)

α2A-L cells
(wild-type α2A-ARs
(low receptor number)

24-hour pre-treatment with 0 or 10 μM mirtazapine
(study objective experiments)

Cell membranes
(control exp.)

mACh-Rs
(full-agonist:
metacholine)

β-ARs
(full-agonist:
iso-proterenol)

α2A-ARs
(full-agonist:
UK-14,304)

18-hour labelling with:
1 μCi/ml myo-[2-3H]-inositol (mACh-Rs)
or 1 μCi/ml [2-3H]-adenine (β-ARs & α2A-ARs)

Conc.-effect curves
([3H]-IPX accumulation) +
myo-[2-3H]-inositol uptake

Emax
([3H]-cAMP accumulation) +
[2-3H]-adenine uptake

Saturation-binding
(KD values)
Yohimbine

Figure 3-2: Schematic layout of experiments conducted for part B of the study.
(α2A-ARs = type-2A alpha-adrenergic receptors; β-ARs = beta-adrenergic receptors; Emax = receptor concentration; CHO-K1 = Chinese hamster ovary; mACh-Rs = muscarinic acetyl choline receptors).
3.1.2 Cell lines

Several cultured cell lines were employed in this study. The Chinese hamster ovary (CHO-K1) cell lines, stably transfected to express the wild-type porcine $\alpha_2A$-AR at relatively high numbers ($\alpha_2A$-H cell line) and relatively low numbers ($\alpha_2A$-L cell line) and the constitutively active mutant $\alpha_2A$-AR ($\alpha_2A$-CAM cell line), as well as the mock-transfected control cells (transfection plasmid, but no cDNA for the $\alpha_2A$-AR − neo cell line) were a kind gift from Dr. Richard Neubig (Department of Pharmacology, University of Michigan, Ann Arbor, USA). The receptors in these cell lines have been previously characterised, with the reported $\alpha_2A$-AR concentration of $19 \pm 3$ pmol/mg for CAM $\alpha_2A$ cells, $25 \pm 4$ pmol/mg for $\alpha_2A$-H (Wade et al., 2000) and $1$ pmol/mg for $\alpha_2A$-L (Wade et al., 1999). These cell lines were used to investigate the mechanism of antagonism of mirtazapine on the $\alpha_2A$-ARs. The $\alpha_2A$-H cells provide a good in vitro biological model for investigating any possible weak / partial agonist activity possessed by $\alpha_2A$-ARs ligands, due to the high $\alpha_2A$-ARs numbers expressed in this cell, so that even very weak agonists would display full agonism. The constitutive activity displayed by the $\alpha_2A$-CAM cell allows investigation of any $\alpha_2A$-AR inverse agonist properties. The neo cells, containing the selection plasmid but not $\alpha_2A$-ARs, were used as a control. The $\alpha_2A$-L cell line was used to investigate the effects of mirtazapine pre-treatment on the $\alpha_2A$-ARs, selected due to its lower receptor expression that resembles expression levels within the normal physiological range. The experiments were conducted as indicated in Figures 3-1 & 3-2.

Another cell line used in this study is the human neuroblastoma (SH-SY5Y) cell line from American Type Culture Collection (ATCC). This cell line provides an in vitro biological model for investigating the modulatory effects of antidepressant drugs, due to its neuronal nature. SH-SY5Y cells are known to endogenously express mACH receptors, predominantly the M3 type (Slowiejkko et al., 1999), with some evidence suggesting the availability of M1 and M2 types (Kukkunen et al., 1992). In addition, the SH-SY5Y cells endogenously express $\beta$-ARs in significant numbers, which was evident from the increase in the $L$-isoproterenol-induced accumulation of [3H]-cAMP in these cells (see Chapter 4).

The $\alpha_2A$-H, $\alpha_2A$-L, $\alpha_2A$-CAM and the neo cells were maintained and grown to about 95% consistency in 150 cm$^2$ cell culture flasks with Ham's F12 medium containing 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin at 37 °C in 5% CO$_2$. In addition, 0.4 mg/ml G418 was used to maintain selection for stable expression. Like the CHO cell lines, SH-SY5Y cells were similarly maintained, although the growth medium used
was a 1:1 ratio mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin.

3.1.3 Materials

3.1.3.1 α2A-adrenergic receptor ligands

Mirtazapine was a kind gift from Organon (Netherlands). Mianserin was obtained from Tocris (Ellisville, USA). Idazoxan hydrochloride, 5-bromo-N-(2-imidazolin-2-yl)-6-quinoxalinamine (brimonidine or UK-14,304) and yohimbine hydrochloride were obtained from Sigma Chemical (St. Louis, USA).

3.1.3.2 Radio chemicals

[2-3H]-adenine (23.0 Ci/mmol) and myo-[2-3H]-inositol (17.0 Ci/mmol) was obtained from Amersham Bioscience (UK). [methyl-3H]-yohimbine (85.0 Ci/mmol) was obtained from PerkinElmer™ Life Sciences (Boston, USA).

3.1.3.3 Cell culture media

Twenty-four well plates and 150 cm² culture flasks were obtained from Corning (New York, 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-verse, 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).

3.1.3.4 Other chemicals

Trichloroacetic acid (TCA), myo-inositol (MI), N-(-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid (HEPES), ethylene glycol-bis[b-amino ethyl ether]-N,N,N',N'-tetra-acetic acid (EGTA), lithium chloride (LiCl), 3-isobutyl-1-methylxanthine (IBMX), serotonin creatinine sulphate, acetyl-β-methylcholine chloride (metacholine chloride), adenosine 3',5'-cyclic monophosphate (cAMP), alumina type WN-3, 1,3-diaza-2,4-cyclopentadiene (imidazole), adenosine triphosphate (ATP), forskolin and isoproterenol were obtained from Sigma Aldrich (Johannesburg, South Africa). Ascorbic acid, Bradford reagent, ethylenediaminetetra-acetic acid (EDTA), HCl, NaHCO₃, NaCl, KCl, Na₂HPO₄, KH₂PO₄, MgCl₂, HCl and NaOH were obtained from Merck (Darmstadt, Germany). Fractioned BSA was obtained from Boehringer Mannheim (Mannheim, Germany). 2-amino-2-hydroxymethylpropan-1, 3-diol (Tris) was obtained from Acros (Geel, Belgium). Formic acid was obtained
3.1.4 Instruments

Tri-carb 2100 TR liquid scintillation analyser (Packard, A.D.P. South Africa), Sigma Laborzentrifuge 3K15 bench-top centrifuge, Sorval® Discovery 90SE ultra-centrifuge, Nikon TMS halogen light microscope, haemocytometer (0.1 mm depth, 0.0025 cm²), Consort P901 electrochemical analyzer (PH meter), Sartorious BP211D (max 210) balance, 96 well plate reader and 560 nM filter (Labsystems multiskan RC), Teflon® homogeniser, HERA cell & Forma Scientific CO₂ incubators were used.

Other apparatus used include Capp autoclavable and Eppendorf research micropipettes.

3.2 ASSAYS

All the experiments requiring an aseptic environment were carried out in the laminar-flow chamber with controlled pressure. Standard aseptic techniques were adhered to.

