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

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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

Abstract

Title: Characterisation of the α_{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 α_{2A}-adrenergic receptors (α_{2A}-ARs), serotonin type-2A receptors (5-HT_{2A}-Rs) and histamine type-1 receptors (H_1-Rs). Its actions at the α_{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 α_2-ARs. The current study aimed to determine the mode of α_{2A}-AR antagonism by mirtazapine, as well as to investigate *in vitro* the modulatory effects of mirtazapine pre-treatments on β-adrenergic receptor (β-AR), muscarinic acetylcholine receptor (mAChR) and α_{2A}-AR functions.

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

The results from the current study show that mirtazapine binds to the α_{2A}-AR with an affinity value in the lower micromolar range ($K_i \approx 0.32$ μM; pK_i = 6.50 ± 0.07). Mirtazapine is not a partial agonist at α_{2A}-ARs as it does not affect [^{3}H]-cAMP accumulation in α_{2A}-H cells. Preliminary results suggest that mirtazapine displays partial inverse agonism in α_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 [^{3}H]-cAMP accumulation in SH-SY5Y cells

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

Key words: mirtazapine, antidepressant, onset of action, inverse agonism, neutral antagonism, α_{2A}-adrenergic receptors, β-adrenergic receptors, muscarinic acetylcholine receptors.

Abstrak

Titel: Karakterisering van die α_{2A}-adrenoseptor antagonisme deur mirtazapien en die modifiserende effekte daarvan op reseptorseining

Mirtazapien is 'n atipiese antidepressant wat gebruik word vir die behandeling van major-depressie. As multipotente antagonis werk dit op α_{2A}-adrenergiese reseptore (α_{2A}-ARe), serotonien tipe-2A reseptore (5-HT_{2A}-Re) en histamien tipe-1 reseptore (H_1-Re). Die werking daarvan op die α_{2A}-AR is voorgestel om 'n rol te speel in die moontlike vroeër aanvang van werking. Dit is egter nie bekend of mirtazapien 'n neutrale antagonis of inverse agonis op α_2-ARe is nie. Die huidige studie poog om die modus van α_{2A}-AR antagonisme deur mirtazapien te bepaal, so wel as om die *in vitro* modulerende effekte van mirtazapien voor-behandelings op β-adrenergiese reseptor- (β-AR), muskariniese asetielkolien reseptor- (mAChR) en α_{2A}-AR-funksies te ondersoek.

Chinese hamster ovariale (CHO-K1) selle wat die vark α_{2A}-AR in hoë getalle uitdruk (α_{2A}-H), 'n konstitutueel aktiewe mutant-α_{2A}-AR (α_{2A}-CAM), of fop-getransfekteerde kontroleselle (neo) was radio-aktief gemerk met [^{3}H]-adenien en konsentrasie-effekskurwes van mirtazapien, johimbien, mianserien of idazoksaan is opgestel deur [^{3}H]-cAMP-akkumulasie te meet. Addisioneel is menslike neuroblastoomselle (SH-SY5Y) en CHO-K1 selle wat die vark α_{2A}-AR in lae getalle uitdruk (α_{2A}-L) gebruik om ondersoek in te stel na die effekte van mirtazapien voor-behandelings op respektiewelik mAChRe en β-ARe of α_{2A}-ARe. Na radio-aktiewe merking met *mio*-[2-^{3}H]-inositol of [2-^{3}H]-adenien, is die opname van die radio-aktiewe merker gemeet en reseptorfunksie is ondersoek deur konsentrasie-effekskurwes op te stel deur die meting van onderskeidelik [^{3}H]-IP_x of [^{3}H]-cAMP akkumulasie.

Die resultate van die huidige studie toon aan dat mirtazapien aan die α_{2A}-AR bind met 'n affiniteitswaarde in die hoër nanomolare orde (pK_i = 6.50 ± 0.07). Mirtazapien is nie 'n gedeeltelike agonis op α_{2A}-ARe nie, aangesien dit nie [^{3}H]-cAMP akkumulasie in α_{2A}-H selle affekteer nie. Voorlopige resultate dui daarop dat mirtazapien gedeeltelike inverse agonisme vertoon in α_2-CAM selle, terwyl mianserien neutrale antagonisme vertoon. Mirtazapien voor-behandeling in SH-SY5Y selle wysig nie muskariniese reseptor-funksie nie (anders as fluoksetien en imipramien), maar verlaag *l*-isoproterenol-geïnduseerde verhoging in [^{3}H]-cAMP akkumulasie in SH-SY5Y selle (tipies geassosieer met kroniese antidepressant-

aktiwiteit). Alhoewel onbeslis, dui die data daarop dat mirtazapien α_{2A}-AR funksie mag verlaag.

Sleutelwoorde: mirtazapien, antidepressant, aanvang van werking, inverse agonisme, neutrale antagonisme, α_{2A}-adrenergiese reseptore, β-adrenergiese reseptore, muskariniese asetielcholienreseptore.

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Introduction

Chapter 1

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 venlafafine (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 α_{2A}-adrenergic auto- and heteroreceptors (α_{2A}-ARs), serotonergic type-2 receptors (5-HT_2-Rs) and histamine type-1 receptors (H_1-Rs). It has been suggested by De Boer (1996) that mirtazapine's principal action involves the blocking of α_2-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 α_{2A}-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 α_{2A}-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 conclussive 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 α_{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 (β-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 α_2-lytic action of mirtazapine), three Chinese hamster ovary (CHO-K1) cell lines were utilised, namely a cell line expressing the wild-type porcine α_{2A}-AR at high numbers (α_{2A}-H cells), a cell line expressing its constitutively active mutant receptor (α_{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 α_{2A}-H and the K_i value of mirtazapine, UK-14,304, mianserin, or idazoxan were determined. The receptor concentrations (B_{max} values) were determined for α_{2A}-H, α_{2A}-L (CHO cell line, expressing the wild-type porcine α_{2A}-AR at lower numbers – also provided by Dr. Neubig) or α_{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 α_{2A}-ARs), idazoxan (neutral antagonist at α_{2A}-ARs) or mianserin (atypical antidepressant with α_{2A}-AR lytic effects).

Human neuroblastoma cells (SH-SY5Y) were used to investigate the modulating effect of mirtazapine pre-treatment on mACh-R and β-AR functions. In addition α_{2A}-L cells were used to investigate the modulatory effects of mirtazapine pre-treatment on the α_{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 ($[^3H]$-IP_x) or $[^3H]$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.

Literature review

Chapter 2

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 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 α_{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 α_{2A}-adrenergic receptor (α_{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 α_{2A}-adrenergic receptors (α_{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.