3.2.1 Cell counting

In all the experiments, cells were counted before seeding into 24 well plates to ensure a uniform cell distribution between the wells and to avoid large variations between experiments. CHO cell lines (α2A-H, α2A-L, α2A-CAM, and Neo cells), were counted and seeded homogenously at 2 million cells/well, while the SH-SY5Y cells were seeded at 8 million cells/well. Unlike the CHO cell lines with good adherence to the surface of the well, the SH-SY5Y cells have a poorer adherence, thus they were thickly seeded in order to compensate for serum-deprivation during pre-treatment, causing cell loss and other losses during the course of the experiments.

Cells were loosened from the culture flask by trypsination for about 10-15 minutes. The suspension was diluted with the normal medium and homogenised by pipetting cells up and down. If necessary (with high cell numbers), the cell suspension was diluted, whereafter 20 µl of the cells suspension was pipetted into the haemocytometer and counted under a Nikon TMS halogen light microscope. The number of cells within each nine blocks was counted, the average calculated and the average counted number of cells was then multiplied with 100 000 (1E5) according to manufacturer specifications of the haemocytometer. The cell
concentration of the mother cell suspension was then adjusted and the cells seeded according to the experiment requirements.

3.2.2 Whole-cell [³H]-cAMP accumulation assay

The aim of this study was to construct concentration-effect curves of UK-14,304, mirtazapine, mianserin, idazoxan and yohimbine in the α₂A-H, CAM α₂A and neo cells. The study also involved investigating the effect of mirtazapine pre-treatment on the β-ARs and α₂A-ARs expressed in the SH-SY5Y and α₂A-L cells respectively. [³H]-cAMP accumulation was determined as described by Wade et al. (1999) and Wong (1994), with minor modifications. For α₂A-H, CAM α₂A, and the Neo cell experiments, the cells were labelled before seeding by adding 300 μl EMEM (37 °C) containing 1 μCi/ml [2-³H]-adenine. It was thereafter incubated for about 18 hours before the assay was conducted, while the SH-SY5Y and α₂A-L cells were pre-treated for 24 hours before labelling.

- After labelling, the cells were rinsed with 0.5 ml DMEM.

- Thereafter 1 ml of stimulation medium (consisting of DMEM with 1 mM IBMX, 30 μM forskolin, and appropriate concentrations of UK-14,304, mirtazapine, mianserin, idazoxan l-isoproterenol or yohimbine) was added and cells were incubated for 20 minutes at 37° C and 5% CO₂.

- The stimulation medium was aspirated and the [³H]-cAMP accumulation reaction terminated by adding 1 ml ice-cold 5% TCA containing 1 mM ATP and 1 mM cAMP to each well.

- The plates were placed in the cold room (4 °C) for 30 minutes for cells to lyse.

- [³H]-cAMP was separated by using two sets of 100 Biorad poly-prep columns fitted into double-layer racks¹.

- The columns were regenerated (see Table 3-1) and the Dowex columns placed over the scintillation vials.

¹ These racks were designed to fit over the collecting buckets and scintillation vial racks. The first set of 100 columns was packed with alumina type WN-3, while the second set was packed with Dowex AG50W×4 resin.
After the lyses period, 1 ml of cell supernatant from each well was transferred to each respective dowex column.

- The columns were diluted with 1 ml water.
- Thereafter 7 ml of Ultima Gold XR scintillation fluid was added to each scintillation vial, thoroughly mixed and counted ([³H]-ATP counts).

- Dowex columns were placed over the alumina columns and washed with 2 x 6 ml of water.
- Alumina columns were placed over the scintillation vials and eluded with 4 ml 0.1 M imidazole.
- Thereafter 7 ml Ultima Gold XR scintillation fluid was added to each scintillated vials.
- Vials were thoroughly mixed and the [³H]-cAMP was counted in a Tri-carb 2100 TR liquid scintillation analyser.

- [³H]-cAMP counts were expressed as % cAMP as follows:

\[
\%[³H]cAMP = \frac{[³H]cAMP}{[³H]cAMP + [³H]ATP} \times 100
\]

\(^2\) The 4 ml 0.1 M imidazole was determined from the experiment done in this study.
Table 3-1 Regenerating the alumina and Dowex columns for cAMP assay

<table>
<thead>
<tr>
<th>Columns</th>
<th>Alumina</th>
<th>Dowex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procedure</td>
<td>Place the columns over the collecting basket</td>
<td>Place the columns over the collecting basket</td>
</tr>
<tr>
<td></td>
<td>❖ Add 8 ml of 0.1 M imidazole and let it run through.</td>
<td>❖ Add 1 ml nM HCl and let it run through.</td>
</tr>
<tr>
<td></td>
<td>❖ Add another 8 ml of 0.1 M imidazole and let it run through.</td>
<td>❖ Add 6 ml double-distilled water and let it run through.</td>
</tr>
<tr>
<td></td>
<td>❖ Then add 8 ml double-distilled water and let it run through.</td>
<td>❖ Add another 6 ml de-ionised water and let it run through to wash off all the unwanted radio-chemicals.</td>
</tr>
<tr>
<td></td>
<td>❖ Add another 8 ml double-distilled water and let it run through.</td>
<td></td>
</tr>
</tbody>
</table>

3.2.3 Whole-cell [³H]-IP₅ accumulation assay

The objective of this study was to investigate the effect of mirtazapine pre-treatments on the mACh-R function by constructing dose-response curves of mACh-R full-agonist metacholine and measuring whole-cell IP₅-accumulation³ in the SH-SY5Y cells. The SH-SY5Y cells were pre-treated for 24 hours as described by Brink et al. (2004). The G₅-mediated [³H]-IP₅ accumulation assay in the SH-SY5Y cells was carried out as described by Casarosa et al. (2001).

❖ After the pre-treatment cells had been rinsed out with 2 X 0.5 ml EMEM (37 °C) and labelled by adding 300 µl EMEM containing 1% BSA and 1 µCi/ml to each well, they were incubated for 18 hours at 37 °C and 5% CO₂ before the assay was initiated.

³ IP₅ includes all the inositolphosphates, i.e. inositolphosphate (IP), inositolidiphosphate (IP₂) and inositoltetriphosphate (IP₃). The study focuses more on IP₃.
After labelling, the cells were rinsed out with $2 \times 0.5$ ml DMEM and incubated for 10 minutes with $0.5$ ml/well assay medium (DMEM + 0.5 M HEPES + 0.4 M LiCl). The assay medium was then aspirated and $0.5$ ml assay medium with appropriate concentrations of metacholine added to each well, hereafter it was incubated for 60 minutes at $37^\circ$C and 5% CO$_2$.

After the incubation period, the medium was aspirated and the reaction terminated by adding $1$ ml ice-cold $10$ mM formic acid. It was then left to stand for 90 minutes to allow cells to lyse.

$100$ Bio-Rad poly-prep filled with Dowex 1 $\times 8$ - 400, 200 - 400 mesh 1-chloride type was used.

The columns were regenerated before use (see Table 3-3).

After the lyses period, $1$ ml lyses solution supernatant from each well was transferred to the corresponding Dowex column.

$1$ ml of ice-cold ($4$ $^\circ$C) was added to each well and transferred again to each corresponding Dowex column.

The columns were then rinsed out with $2 \times 5$ ml solution 3 (see Table 3-2 for composition).