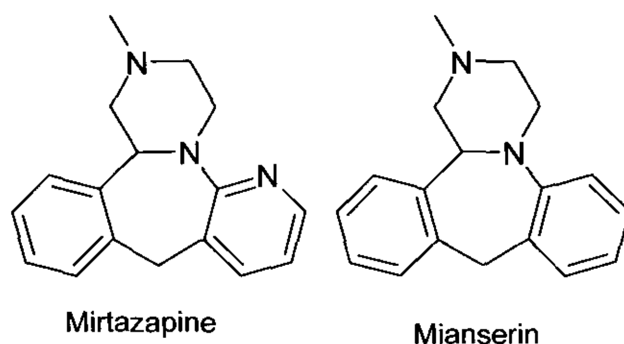

Figure 2-1: Chemical structures of mirtazapine and mianserin

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 α_{2A}-adrenergic auto and heteroreceptors, as well as by blocking serotonin (5-HT) type-2 (5-HT_2) and type-3 (5-HT_3) and H_1 receptors (De Boer, 1996; Danilevicuite & Sveikata, 2002; Blier, 2003). It is believed that its actions at both α_{2A}-ARs and 5-HT_2 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 α_{2A}-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 α_2-adrenergic autoreceptors leads to the

enhancement of noradrenergic neurotransmission through increased noradrenergic cell firing and *l*-NA release (Danilevicuite & Sveikata, 2002). It has been proposed by De Boer (1996) that mirtazapine's principal action is the blockade of α_2-ARs, and it is believed that this feature underlies much of its antidepressant activity.

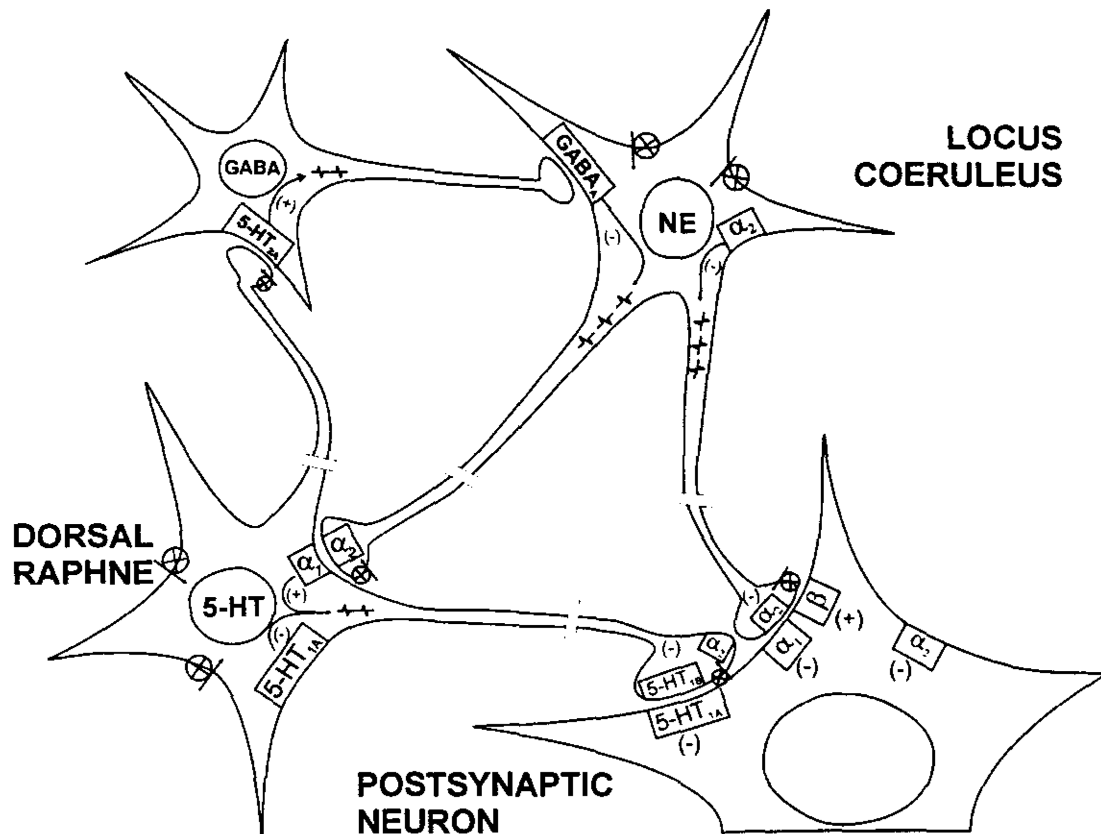

Figure 2-2: Interaction between 5-HT and NA neurones and their projections to pyramidal neurons of the hippocampus. The cog-wheels represent the reuptake transporters responsible for the synaptic inactivation of the various neurotransmitters. The pharmacological subtypes of the pre- and postsynaptic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

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 α_{2A}-adrenergic auto receptors on the noradrenergic terminals, which inhibit the release of *l*-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 *l*-NA in the synapse. This then results in the α_1-AR-mediated firing at the serotonergic neurons, with the α_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 α_{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 paroxitine (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 contra-indicated 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 (MAOIs), or within 14 days of initiating or discontinuing therapy with MAOIs. 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 α_1-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 α_2-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 α_{2A}-auto and heteroreceptors. Most recently, Sanacora *et al.* (2004) reported that co-administering of yohimbine, an α_2-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 α_2-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 α_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 α_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 α_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 α_{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 (α), beta (β) and gamma (γ) subunits. G-proteins are further classified according to their α-subunits into three families, namely $G_{i/o}$ (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 ($i_{1\text{-}3}$) and three extracellular ($e_{1\text{-}3}$) peptide loops with an extracellular N-terminal and cytosolic carboxy-terminal (Parnot *et al.*, 2002) (see Figure 2-3).

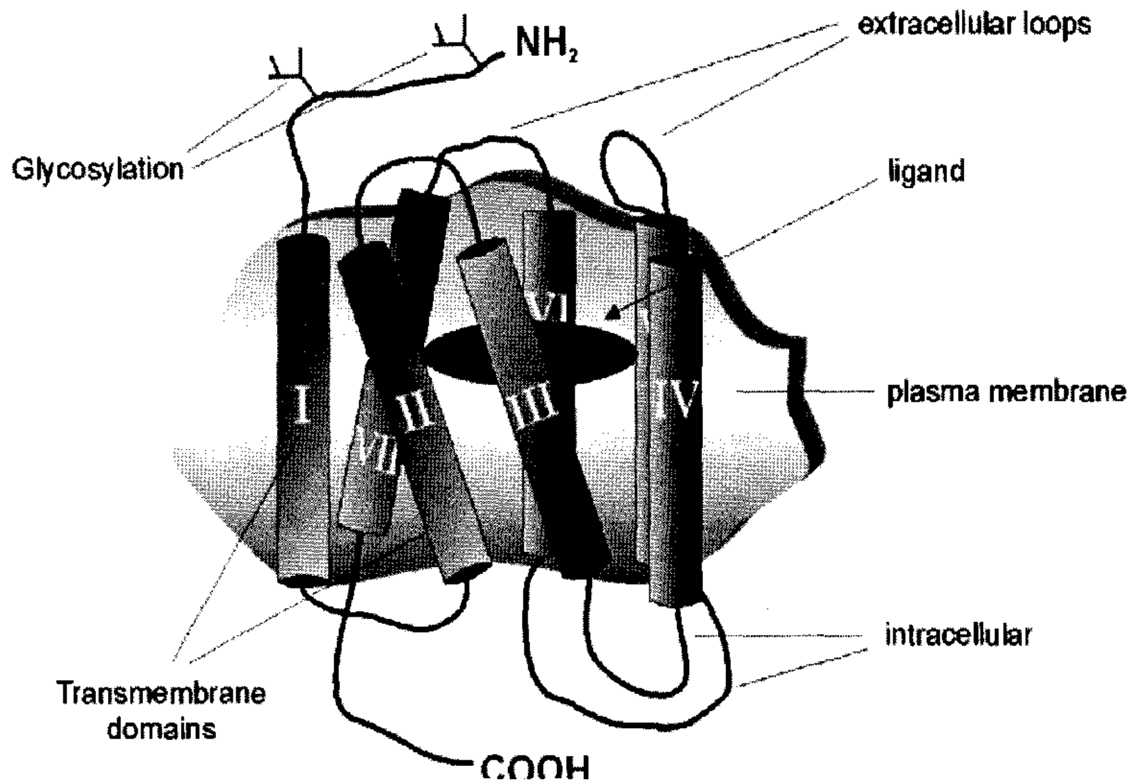

Figure 2-3: Counter-clockwise orientation of GPCR from transmembrane domains I-VII. The closed-loop structure is representative of receptors for small ligands such as biogenic amines and nucleosides. In this arrangement, the core is comprised mainly of TM domains II, III, V, and VI, whereas TM domains 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

Class	Source	Ligand (receptor)
A	Mammalian	Endocrine Oxytocin, gonadotropin, prostaglandin, lutenizing hormone, melanocortin, thyrotropin, adrenomedullin, mwlatonin, gonadotropin-releasing hormone, thyrotropin-releasing hormone, follicle-stimulating hormone, somatostatin. Neurotransmitters Acetylcholine (e.g. **muscarinic)**, neuropeptide Y, neurotensin, **serotonin**, opioid, ***l*-NA (α- and β-adrenergic)**, **dopamine** Cardiovascular Angiotensine, bradykinin, endothelin, tachykinin, vasopressin, thrombin Others Histamine, chemokine, interleukin, olfactory receptors, rhodopsin (light-sensing receptors), chemoattactant C5a, purine receptors
B		Calcitonin, parathyroid hormone, growth hormone-releasing hormone, corticotrophin-releasing factor, PACAP, secretin, glucagons, vasoactive intestinal peptide
C		Metabotropic glutamate receptors, calcium-sensing receptor, GABA-B
D	Fungal	Yeast α–factor receptor, yeast a-factor
E		Dictyostellium chemoattractant receptors

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 α-subunit and the subsequent binding of GTP. This is followed by the dissociation of the G-protein trimeric protein into its α- 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 α-subunit), resulting in the re-association of the α 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.

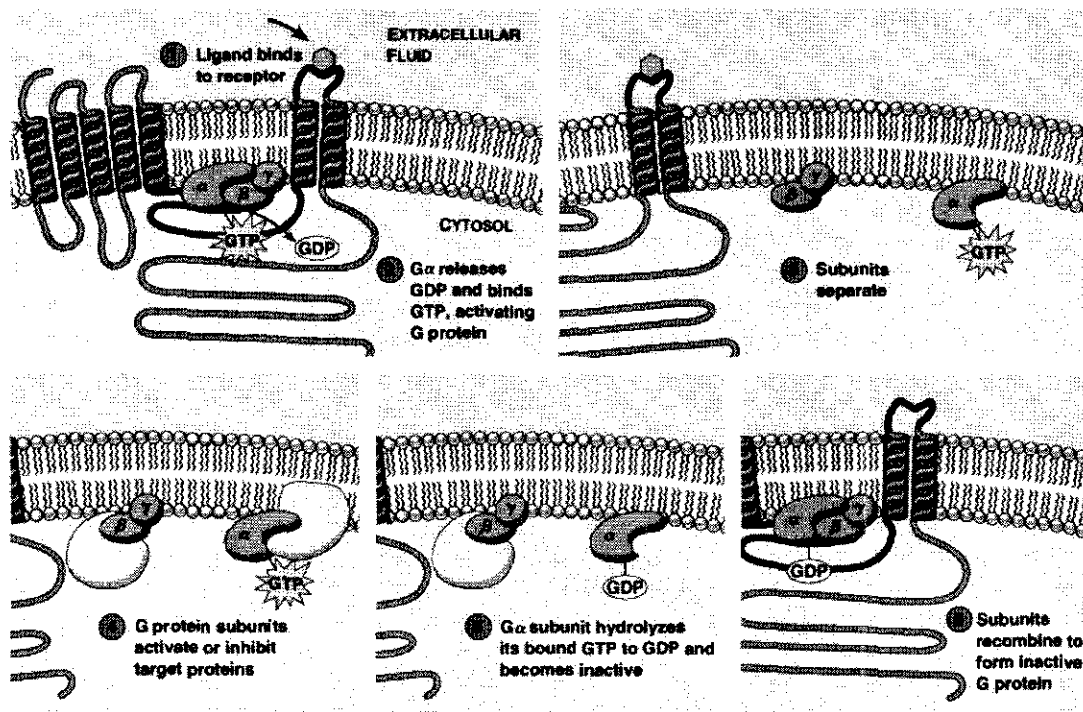

Figure 2-4: Schematic illustration of the G-protein activation/inactivation cycle [obtained from Addison Wesley Longman, (2003)]

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 (M_1 & M_2) acetylcholine receptors, adrenergic ($\alpha_{1A,\ 1B\ \&\ 1D}$) receptors, and serotonergic (5-$HT_{2A,\ 2B,\ \&\ 2C}$) receptors regulate the activity of PLC through interaction with all four members of the G_q subfamily ($G\alpha_q$, $G\alpha_{q/11}$, $G\alpha_{q/14}$ & $G\alpha_{q/16}$) and Gβγ subfamily (Sternweis & Smrcka, 1992; Berridge, 1993).

Following the stimulation of these receptor types, the G-protein is then activated and $G\alpha_{q(GTP)}$ binds to PLC-β (PLC isoform commonly modulated by GPCRs), thus catalysing the hydrolysis of intracellular-associated phosphatidylinositol-4,5-bisphosphate (PIP_2) to form diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Gβγ-GTP subunit also binds to certain PLC-β isoforms, resulting in activation of the enzyme and formation of IP_3 and DAG (Rhee & Choi, 1992).

IP_3 is a soluble molecule that diffuses through the cytosol and binds to IP_3 receptors on the endoplasmic reticulum, causing the release of calcium ions (Ca^{2+}) 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).