Columns were placed over the scintillation vials and the $[^3H]$$\text{IP}_x$ in each column was diluted with $3$ ml solution 4 (see Table 3-2 for composition).

$7$ ml Ultima Gold XR scintillation fluid was added to each vial, mixed thoroughly, after which $[^3H]$$\text{IP}_x$ was counted in a Tri-carb TR liquid scintillation analyser.

$^4$ Li$^+$ inhibit the enzyme which metabolises $\text{IP}_x$. 
Table 3-2: Chromatographic solutions (All the solutions were made up by using double-distilled water)

<table>
<thead>
<tr>
<th></th>
<th>Solution Composition</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 M Ammonium for and 3 M formic acid</td>
<td>Dowex column regeneration</td>
</tr>
<tr>
<td>2</td>
<td>0.1 M myo-inositol and 3 M formic acid</td>
<td>Dowex column regeneration</td>
</tr>
<tr>
<td>3</td>
<td>1 M Sodium formate and 0.1 M borax decahydrate</td>
<td>Washing through unwanted $[^3]$-biochemicals</td>
</tr>
<tr>
<td>4</td>
<td>5 M Ammonium formate and 3 M formic acid</td>
<td>Washing through $[^3]$-IP$_x$</td>
</tr>
</tbody>
</table>

Table 3-3: Regenerating the Dowex columns for IP$_x$ assay

<table>
<thead>
<tr>
<th>Procedure</th>
<th>The columns were placed over the collecting basket and regenerated as follows:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>◇ Add 5 ml water and let it run through.</td>
</tr>
<tr>
<td></td>
<td>◇ Add 2.5 ml solution 1 (see Table 3.2 for composition) and let it run through.</td>
</tr>
<tr>
<td></td>
<td>◇ Add 10 ml water and let it run though.</td>
</tr>
<tr>
<td></td>
<td>◇ 2 × Solution 2 (see Table 3.2 for composition) and let it run through.</td>
</tr>
</tbody>
</table>
3.2.4 Total [2-³H]-adenine & myo-[2-³H]-inositol uptake

The study was aimed at investigating the possible effect of mirtazapine pre-treatment on the total myo-[2-³H]-inositol and [2-³H]-adenine uptake into the SH-SY5Y and the α₂A-L cells during labelling. This study was conducted concurrently with [³H]-IP₃ and the [³H]-cAMP assays respectively.

- Cells were seeded in 24 well plates, pre-treated, labelled and lysed (see § 3.2.2. and 3.2.3 above).
- Thereafter 1 ml lysates solution supernatant was transferred directly to the scintillation vials.
- Then 7 ml Ultima Gold XR scintillation liquid was added to each vial and the total radio-activity was counted in the Tri-carb TR liquid scintillation analyser.

3.2.5 Preparing membranes from α₂A-H and CAM α₂A cells

The aim of this study was to prepare, separate, purify and quantify membrane proteins from cultured cells for the purpose of determining receptor concentration in the cells by using appropriate radio-ligand-binding studies.

- 5 x 150 cm² confluent culture flasks with either α₂A-H or CAM α₂A cells were fed 24 hours before membrane preparation.
- The culture medium was aspirated and washed with 2 x 5 ml phosphate-buffered saline (PBS) (see Table 3-5 for composition), and then loosened with 2 ml trypsine-versine. A cell scraper was used to ensure that all the cells were detached from the flask surface.
- Cell suspension was transferred to a 50 ml centrifuge tube and 20 ml PBS was added and mixed thoroughly with a vortex. The 50 ml centrifuge tube was spun in the Sigma Laborzentrifuge 3K15 bench-top centrifuges at 5000 rpm for 10 minutes at a temperature of 4 °C.
- After 10 minutes spinning, the supernatant (PBS) was carefully aspirated and the pellet re-suspended in 20 ml PBS. This process was repeated another 2 x to ensure that the trypsine-versine had been cleaned from the cells.
After the washing cycles had been completed, the pellet was re-suspended in 15 ml of 1 M ice-cold Tris buffer (see Table 3-5 for composition) and rotated in the cold-room (4 °C) for 15 minutes.

The suspension was then homogenised with a Teflon® homogeniser 5 x up and down until the suspension had no visible particles.

The suspension was then spun in the Sigma Laborzentrifuge 3K15 bench-top centrifuge at 1000 x g (3321.82 rpm in type 40 rotor or 2877.177 rpm in type 50 rotor) for 10 minutes at 4 °C.

The supernatant (containing the membrane) was collected and transferred to a special ultracentrifuge tube (kept on ice). The pellet containing the unwanted cell nuclei was then re-suspended in 25 ml of 1 mM tris buffer and the rotating, homogenising and spinning cycle repeated.

The cycle was then completed, the supernatant was collected, combined with the previously separated supernatant and then ultracentrifuged at 40 000 x g for 60 minutes.

The supernatant (unwanted chemicals) was carefully aspirated and the pellet (containing membrane) was re-suspended in 1 ml TME buffer (see Table 3-5 for composition) and homogenised with Teflon® homogeniser.

The membrane-suspension was transferred to 1 ml aliquots (50 µl each) and properly marked, hereafter the snap frozen in liquid N2 and stored in -80 °C freezer until used.

15 ml of the membrane suspension was used to determine the protein concentration by using the Bradford method (Bradford, 1976) as follows:

- Protein standards were prepared by weighing 2 mg BSA, then dissolving it in 1 ml double-distilled water (exactly 2 mg/ml).

- Thereafter 1 x 100 µl of each of the following dilutions of BSA in test tubes were measured very accurately as indicated in Table 3-4.
Table 3-4: Protein concentration dilutions

<table>
<thead>
<tr>
<th>Protein concentration</th>
<th>Dilution in test tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume of 2 mg/ml BSA</td>
</tr>
<tr>
<td>0 mg/ml (blank)</td>
<td>0 μl</td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>5 μl</td>
</tr>
<tr>
<td>0.4 mg/ml</td>
<td>20 μl</td>
</tr>
<tr>
<td>0.7 mg/ml</td>
<td>35 μl</td>
</tr>
<tr>
<td>1.0 mg/ml</td>
<td>50 μl</td>
</tr>
<tr>
<td>1.4 mg/ml</td>
<td>70 μl</td>
</tr>
</tbody>
</table>

- Then 2 × 5 μl of each tube (from blank, standard and unknown membrane concentration) were added to separate wells of a 96-well plate, i.e. all in duplicate.

- Thereafter 250 μl of Bradford reagent was added to each well, using a micropipette and immediately mixing it by using a 96-well plate reader for 30 seconds. It was thereafter incubated at room temperature for 15 minutes.

- The absorbance in each well was determined in the plate reader, using a 560 nM filter. The concentration of protein in the cell suspension was determined from the plotted net absorbance against the protein concentration of the standard.

3.2.6 Radio-ligand competition-binding studies

The aim of this study was to construct competition-binding curves of mirtazapine, idazoxan, mianserin or UK-14,303 on the α2A-ARs expressed in the α2A-H cells to determine their pKᵢ values. The concentration of 5 nM [methyl-³H]-yohimbine ("hot yohimbine") was used to define total binding.
Cells were grown to 95% consistency in 150 cm² cell culture flasks with Ham's F-12 medium containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in 5% CO₂.