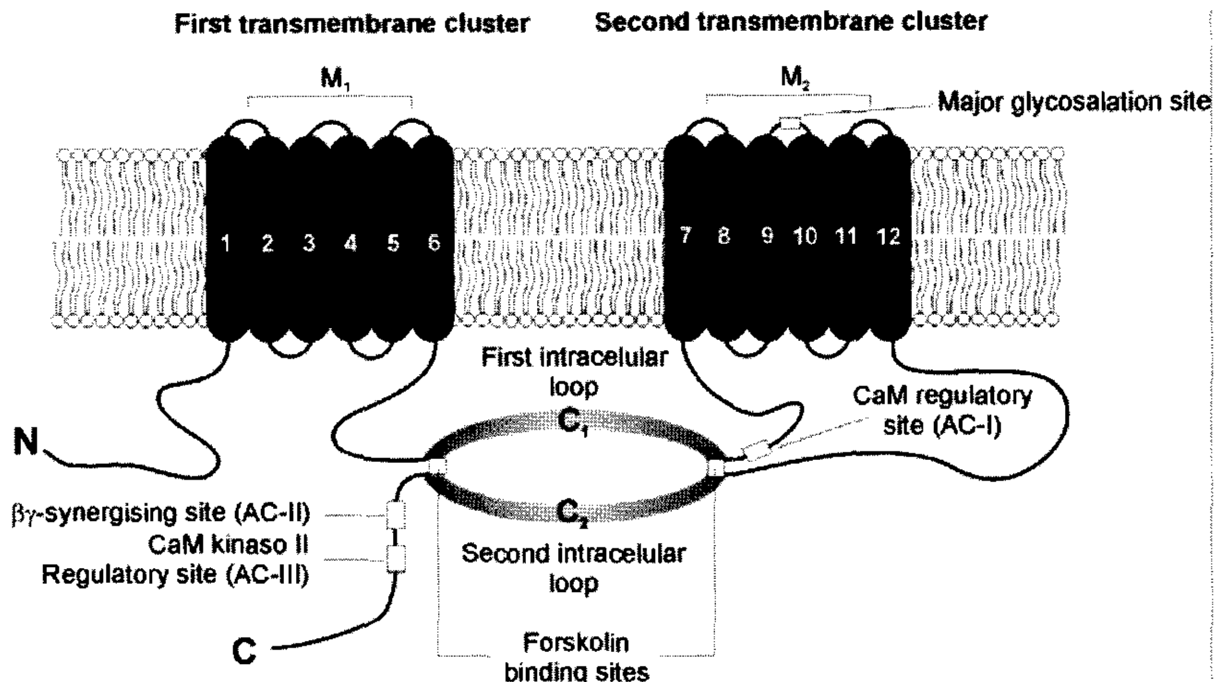

Figure 2-5: Schematic representation of adenylyl cyclase (modified from Houslay & Milligan, 1997)

Several GPCRs, including cholinergic (M_2) receptors, serotonergic (5-HT_1) receptors and adrenergic (α_2) receptors couple to G_i proteins. $G\alpha_i$ interacts with either of the two soluble active sites of the adenlylyl 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 Ca^{2+} 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 ($\beta_{1,2\,\&\,3}$) results in coupling of the receptor to the $G\alpha_s$, 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 α_1-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).

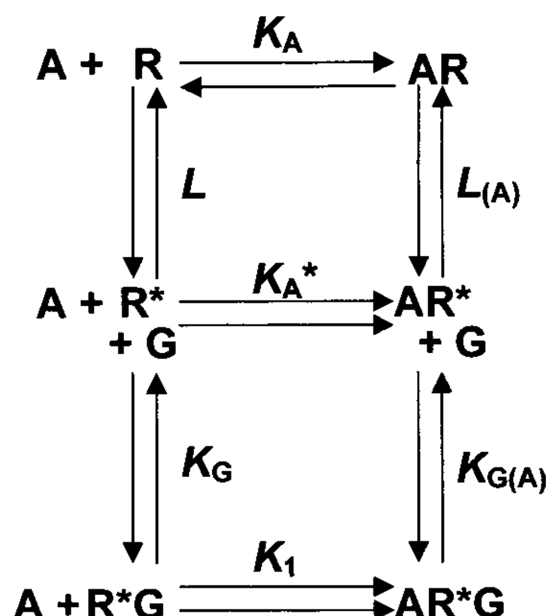

Figure 2.6: Extended ternary complex model. Simple version of the extended ternary complex model in which the receptor exists in two states, an inactive **R** and an active **R*** state. K_A and K_A* and K_1 represent the affinities of the ligand **A** for **R**, **R*** and **R*G**, respectively, whereas 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 α_{1A}-, α_{1B}-, α_{2A}- and β_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 β_2-AR inverse agonists ICI-118,551 and propranolol in transgenic mice (mutants with cardiac overexpresion of human β_2-ARs) enhanced baseline atrial contractility (Nagajara *et al.,* 1999). Also, the prolonged treatment of about 24 hours with inverse agonists in human 5-HT_{2c} receptor (expressed stably in CHO-K1 cells with no receptor reserve), selectively enhance 5-HT_{2C}-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 andidepressants (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).

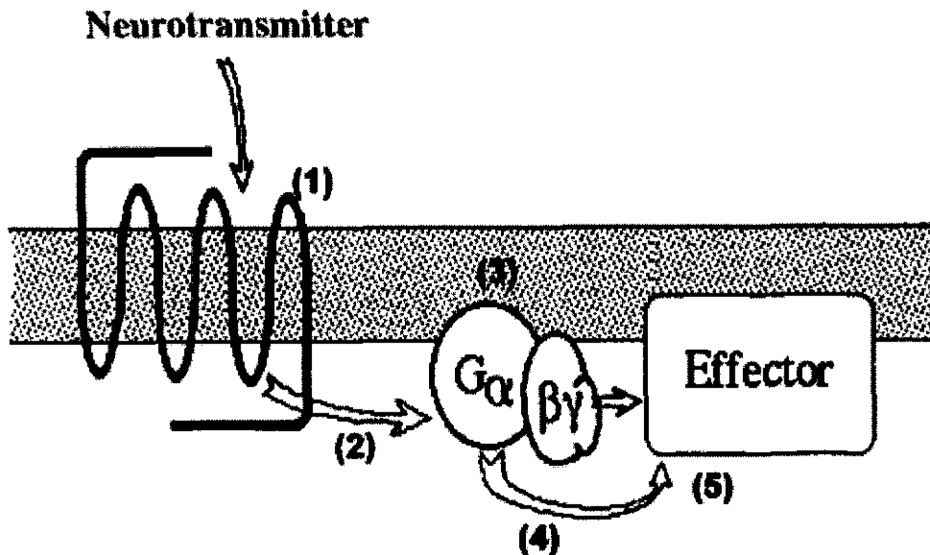

Figure 2-7: Schematic representation of possible targets of antidepressant action (Donati & Rasenick, 2003).

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

2.51 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).

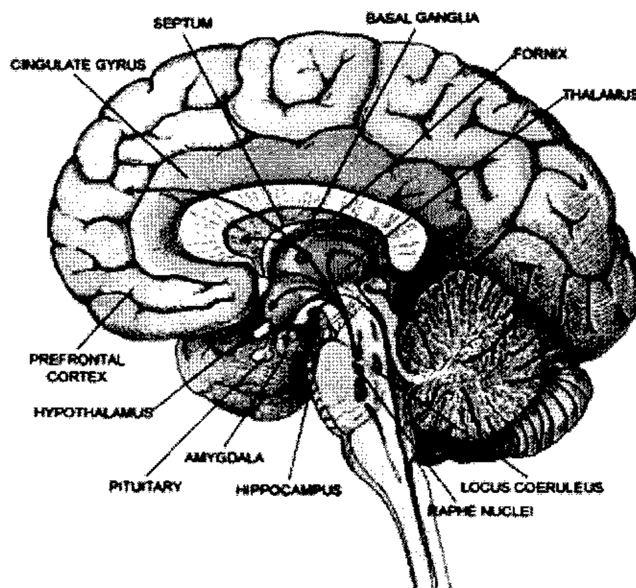

Figure 2.8: Anatomical layout of the limbic system (Albany, 2004)

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), acetylcoline (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 Keltner *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, Fe^{2+} 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-

p-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, Cu^{2+}, 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).