The cells were then seeded as described in 24 well plates as described in § 3.5.1.1, although in this study they were seeded for at least 18 hours before the assay was initiated.

After seeding, the normal medium was aspirated and cells were rinsed with 2 × 0.5 ml PBS (37 °C) to wash off the medium.

Then 300 µl of appropriate drug dilutions⁶ were added to each well and the cells incubated for 60 minutes at 37 °C in 5% CO₂.

After 60 minutes incubation, the stimulation medium was aspirated from each well and the reaction terminated by rinsing the cells out with 2 X ice-cold PBS.

In the next step 1 ml lyses solution (5% TCA) was added to each well and the cells were left to stand for 60 minutes at room temperature for complete lyses.

After a 60 minutes lyses period, the supernatant was collected from each well and transferred to scintillation vials.

Lastly 7 ml Ultima Gold XR scintillation liquid was added to each vial and thoroughly mixed. The total radio-activity was then counted in the Tri-carb TR liquid scintillation analyser.

3.2.7 Radio-ligand saturation-binding studies

The aim of this study was to determine the concentration of α₂A-ARs from the α₂A-H, α₂A-L, or α₂A-CAM cells through radio-ligand-binding studies. The receptor concentration (Bₘₐₓ) was determined by employing α₂A-ARs from the α₂A-H, α₂A-L, or CAM α₂A membranes. The concentration of 1, 2, 5, 10, 20 and 40 nM [methyl-³H]-yohimbine ("hot yohimbine") was used to define total binding, while non-specific binding was determined by adding 10 µM yohimbine ("cold yohimbine") to each of the above radio-ligand concentrations. Concentration-effect curves were constructed by correcting free [methyl-³H]-yohimbine

⁶ Appropriate drug dilutions of mirtazapine, mianserin, idazoxan or UK-14,304 were diluted in DMEM containing 5 nM "hot yohimbine".
concentrations with bound drug, that reduced the free concentrations significantly. All the
dilutions were done in TME buffer (see Table 3-5 for composition). The assay was done as
follows:

- After the membranes had been prepared and the protein concentration determined by
means of the Bradford method, the assay was initiated by diluting the membranes to
0.05 mg/ml protein in TME buffer and then kept on ice.

- Appropriate concentrations of "hot yohimbine" were prepared and kept on ice.

- Three (triplicates) x 50 μl membrane suspension was transferred to properly labelled
 test tubes and kept on ice.

- Then 50 μl of appropriate drug dilution was added to each tube, thoroughly mixed and
 left to stand at room temperature for 30 minutes.

- The solution was added to Beckman GF/B filters and washed with 2 X 2 ml TME
 buffer.

- The filters (containing membranes bound to "hot yohimbine") were left to dry and
 placed in the 5 ml scintillation vials.

- After drying period, 3 ml Ultima Gold XR scintillation liquid was added to each and the
 vials were shaken for 60 minutes using an empty water bath.

- After 60 minutes shaking, the vials were counted by using the Tri-carb TR liquid
 scintillation analyser.
Table 3-5: Composition of buffers (all the buffers were prepared in de-ionised water)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-buffer</td>
<td>1 mM Tris acid</td>
</tr>
<tr>
<td>TME buffer</td>
<td>50 mM Tris, 10 mM MgCl₂, &amp; 1 mM EGTA</td>
</tr>
<tr>
<td>PBS</td>
<td>80 g of NaCl, 2 g of KCl, 9 g of Na₂HPO₄, 2 g of KH₂PO₄ in 1000 ml of double-distilled water, to be diluted to 1 part to 9 parts water</td>
</tr>
</tbody>
</table>

3.3 DATA ANALYSIS

Data from all assays was obtained as triplicate observations from at least three separate experiments and expressed as mean \( \pm \) S.E.M., unless stated otherwise. Semi-logarithmic concentration-effect curves were constructed as non-linear least square fit, by using the computer software Graph Pad Prism® (version 4.01 for Windows®, Graphpad software, San Diego, CA, USA, www.graphpad.com). The Hill-Slope factor was at 1 and the bottom constant at 100%. The student two-tailed unpaired t test was implemented to compare relevant values, where all the values were expressed relative to one control value. For all reported statistical probability \( (P) \) values \( P < 0.05 \) was taken as statistically significant.
4.1 INTRODUCTION

In this chapter all the results of the experiments conducted in the study are presented and discussed. All the experiments were conducted in the Laboratory for Applied Molecular Biology at the North-West University (Potchefstroom Campus), Republic of South Africa.

4.2 RESULTS OF CONTROL EXPERIMENTS

Saturation-binding assays were conducted, utilising [methyl-\(^{3}\)H]-yohimbine \(\pm\) 10 \(\mu\)M yohimbine to determine the \(\alpha_2A\)-adrenergic receptor (\(\alpha_2A\)-AR) concentrations in three Chinese hamster ovary (CHO-K1) cell lines utilised in this study. These include CHO-K1 cells transfected with the wild-type \(\alpha_2A\)-ARs at high numbers (\(\alpha_2A\)-H) and low numbers (\(\alpha_2A\)-L) and with the constitutively-active mutant of the \(\alpha_2A\)-AR (\(\alpha_2A\)-CAM). The receptor concentrations were determined to confirm the reported receptor expression levels (Brink et al., 2000; Wade et al., 2000) in these cell lines.

Figure 4-1 depicts saturation binding of [methyl-\(^{3}\)H]-yohimbine in the \(\alpha_2A\)-H, \(\alpha_2A\)-L and \(\alpha_2A\)-CAM cell lines.
Figure 4-1: Saturation-binding of [methyl-\textsuperscript{3}H]-yohimbine in $\alpha_{2A}$-H, $\alpha_{2A}$-CAM and $\alpha_{2A}$-L cells. Non-specific binding was defined by 10 $\mu$M yohimbine ($>2000 \times K_d$). Total and non-specific binding is presented for (A) $\alpha_{2A}$-H, (C) $\alpha_{2A}$-CAM and (E) $\alpha_{2A}$-L cells. Corresponding specific binding was calculated and is presented for (B) $\alpha_{2A}$-H, (D) $\alpha_{2A}$-CAM and (F) $\alpha_{2A}$-L cells. The data are represented as mean ± S.E.M and expressed as percentage of control. All data points were obtained from triplicate observation from three independent experiments ($n = 3$).
In Figure 4-1A and 4-1C it is evident that the non-specific binding in both the \( \alpha_{2A} \)-H and \( \alpha_{2A} \)-CAM cells was substantial, but resolution was sufficient to calculate specific binding. Specific binding was calculated by assuming linearity of non-specific binding with concentration and subtracting the predicted value from total binding. The \( K_D \) value of [methyl-\(^3\)H]-yohimbine in the \( \alpha_{2A} \)-H cells (wild type \( \alpha_{2A} \)-AR expressed at relatively high numbers) was calculated as 6.72 ± 2.05 nM and the \( B_{\text{max}} \) value was calculated as 46.18 ± 5.23 pmol/mg protein (see Figure 4-1B), while \( K_D \) value of [methyl-\(^3\)H]-yohimbine in the \( \alpha_{2A} \)-CAM cells was calculated as 2.90 ± 1.22 nM and \( B_{\text{max}} \) value was estimated as 25.37 ± 3.59 pmol/mg protein (see Figure 4-2D). The \( B_{\text{max}} \) values in these cell lines corresponded with what was reported by Wade et al. (2000) with minor variations.