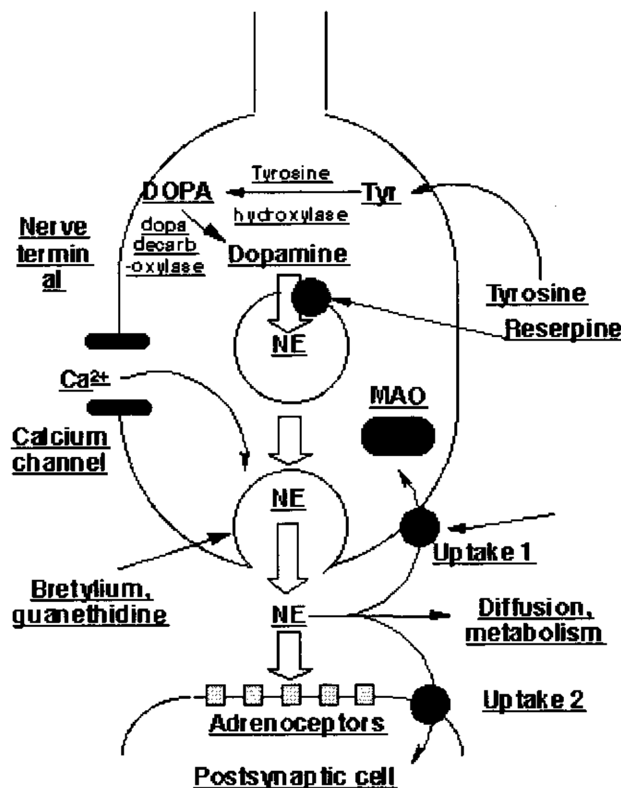

Figure 2-9: Biosynthesis and transmission at the adrenergic nerve terminal (University of Guelph, 2004). (DOPA = 3,4-dihydroxyphenylalanine; MAO = monoamine oxidase inhibitor; NE = norepinephrine; Tyr = tyrosine)

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, *l*-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 *l*-NA is rapidly released into the synaptic cleft through exocytosis following nerve stimulation. The mechanism by which *l*-NA is released is dependent on the Ca^{2+} 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). *l*-NA at the synaptic cleft regulates its own release by interacting with presynaptic α_2-adrenergic autoreceptors, thus inhibiting further release via (1) inhibition of the voltage-sensitive Ca^{2+} 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 Ca^{2+} levels (Cooper *et al.*, 1996). For example, mirtazapine is an atypical antidepressant believed to exert part of its antidepressant actions by antagonising the α_{2A}-adrenergic autoreceptors, thus increasing *l*-NA neurotransmission.

Removal: Synaptic *l*-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, α_1 and α_2, have been reported to be present in the CNS (Keltner *et al.*, 2001). These receptors are further subdivided into several subtypes designated α_{1A}, α_{1B}, α_{2A}, α_{2B}, for α-AR class, while β-ARs are divided into β_1, β_2, & β_3. All known adrenergic receptors are coupled to G-proteins and modulate either AC (β_1, β_2, β_3 & α_2-ARs), thereby influencing the formation of second messenger c-AMP, or PLC (α_1-ARs), leading to the formation of second messenger IP_3 and DAG (Taussig & Gilman, 1995; Lingett, 1996).

α_1-ARs are generally postsynaptic and excitatory, while α_2-ARs are inhibitory in nature (Elhwuegi, 2004). However, α_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 α_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 α_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 α_2-ARs in major depression (Garcia-Sevilla *et al.,* 1999). Desipramine, a selective *l*-NA reuptake inhibitor, has been reported to induce α_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 α_2-ARs in the dorsal raphe nuclei (Szabo & Blier, 2001). Amitryptyline has also been reported to induce α_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 α_2-ARs, suggesting that desensitisation of α_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 β-ARs is often regarded as an indication of antidepressant potential for new agents (Leonard, 2003). Consistent decrease in the β-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 β-AR levels and function are restored following antidepressant therapy (Leonard, 2003).

However, the β-AR hypothesis has several drawbacks. Firstly, there is evidence suggesting that the down-regulation of β-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 ($5\text{-}HT_{1\text{-}7}$) have been recognised, although only the $5\text{-}HT_1$, $5\text{-}HT_2$ and $5\text{-}HT_3$ receptor classes have been defined well (Martin & Humphrey, 1994).

The desensitation of $5\text{-}HT_{1A}$ 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 $5\text{-}HT_{2A}$ 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 (D_1) and type-2 (D_2) 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 (Elhwuegi, 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, nefadazone 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):

- 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 associate 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 *l*-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 *l*-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, tolaxatone 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 ethyline 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. *l*-NA, 5-HT and sometimes DA, enhancing the availability of these neurotransmitters. Imipramine-like TCAs inhibit both the *l*-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-demetylated, such as amoxapine, desipramine. maprotiline, norclomipramine, nordoxepin and nortriptyline, are relatively selective for *l*-NA transport (Baldessarini, 2001).

In addition to the blockade of neurotransmitter uptake, TCAs also have an effect on several heterogenous receptors, including the H_1, α_1-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).

SSRIs 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 *l*-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 Gullian-Barre ascending paralysis (Baldessarini, 2001; Carlsson & Wong, 1997). SSRIs that are currently on the market include fluoxetine, sertraline, paroxetine, citalopram and fluvoxamine (Ables & Baughman, 2003).

SSRIs 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-HT_3 receptors is suspected of being responsible for the common adverse affects characteristic in this class of drugs, including gastrointestinal (nausea and vomiting) and sexual effects (delayed or impaired orgasm). Stimulation of 5-TH_{2C} receptors is suggested to contribute to the risk of agitation or restlessness, usually induced by SSRIs (Baldessarini, 2001). Their repeated treatment leads to secondary changes, such as gradual down-regulation of postsynaptic 5-HT_{2A} 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 SSRIs is thought to be due to stimulation of 5-HT_2 receptors (Thase, 1999). Concomitant, treatment with low doses of trazodone (a 5-HT_2 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, threohydroxydrbupropion 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)

Reboxitine 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 fluoxitine

(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 *l*-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 *l*-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 *l*-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 nefadazone 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 nefadazone 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 α_2-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.

Materials and Methods

Chapter 3

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-^{3}H]-inositol and [2-^{3}H]-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 α_{2A}-adrenergic receptors (α_{2A}-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 β-adrenergic receptor (β-AR) function (see Figure 3-2).