The \( B_{\text{max}} \) value for \( \alpha_{2A} \)-L cells could not be determined (see Figure 4-1E & F), since the non-specific binding was proportionally too large compared to total binding. This may be due to low expression of the \( \alpha_{2A} \)-AR numbers in this cell line that would require better resolution of measurements. Wade et al., (1999), however reported the \( B_{\text{max}} \) value for \( \alpha_{2A} \)-ARs in this cells line as 1 pmol/mg protein.

### 4.3 RESULTS OF STUDY OBJECTIVE EXPERIMENTS

In this study, experiments were conducted to address the main objectives, i.e. firstly to characterise the \( \alpha_{2A} \)-ARs antagonism by mirtazapine, and secondly to investigate the possible modulatory effects of mirtazapine pre-treatment on the muscarinic acetylcholine receptors (mACHRs), \( \beta \)-adrenergic receptors (\( \beta \)-ARs) and \( \alpha_{2A} \)-ARs in the SH-SY5Y human neuroblastoma and Chinese hamster ovary cells transfected to express \( \alpha_{2A} \)-ARs at relatively low numbers (\( \alpha_{2A} \)-L).

#### 4.3.1 Characterisation of the mode of \( \alpha_{2A} \)-adrenoceptors antagonism by mirtazapine

##### 4.3.1.1 Characterisation of binding to \( \alpha_{2A} \)-adrenergic receptors

Reports that mirtazapine antagonises the \( \alpha_{2A} \)-ARs were confirmed by determining the affinity value (\( pK_i \)) of mirtazapine in competition-binding experiments against [methyl-\(^3\)H]-yohimbine. In addition the \( pK_i \) values of other selected \( \alpha_{2A} \)-AR ligands including, UK-14,304 (\( \alpha_{2A} \)-AR full-agonist), idazoxan (neutral antagonist) and mianserin (atypical antidepressant with \( \alpha_{2A} \)-AR lytic properties) were determined.
Figure 4-2 illustrates competition-binding of mirtazapine, UK-14,304, idazoxan or mianserin against 5 nM [methyl-^3H]-yohimbine in the Chinese hamster ovary cells transfected to express $\alpha_{2A}$-ARs at high numbers ($\alpha_{2A}$-H cell line).

Results of competition-binding studies illustrated in Figure 4-2A and 2B shows that mirtazapine and UK-14,304 have similar affinity values at the porcine $\alpha_{2A}$-ARs. The $pK_i$ values were determined as $pK_i = 6.39 \pm 0.06$ for UK 14,304 and $6.50 \pm 0.07$ for mirtazapine and do not differ statistically significantly. Figure 4.1 (C & D) shows the affinity values of other $\alpha_{2A}$-ARs ligands used in this study, i.e. idazoxan, ($pK_i = 7.48 \pm 0.03$) and mianserin ($pK_i = 6.94 \pm 0.14$). From these results it can be seen that mirtazapine binds to the $\alpha_{2A}$-ARs with an affinity similar to that of the full agonist UK-14,304 and $\alpha_{2A}$-ARs antagonist mianserin.
For the characterisation of the mode of $\alpha_{2A}$-ARs action by mirtazapine, any possible partial agonism was investigated in the $\alpha_{2A}$-H cells, while the possibility of inverse agonism was investigated in the $\alpha_{2A}$-CAM cells.

### 4.3.1.2 Whole-cell $[^3H]$-cAMP accumulation assay in the $\alpha_{2A}$-H cells in response to mirtazapine and UK-14,304 treatment

The $\alpha_{2A}$-H cell line is known to express the $\alpha_{2A}$-ARs at high numbers (Wade et al., 1999; Brink et al., 2000), so that even weak partial agonist effects at these receptors would be expected to display full agonism (i.e. maximal $G_{i}$-mediated decrease in whole-cell $[^3H]$-cAMP accumulation). Figure 4-3 depicts the concentration-effect curves of the classical $\alpha_{2A}$-ARs full agonist UK-14,304 and atypical antidepressant mirtazapine measuring the whole-cells $[^3H]$-cAMP accumulation in the $\alpha_{2A}$-H and Neo cells.

**Figure 4-3**: Concentration-effect curves of mirtazapine and UK-14,304, measuring the whole-cell $[^3H]$-cAMP accumulation in (A) Neo cells (B) and $\alpha_{2A}$-H cells. The data are represented as mean ± S.E.M and expressed as percentage of control. The data represent triplicate observations from three independent experiments ($n = 3$).

In Figure 4-3A it can be seen that both $\alpha_{2A}$-ARs full agonist UK-14,304 and mirtazapine do not elicit any change in cAMP production in the control Neo cells (transfected with the selection plasmid without cDNA for the $\alpha_{2A}$-AR). This would confirm that any observed responses in the transfected $\alpha_{2A}$-H cells result from interaction with the $\alpha_{2A}$-ARs.

In Figure 4-3B it is evident that the $\alpha_{2A}$-AR full agonist UK-14,304 causes a concentration-dependent decrease in cAMP accumulation. The $EC_{50}$ value of the concentration-effect curve is 0.55 nM (i.e. $EC_{50} = 0.001 \times K_{i}$), suggesting a large proportion of spare receptors in
the \( \alpha_{2A} \)-H cell line. This would correlate well with the expression of 46 pmol/mg protein, as determined in Figure 4-2. However, mirtazapine did not elicit any effect on the \( \alpha_{2A} \)-ARs even at the concentration of 0.1 mM (\( \pm 330 \times K_i \)). These results suggest that mirtazapine lacks any weak partial agonist effect at \( \alpha_{2A} \)-ARs and that it may either act as a neutral antagonist or as an inverse agonist at \( \alpha_{2A} \)-ARs.

4.3.1.3. **Inverse efficacy at the \( \alpha_{2A} \)-CAM cells**

Figure 4-4 depicts DRCs of idazoxan, yohimbine, mianserin, mirtazapine and UK 14,304 measuring the whole-cell \([\text{H}]\)-cAMP accumulation on the CAM \( \alpha_{2A} \)-H cells.
Figure 4-4: Ligand activity at α2A-ARs. Concentration-effect curves of (A) idazoxan, (B) yohimbine, (C) mianserin, (D) mirtazapine and (E) UK-14,304 in the neo, α2A-H and α2A-CAM cells, measuring the whole-cell [3H]-cAMP accumulation. The data are represented as mean ± S.E.M and expressed as percentage of control. The data represent triplicate observations from three independent experiments (n = 3). (F) Corresponding concentration-effect curves of yohimbine and idazoxan in α2A-CAM cells for comparison, as obtained by Wade et al. (2000).
Figure 4.4A illustrates that idazoxan elicits a dose dependant decrease in cAMP accumulation from 103.4 ± 6.45 (at 0 M) to 39.8 ± 3.97 (at 0.1 mM) (P = 0.0001), which would resemble normal agonism at α2A-ARs. These results are different from what was found by Wade et al. (2000) (see Figure 4-4F). No effects were observed in control neo cells or in α2A-H cells containing the wild-type α2A-AR. It can be seen in Figure 4-4B that yohimbine elicits a slight increase in cAMP accumulation from 100.0 ± 4.68 (at 0 M) to 127.7 ± 2.89 (at 10 nM) (P = 0.0001) in α2A-CAM cells, which would indicate inverse agonism at α2A-ARs, as was found by Wade et al. (2000) (see Figure 4-4F). The maximal inverse effect was, however, much smaller (±28% over basal) than what was found by Wade and et al. (2000) (±650% over basal). At higher concentrations yohimbine displays auto-inhibition. No effects were observed in control neo cells or in α2A-H cells containing the wild-type α2A-AR. These results, as illustrated in Fig. 4-4A and Fig. 4-4B, suggest reduced signalling by the CAM α2A-ARs (not reduced receptor expression, as determined and presented in Fig. 4-2) and may possibly be explained by changes/damage of cells during import. It follows that results using the α2A-CAM cell line cannot be conclusive, but comparison with the results obtained by Wade et al. (2000) may provide tentative answers.