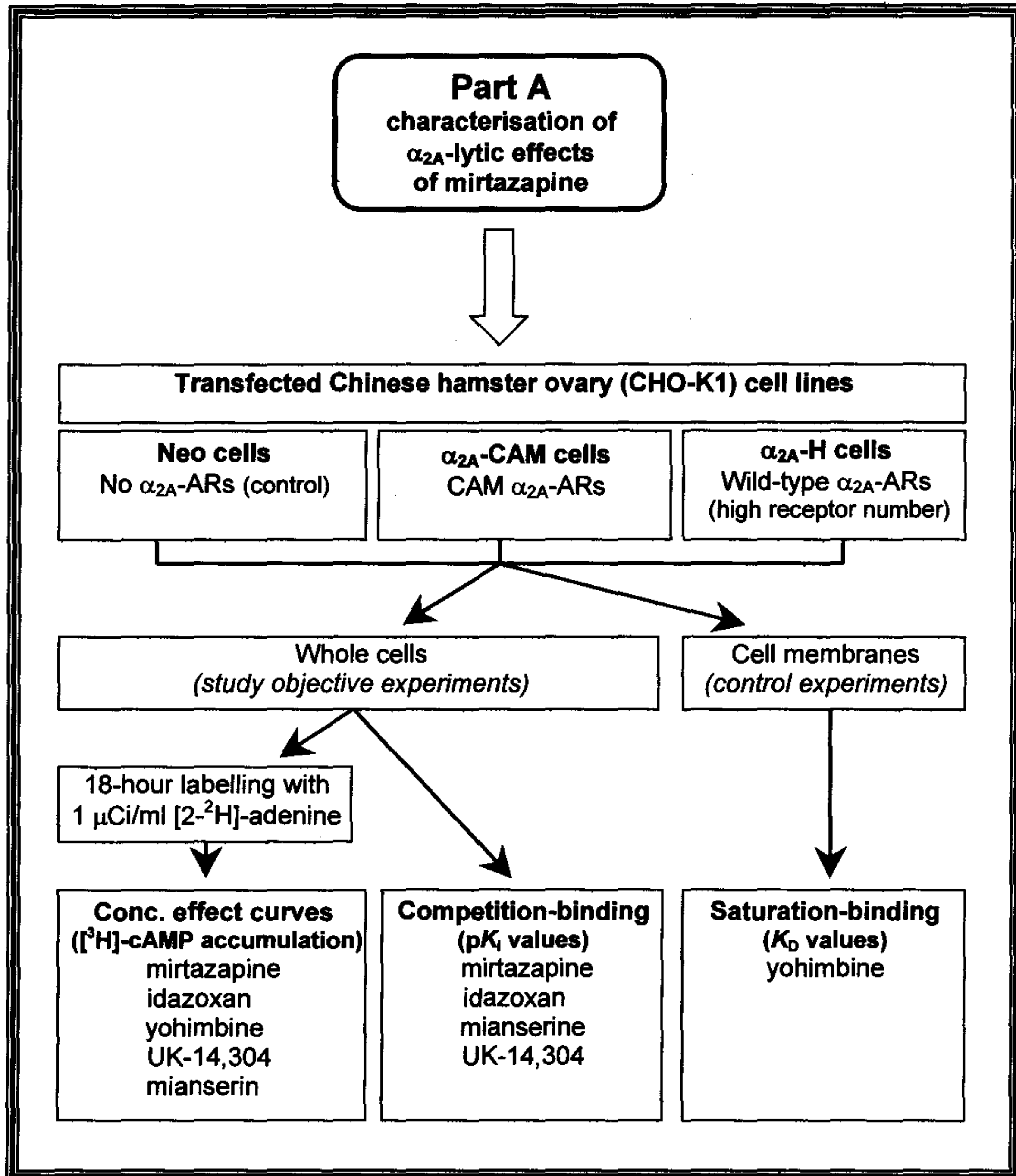

Figure 3-1 Schematic layout of the experiments conducted for part A of the study. (B_{max} = receptor concentration; CAM = constitutively active mutant).

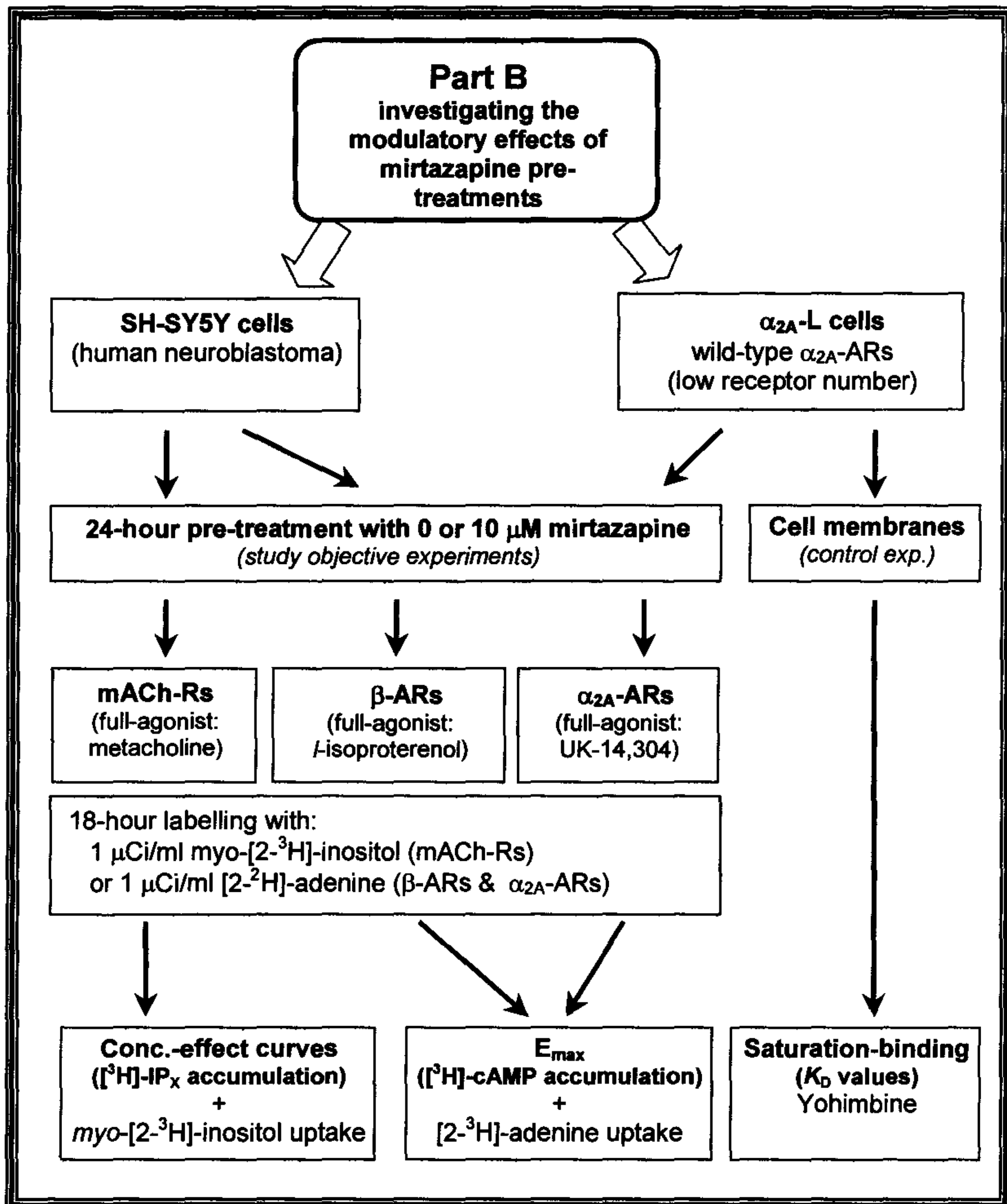

Figure 3-2: Schematic layout of experiments conducted for part B of the study. (α_{2A}-ARs = type-2A alpha-adrenergic receptors; β-ARs = beta-adrenergic receptors; B_{max} = receptor concentration; CHO-K1 = Chinese hamster ovary; mACh-Rs = muscarinic acetyl choline receptors).

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 α_{2A}-AR at relatively high numbers (α_{2A}-H cell line) and relatively low numbers (α_{2A}-L cell line) and the constitutively active mutant α_{2A}-AR (α_{2A}-CAM cell line), as well as the mock-transfected control cells (transfection plasmid, but no cDNA for the α_{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 α_{2A}-AR concentration of 19 ± 3 pmol/mg for CAM α_{2A} cells, 25 ± 4 pmol/mg for α_{2A}-H (Wade *et al.*, 2000) and 1 pmol/mg for α_{2A}-L (Wade *et al.*, 1999). These cell lines were used to investigate the mechanism of antagonism of mirtazapine on the α_{2A}-ARs. The α_{2A}-H cells provide a good *in vitro* biological model for investigating any possible weak / partial agonist activity possessed by α_{2A}-ARs ligands, due to the high α_{2A}-ARs numbers expressed in this cell, so that even very weak agonists would display full agonism. The constitutive activity displayed by the α_{2A}-CAM cell allows investigation of any α_{2A}-AR inverse agonist properties. The neo cells, containing the selection plasmid but not α_{2A}-ARs, were used as a control. The α_{2A}-L cell line was used to investigate the effects of mirtazapine pre-treatment on the α_{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-SY5H cells are known to endogenously express mACh receptors, predominantly the M_3 type (Slowiejko *et al.*, 1999), with some evidence suggesting the availability of M_1 and M_2 types (Kukkunen *et al.*, 1992). In addition, the SH-SY5Y cells endogenously express β-ARs in significant numbers, which was evident from the increase in the *l*-isoproterenol-induced accumulation of [^{3}H]-cAMP in these cells (see Chapter 4).

The α_{2A}-H, α_{2A}-L, α_{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 α_{2A}-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-^{3}H]-adenine (23.0 Ci/mmol) and *myo*-[2-^{3}H]-inositol (17.0 Ci/mmol) was obtained from Armershan Bioscience (UK). [methyl-^{3}H]-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 cm^2 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-versine, bovine serum albumin (BSA), and DMEM were obtained from Highveld Biological (Johannesburg, South Africa). Foetal bovine serum (FBS) and penicillin-streptomycin mixture were obtained from Gibco™ Invitrogen Life Technologies (California, USA).

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 monophophate (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, ethyllenediaminetetra-acetic acid (EDTA), HCl, $NaHCO_3$, NaCl, KCl, Na_2HPO_4, KH_2PO_4, $MgCl_2$, HCl and NaOH were obtained from Merck (Darmstadt, Germany). Fractioned BSA was obtained from Boehringer Mannheim (Mannheim, Germany). 2-amino-2-hydroxymethyl-propan-1, 3,-diol (Tris) was obtained from Acros (Geel, Belgium). Formic acid was obtained

from Saarchem-Holpro Analytic (Krugersdorp, South Africa). Liquid N_2 was obtained from Afrox (Johannesburg, South Africa). Ultima Gold XR scintillation fluid was obtained from Packard BioScience (Meriden, CT, USA). Dowex AG50W×4 resin was obtained from Biorad (USA).

3.1.4 Instruments

Tri-carb 2100 TR liquid scintillation analyser (Packard, A.D.P. South Africa), Sigma Laborzentrifuge 3K15 bench-top centrifuge, Sorvall® Discovery 90SE ultra-centrifuge, Nikon TMS halogen light microscope, haemocytometer (0.1 mm depth, 0.0025 cm^2), 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 CO_2 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 (α_{2A}-H, α_{2A}-L, α_{2A}-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, wherafter 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 [^{3}H]-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 α_{2A}-H, CAM α_{2A} and neo cells. The study also involved investigating the effect of mirtazapine pre-treatment on the β-ARs and α_{2A}-ARs expressed in the SH-SY5Y and α_{2A}-L cells respectively. [^{3}H]-cAMP accumulation was determined as described by Wade *et al,* (1999) and Wong (1994), with minor modifications. For α_{2A}-H, CAM α_{2A}, and the Neo cell experiments, the cells were labelled before seeding by adding 300 µl EMEM (37 °C) containing 1 µCi/ml [2-^{3}H]-adenine. It was thereafter incubated for about 18 hours before the assay was conducted, while the SH-SY5Y and α_{2A}-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% CO_2.

- The stimulation medium was aspirated and the [^{3}H]-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.

- [^{3}H]-cAMP was separated by using two sets of 100 Biorad poly-prep columns fitted into double-layer racks[1].

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

[1] These racks where 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 × 6 ml of water.
- Alumina columns were placed over the scintillation vials and eluded with 4 ml 0.1 M imidazole[2].
- 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

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

3.2.3 Whole-cell [^{3}H]-IP$_x$ 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 IP$_x$-accumulation[3] in the SH-SY5Y cells. The SH-SY5Y cells were pre-treated for 24 hours as described by Brink *et al.* (2004). The G$_q$-mediated [^{3}H]-IP$_x$ 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% CO_2 before the assay was initiated.

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[3] IP$_x$ includes all the inositolphosphates, i.e. inositolphosphate (IP), inositoldiphosphate (IP$_2$) and inositoltriphosphate (IP$_3$). The study focuses more on IP$_3$.

- After labelling, the cells were rinsed out with 2 × 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[4]).

- 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 °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 × 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 °C) was added to each well and transferred again to each corresponding Dowex column.

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

- Columns were placed over the scintillation vials and the [^{3}H]-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 [^{3}H]-IP_x was counted in a Tri-carb TR liquid scintillation analyser.

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[4] Li^+ inhibit the enzyme which metabolises IP_x

Table 3-2: Chromatographic solutions (All the solutions were made up by using double-distilled water)

1	5 M Ammonium for and 3 M formic acid	Dowex column regeneration
2	0.1 M *myo*-Ionositol and 3 M formic acid	Dowex column regeneration
3	1 M Sodium format and 0.1 M borax decahydrate	Washing through unwanted [^{3}H]-biochemicals
4	5 M Ammonium format and 3 M formic acid	Washing through [^{3}H]-IP_x

Table 3-3: Regenerating the Dowex columns for IP_x assay

Procedure	The columns were placed over the collecting basket and regenerated as follows: ❖ Add 5 ml water and let it run through. ❖ Add 2.5 ml solution 1 (see Table 3.2 for composition) and let it run through. ❖ Add 10 ml water and let it run though. ❖ 2 × Solution 2 (see Table 3.2 for composition) and let it run through.

3.2.4 Total [2-^{3}H]-adenine & *myo*-[2-^{3}H]-inositol uptake

The study was aimed at investigating the possible effect of mirtazapine pre-treatment on the total *myo*-[2-^{3}H]-inositol and [2-^{3}H]-adenine uptake into the SH-SY5Y and the α_{2A}-L cells during labelling. This study was conducted concurrently with [^{3}H]-IP_x and the [^{3}H]-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 lyses 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 α_{2A}-H and CAM α_{2A} 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 cm^2 confluent culture flasks with either α_{2A}-H or CAM α_{2A} cells were fed 24 hours before membrane preparation.
- The culture medium was aspirated and washed with 2 × 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 × 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 × 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 × 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 × 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 N_2 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 × 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

Protein concentration	Dilution in test tubes	
	Volume of 2 mg/ml BSA	Volume of TME Buffer
0 mg/ml (blank)	0 µl	100 µl
0.1 mg/ml	5 µl	95 µl
0.4 mg/ml	20 µl	80 µl
0.7 mg/ml	35 µl	65 µl
1.0 mg/ml	50 µl	50 µl
1.4 mg/ml	70 µl	30 µl

- 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 α_{2A}-ARs expressed in the α_{2A}-H cells to determine their pK_i values. The concentration of 5 nM [methyl-^{3}H]-yohimbine ("hot yohimbine") was used to define total binding.