In Figure 4-4D it can be seen that mirtazapine does not display any intrinsic activity at the CAM α2A-ARs. However, its concentration-effect curve is compared with that of idazoxan (Fig. 4-4A, neutral antagonist according to Wade et al. (2000), and of yohimbine (strong inverse agonist according to Wade et al. (2000)), it is clear that the response lies between that of yohimbine and idazoxan, which suggests that the action of mirtazapine at the CAM α2A-ARs are different from both yohimbine and idazoxan and it may be possible that mirtazapine would display partial inverse agonism under the same experimental conditions as implemented by Wade et al. (2000). No effects were observed in control neo cells or in α2A-H cells containing the wild-type α2A-AR. As illustrated in Figure 4-4D, mianserin display the same activity as idazoxan, which would suggest that it would act as a neutral antagonist under the same experimental conditions as implemented by Wade et al. (2000). No effects were observed in control neo cells or in α2A-H cells containing the wild-type α2A-AR, which would exclude partial agonism at wild-type receptors or effects resulting from endogenous signalling mechanisms. These results may also explain the putative difference in affectivity / rapidity of onset of action between mirtazapine and mianserin.

1 Means are significantly different if p < 0.05
In conclusion, the results, although inconclusive, suggest that mirtazapine may be a partial inverse agonist and mianserin may be a neutral antagonist at $\alpha_2$-ARs.

### 4.3.2. Modulatory effects of mirtazapine pre-treatments on the muscarinic acetyl choline receptor function

Figure 4.5 depicts the modulatory effects of mirtazapine pre-treatment on the mAChRs mediated $[^{3}H]$-IP$_{3}$ production and the total cellular myo-$[^{2-3}H]$-inositol uptake during the radio-labelling of the SH-SY5Y neuroblastoma cells.

![Figure 4.5: The modulatory effects of 24-hour pre-treatment with 0 M or 10 $\mu$M mirtazapine on the mAChR function.](image)

In Figure 4-5A it can be seen that 24-hour pre-treatment with mirtazapine does not greatly affect endogenously expressed mAChRs expressed on the SH-SY5Y cells ($E_{\text{max}}$ and $E_{50}$ values not statistically significantly different). As depicted in Fig 4-5B, there is a significant decrease in total $[^{2-3}H]$-myo-Inositol uptake following 24-hour pre-treatment with mirtazapine from 100.0 $\pm$ 3.655 to 84.20 $\pm$ 3.748 ($P = 0.008$). This decrease in total $[^{2-3}H]$-myo-Inositol uptake might explain the slight decrease in $[^{3}H]$-IP$_{3}$ accumulation following 10 $\mu$M mirtazapine pre-treatment (although not statistically significant). From the above data it can be speculated that mirtazapine does not affect mACh-R function.
In previous studies conducted in the same laboratory, it was shown that 24-hour pre-treatment of the SH-SY5Y cells with fluoxetine, imipramine or myo-inositol reduces endogenous mACHR function (Brink et al., 2004). The data in the current study shows that mirtazapine does not influence endogenous mACH-Rs function as these antidepressants and would suggest that it will not influence central cholinergic activity, so that the data is not supportive of anticholinergic activity (according to the cholinergic hypothesis of depression) as part of the mechanism of the antidepressant action of mirtazapine.

4.3.3. Modulatory effects of mirtazapine pre-treatments on the β-adrenergic receptor function

Figure 4-6 illustrates the modulatory effects of mirtazapine pre-treatment on the β-ARs mediated [3H]-cAMP production and the total cellular [2-3H]-adenine uptake during the radiolabelling of the SH-SY5Y neuroblastoma cells.

In Figure 4-6A it can be seen that 24-hours pre-treatment of the SH-SY5Y cells with mirtazapine reduces endogenous β-AR function (196.5 ± 13.69 % of baseline control versus 150.5 ± 9.89% of baseline control (P = 0.015)). As depicted in Figure 4-6B, it is evident that mirtazapine pre-treatment has no effect of the total Total [2-3H]-adenine uptake during radiolabelling, thus suggesting that the reduced cAMP seen in Figure 4-7A could be mediated by mirtazapine-induced reduction of β-AR function. These results were expected, since a
reduction in β-AR function/ expression is well documented after chronic treatment with antidepressants. In this regard a consistent decrease in β-AR number and function following chronic treatment with antidepressants was reported in rat models of depression, especially in the rat cortex with desipramine (Heal et al., 1989), electroconvulsive therapy (Heal et al., 1989) and reboxetine (Harkin et al., 2000). As a result the finding that most antidepressants cause down-regulation of the β-ARs is often regarded as an indicator for antidepressant potential for new agents (Leonard, 2003).

4.3.4. Modulatory effects of mirtazapine pre-treatments on the α2A-adrenoceptors

Figure 4-7 illustrates the modulatory effects of mirtazapine pre-treatment on the α2A-ARs mediated [3H]-cAMP production and the total cellular [2-3H]-adenine uptake during the radio-labelling of the α2A-L cells.

![Figure 4-7](image)

**Figure 4-7:** The modulatory effects of 24-hour pre-treatment with 10 μM mirtazapine on the α2A-AR function. (A) Concentration-effect curves of the full agonist UK-14,304-induced decrease in [3H]-cAMP accumulation on the α2A-ARs in the α2A-L cells with either 0 M or 10 μM mirtazapine pre-treatment. (B) Total cellular [2-3H]-adenine uptake during the radio-labelling of the α2A-L cells. The data are represented as mean ± S.E.M and expressed as percentage of control. The data represent triplicate observations from four independent experiments (n = 4).

In Figure 4-7A it can be seen that 24-hours pre-treatment of the α2A-L cells with mirtazapine significantly reduces the full-agonist UK-14,304-induced reduction in [3H]-cAMP accumulation (29.69 ± 6.11 % of 0 M mirtazapine versus 74.59 ± 14.38% of 10 μM mirtazapine (P = 0.074)). However on the other hand, (see Figure 4-7B) it can be seen that there is a decrease in cellular [2-3H]-adenine uptake following 10 μM mirtazapine pre-treatment on the
$\alpha_{2A}$-L cells from 100.0 ± 2.75 of baseline control to 76.3 ± 4.88% of baseline control ($P = 0.001$). The data presented in Figure 4-7B complicates the interpretation of the results in A, since it is not clear whether the reduction in the agonist-induced inhibition of cAMP accumulation following mirtazapine pre-treatment in Figure (A) is due to the reduced cellular $[2^{-3}H]$-adenine uptake following mirtazapine pre-treatment during radio-labelling or due to mirtazapine-induced $\alpha_{2A}$-AR function.

However in previous studies it was shown that desipramine and selective l-NA reuptake inhibitors induce $\alpha_{2A}$-AR desensitisation after chronic treatment in rats at several brain areas such as hypothalamus, corpus striatum, brainstem, cerebral cortex and the hippocampus (Barturen & Garcia-Sevilla, 1992).

4.4. SUMMARY

Experimental data in this study can be summarised as follows:

Data from control experiments suggest that $\alpha_{2A}$-ARs in $\alpha_{2A}$-H and $\alpha_{2A}$-CAM are expressed at similar numbers as reported in literature (see § 4.2). The data from study aims experiments suggest that mirtazapine is not a partial agonist at $\alpha_{2A}$-ARs and that it may be a partial inverse agonist at the these receptors (see § 4.3.1.3.).

In contrast to fluoxetine and imipramine, the current data (see § 4.3.2.) does not support any modulatory effect of mirtazapine on the mAChRs. However, mirtazapine reduced $\beta$-AR function (see § 4.3.3), a property usually regarded as a marker for antidepressant action. Although inconclusive, mirtazapine pre-treatment apparently seems to reduce $\alpha_{2A}$-ARs function.
5.1 SUMMARY

The current study had two main objectives. The first objective entailed the characterisation of the $\alpha_{2A}$-adrenergic receptor ($\alpha_{2A}$-AR) antagonism by mirtazapine. For this purpose Chinese hamster ovary cells (CHO-K1) expressing the porcine $\alpha_{2A}$-ARs at high numbers ($\alpha_{2A}$-H), the constitutively active mutant of $\alpha_{2A}$-ARs ($\alpha_{2A}$-CAM), and mock-transfected control (Neo) cells were used. The second objective entailed an investigation into mirtazapine's relevance to antidepressant actions, i.e. investigating the effect of mirtazapine pre-treatment on muscarinic acetylcholine receptor (mACHR) and beta-adrenergic receptor (\(\beta\)-AR) functions, using SH-SY5Y human neuroblastoma cells.

Control experiments were performed to determine the $\alpha_{2A}$-AR concentrations ($B_{max}$ values) in the three CHO-K1 cell lines. Table 5-1 summarises the $B_{max}$ values and $K_D$ values of the three cell lines.
<table>
<thead>
<tr>
<th>$\alpha_{2A}$-H</th>
<th>46.18 ± 5.23</th>
<th>6.72 ± 2.05</th>
<th>The $B_{max}$ value is relatively high, corresponding with the value reported by Wade et al. (2000), thus supporting the concept of spare receptors in the $\alpha_{2A}$-H cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{2A}$-CAM</td>
<td>25.37 ± 3.59</td>
<td>2.90 ± 1.22</td>
<td>The $B_{max}$ value is relatively high, corresponding with the value reported by Wade et al. (2000).</td>
</tr>
<tr>
<td>$\alpha_{2A}$-L</td>
<td>-</td>
<td>-</td>
<td>The $B_{max}$ &amp; $K_0$ values could not be determined, since the non-specific binding was proportionally too large compared to total binding.</td>
</tr>
</tbody>
</table>

Competition-binding studies were performed, using 5 nM [methyl-3H]-yohimbine on $\alpha_{2A}$-H cells to determine the affinity values of the $\alpha_{2A}$-ARs ligands, mirtazapine, UK-14,304, idazoxan and mianserin for $\alpha_{2A}$-ARs. Table 5-2 summarises these affinity values:
Table 5-2: The affinity values at the \( \alpha_{2A} \)-ARs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Affinity Value (IC(_{50}) ± SEM)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mirtazapine (atypical antidepressant)</td>
<td>6.50 ± 0.07</td>
<td>All the values are in the low micro molar concentration range, suggesting intermediate affinity for the wild-type ( \alpha_{2A} )-AR</td>
</tr>
<tr>
<td>UK-14,304 (full-agonist)</td>
<td>6.39 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Idazoxan (neutral antagonist)</td>
<td>7.48 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Mianserin (atypical antidepressant)</td>
<td>6.94 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

The \( \alpha_{2A} \)-H cell line was used to investigate whether mirtazapine possesses any partial agonist properties at the \( \alpha_{2A} \)-AR. Since this cell line is known to express the \( \alpha_{2A} \)-ARs at high numbers, even a weak partial agonist would be expected to display full agonism (i.e. maximal decrease in whole-cell [\(^3\)H]-cAMP accumulation). The results show that mirtazapine has no effect on the whole-cell [\(^3\)H]-cAMP accumulation, thus suggesting that mirtazapine does not possess any weak partial agonist properties at \( \alpha_{2A} \)-ARs.

The \( \alpha_{2A} \)-CAM cell line was used to investigate whether mirtazapine possesses any inverse agonism at the \( \alpha_{2A} \)-AR by constructing concentration-effect curves, measuring whole-cell [\(^3\)H]-cAMP accumulation. However, the CAM \( \alpha_{2A} \)-ARs in the \( \alpha_{2A} \)-CAM cell line did not show similar high activity as previously reported by Wade et al. (2000) due to unknown reasons, although not due to reduced \( \alpha_{2A} \)-ARs number (see Table 5-1). Mianserin displays activity similar to idazoxan, which has been classified by Wade et al. (2000) as a neutral antagonist. Mirtazapine displays activity between idazoxan and yohimbine, which has been classified by Wade et al. (2000) as a strong inverse agonist. Therefore, the results suggest that mirtazapine may be a partial inverse agonist. No activity for any of these drugs was observed in control neo cells or in \( \alpha_{2A} \)-H cells containing the wild-type \( \alpha_{2A} \)-ARs, confirming that the activity observed in \( \alpha_{2A} \)-CAM cells could be interpreted as resulting from interaction with CAM \( \alpha_{2A} \)-ARs.

The effect of mirtazapine pre-treatment on mAChR, \( \beta \)-AR and \( \alpha_{2A} \)-AR functions was also investigated, using the SH-SYSY neuroblastoma cells (for mAChRs, \( \beta \)-ARs) and \( \alpha_{2A} \)-L cells (for wild-type \( \alpha_{2A} \)-AR). The cells were pre-treated with either 0 M or 10 \( \mu \)M mirtazapine for 24 hours a. After pre-treatment, receptor function was determined by measuring whole-cell [\(^3\)H]-IP\(_x\) accumulation (mAChRs, agonist = metacholine) and [\(^3\)H]-cAMP accumulation (\( \beta \)-AR
agonist = l-isoproterenol and \( \alpha_{2A} \)-AR agonist = UK-14,304). In addition, the effect of mirtazapine pre-treatment on the total \( \text{myo-}[2-\text{H}]\)-inositol or \( [2-\text{H}]\)-adenine uptake into the cells during radio-labelling was also investigated. The results are summarised in Table 5-3.

**Table 5-3: The effect of mirtazapine pre-treatment on the mAChR, \( \beta \)-AR and \( \alpha_{2A} \)-AR functions**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Mirtazapine Effect</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAChR</td>
<td>No change</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unlike fluoxetine and imipramine (Brink et al., 2004), mirtazapine has no effect on the mAChR function</td>
</tr>
<tr>
<td>( \beta )-AR</td>
<td>Decreased</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>As commonly associated with chronic antidepressant treatment, mirtazapine reduces ( \beta )-ARs function</td>
</tr>
<tr>
<td>( \alpha_{2A} )-AR</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inconclusive, although mirtazapine apparently reduces ( \alpha_{2A} )-AR function</td>
</tr>
</tbody>
</table>

### 5.2 CONCLUSIONS

Mirtazapine has been reported in several studies to have an onset of action earlier than that of selective serotonin reuptake inhibitors (SSRIs), the current drugs of choice in the treatment of depression (Benkert et al., 2000; Leinonen et al., 1999; Wheatley et al., 1998; Van Oers et al., 2002). De Boer (1996) and (Blier, 2003) propose that mirtazapine's principal therapeutic actions are mediated by its antagonism at \( \alpha_{2A} \)-ARs.

The current study has provided useful novel information for understanding the mechanism of action of mirtazapine. Mirtazapine does not possess any partial agonistic properties at \( \alpha_{2A} \)-ARs, and, although the current data is inconclusive, it suggests that mirtazapine may be a partial inverse agonist at these receptors. It is clear though, that mirtazapine induces an effect between that of mianserin / idazoxan and yohimbine at CAM-\( \alpha_{2A} \)-ARs, making it possible to deduce that it acts differently from its older analogue mianserin. The latter conclusion may also explain the putative difference in affectivity / rapidity of onset of
antidepressant actions between mirtazapine and mianserin. In addition, as indicated most recently in the study by Sanacora et al. (2004), it was shown that yohimbine (α2-ARs antagonist / inverse agonist (Wade et al., 2000)) shortens the delayed onset of symptom relief when combined with fluoxetine.

It can therefore be concluded that inverse agonism, as opposed to neutral agonism at the α2-ARs may play a crucial role in reducing the common latent period in attaining a therapeutic antidepressant response. In addition, it has been described that mirtazapine antagonises both the auto- and hetero α2-ARs, thus enhancing the activity at both serotonergic and noradrenergic nerve terminals. It has also been shown that chronic antidepressant therapy down-regulates α2-ARs (Garcia-Sevilla et al., 1999; Barturen & Garcia-Sevilla, 1992; Szabo & Blier, 2001; Charney et al., 1983). Although the data is inconclusive, the current study shows that mirtazapine pre-treatment apparently reduces α2-AR function. Whether an inverse agonist would regulate receptor trafficking differently from a neutral antagonist at α2-ARs has not yet been established, so that it would be speculative to make any suggestions in this regard. It should be noted that it cannot be assumed that receptor down- and up-regulation are mediated by agonists and antagonists respectively, since it has been shown for 5HT2A-Rs, as an example, that both agonists and antagonists paradoxically cause 5HT2A-R down-regulation (Gray & Roth, 2001). This phenomenon at α2-ARs, however, may be investigated in future studies by performing appropriate saturation-binding studies before and after mirtazapine (or other ligand) pre-treatments to determine any changes in receptor number (from Bmax values). Such studies may help to explain the putative earlier onset of action by mirtazapine and may also suggest a role for inverse agonism, as opposed to neutral antagonism at α2-ARs.

Mirtazapine has a unique mechanism of action. As indicated in this study and unlike fluoxetine and imipramine, mirtazapine does not affect mAChR function. The current data on the action of mirtazapine therefore does not seem to support the cholinergic hypothesis of depression. This hypothesis states that depression is associated with cholinergic super sensitivity, which is normalised by chronic antidepressant treatment. The current data, however, rather supports the idea that anticholinergic activity (directly or indirectly) is not a pre-requisite for antidepressant action. Rather, a drug that is devoid of this feature (e.g. mirtazapine) may have a better tolerability profile due to its lack of anticholinergic-related adverse effects.

As commonly associated with several antidepressants, mirtazapine reduces β-AR function, a property believed to act as a marker for antidepressant potential. Even though the β-AR
hypothesis has several drawbacks (see § 2.5.3.1.2.), the results of this study shows that mirtazapine is in agreement with this hypothesis.

5.3 RECOMMENDATIONS

The results in the current study suggesting possible inverse agonism of mirtazapine at α2A-ARs were inconclusive, since the α2A-CAM cells did not show similar response to yohimbine and idazoxan as previously reported by Wade et al. (2000). It is not clear whether this may be due to factors arising from the shipment of the cells or due to loss of constitutive activity or receptor coupling efficiency of the α2A-ARs with time. To confirm the tentative results, the cells may have to be re-imported or the plasmid obtained in order to repeat the experiments.

In addition in vitro studies utilising appropriate cell lines should be conducted to investigate the modulatory effect of mirtazapine on other receptor types implicated in the pathophysiology of depressions, e.g. on 5-HT2A- or dopaminergic-receptor function.

In vivo animal behavioural studies should also be conducted to investigate the role of α2A-AR inverse agonism or neutral antagonism in the treatment of depression. This may be done by employing an appropriate animal model of depression, e.g. learned helplessness model or the Flinders sensitive line rats. The investigation into how yohimbine (α2A-AR inverse agonist) or idazoxan (α2A-AR neutral antagonist) affect the onset of action and efficacy of antidepressants such as fluoxetine or imipramine should then be compared with that of mirtazapine.


and remission with high sensitivity in patients with major depression. *Journal of clinical psychiatry*, 64:413-420.


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>α₂A-ARs</td>
<td>Type-2A α-adrenergic receptors</td>
</tr>
<tr>
<td>α₂A-H</td>
<td>Chinese hamster ovary cells transfected to express Type-2A α-adrenergic receptors in high numbers</td>
</tr>
<tr>
<td>α₂A-L</td>
<td>Chinese hamster ovary cells transfected to express Type-2A α-adrenergic receptors in low numbers</td>
</tr>
<tr>
<td>AC</td>
<td>Adenyl cyclase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450 enzyme system</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnosis and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>ECT</td>
<td>Electroconvulsive shock therapy</td>
</tr>
<tr>
<td>EMEM</td>
<td>Minimum essential medium (Earle's base)</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(-Hydroxyethyl)piperazine-N’-(2-ethanesulphonic acid)</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium Chloride</td>
</tr>
<tr>
<td>mACH-Rs</td>
<td>Muscarinic acetylcholine receptors</td>
</tr>
<tr>
<td>MAOs</td>
<td>Monoamine oxidase inhibitors</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NARI</td>
<td>Selective Noradrenaline Reuptake Inhibitors</td>
</tr>
<tr>
<td>NaSSA</td>
<td>Noradrenaline and specific serotoninergic antidepressant</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein-kinase C</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error on the mean</td>
</tr>
<tr>
<td>SNRI</td>
<td>Serotonin and Noradrenaline reuptake inhibitors</td>
</tr>
<tr>
<td>SSRIs</td>
<td>Selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCAs</td>
<td>Tricyclic antidepressants</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxilase</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-propan-1,3,-dial</td>
</tr>
</tbody>
</table>

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