- Cells were grown to 95% consistency in 150 cm^2 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% CO_2.

- 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 dilutions[5] were added to each well and the cells incubated for 60 minutes at 37 ° C in 5% CO_2.

- 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 α_{2A}-ARs from the α_{2A}-H, α_{2A}-L, or α_{2A}-CAM cells through radio-ligand-binding studies. The receptor concentration (B_{max}) was determined by employing α_{2A}-ARs from the α_{2A}-H, α_{2A}-L, or CAM α_{2A} membranes. The concentration of 1, 2, 5, 10, 20 and 40 nM [methyl-^{3}H]-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

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[5] Aappropriate 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) × 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)

Tris-buffer	1 mM Tris acid
TME buffer	50 mM Tris, 10 mM $MgCl_2$, & 1 mM EGTA
PBS	80 g of NaCl, 2 g of KCl, 9 g of Na_2HPO_4, 2 g of KH_2PO_4 in 1000 ml of double-distilled water, to be diluted to 1 part to 9 parts water

3.3 DATA ANALYSIS

Data from all assays was obtained as triplicate observations from at least three separate experiments and expressed as mean ± 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.

Results and discussion

Chapter 4

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 ± 10 μM yohimbine to determine the α_{2A}-adrenergic receptor (α_{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 α_{2A}-ARs at high numbers (α_{2A}-H) and low numbers (α_{2A}-L) and with the constitutively-active mutant of the α_{2A}-AR (α_{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 α_{2A}-H, α_{2A}-L and α_{2A}-CAM cell lines.

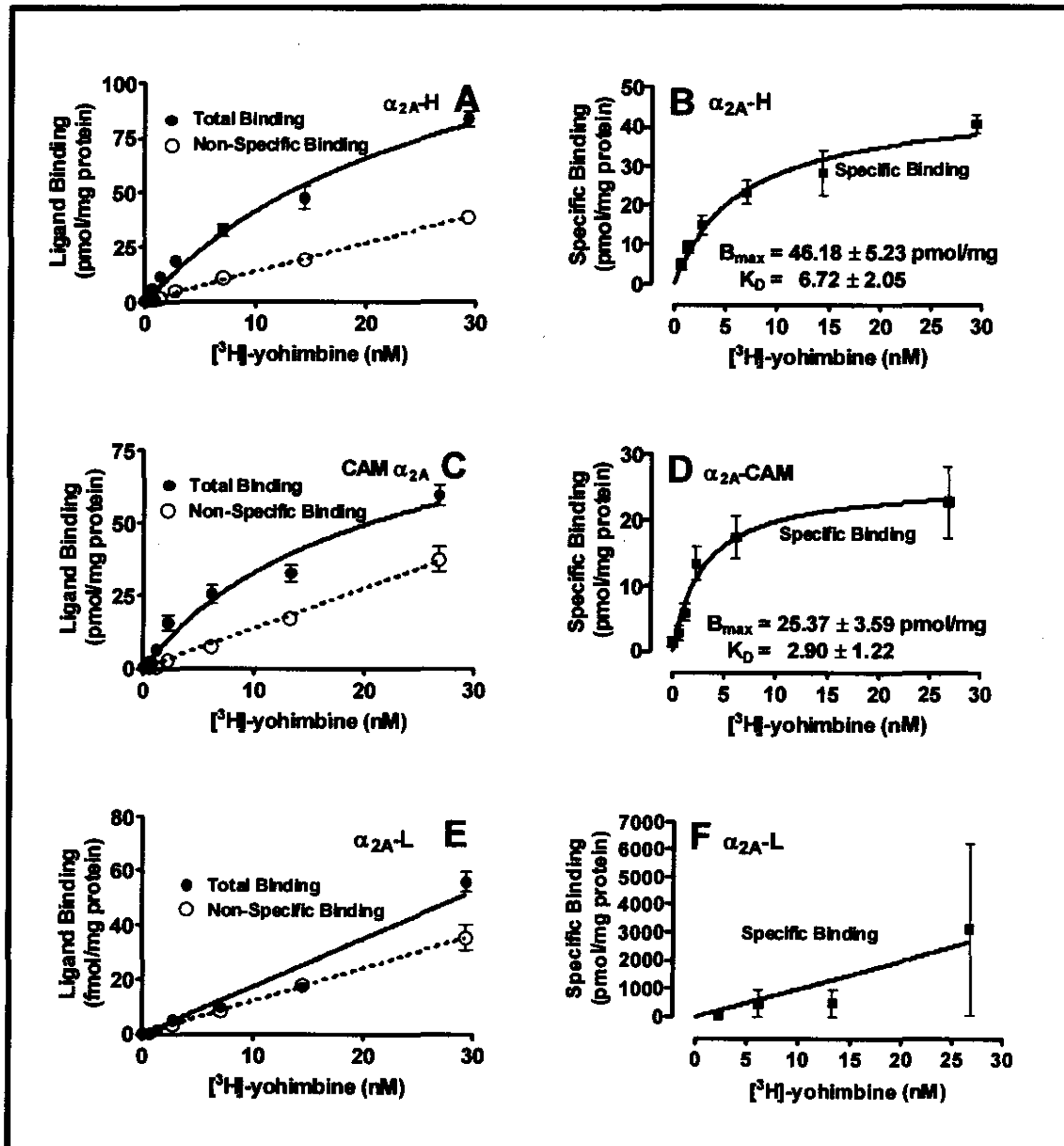

Figure 4-1: Saturation-binding of [methyl-^{3}H]-yohimbine in α_{2A}-H, α_{2A}-CAM and α_{2A}-L cells. Non-specific binding was defined by 10 μM yohimbine (>2000 x K_D). Total and non-specific binding is presented for (A) α_{2A}-H, (C) α_{2A}-CAM and (E) α_{2A}-L cells. Corresponding specific binding was calculated and is presented for (B) α_{2A}-H, (D) α_{2A}-CAM and (F) α_{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 Fiqure 4-1**A** and 4-1**C** it is evident that the non-specific binding in both the α_{2A}-H and α_{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 α_{2A}-H cells (wild type α_{2A}-AR expressed at relatively high numbers) was calculated as 6.72 ± 2.05 nM and the B_{max} value was calculated as 46.18 ± 5.23 pmol/mg protein (see Figure 4-1**B**), while K_D value of [methyl-^{3}H]-yohimbine in the α_{2A}-CAM cells was calculated as 2.90 ± 1.22 nM and B_{max} value was estimated as 25.37 ± 3.59 pmol/mg protein (see Figure 4-2**D**). The B_{max} values in these cell lines corresponded with what was reported by Wade *et al.* (2000) with minor variations.

The B_{max} value for α_{2A}-L cells could not be determined (see Figure 4-1**E** & **F**), since the non-specific binding was proportionally too large compared to total binding. This may be due to low expression of the α_{2A}-AR numbers in this cell line that would require better resolution of measurements. Wade *et al,* (1999), however reported the B_{max} value for α_{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 α_{2A}-ARs antagonism by mirtazapine, and secondly to investigate the possible modulatory effects of mirtazapine pre-treatment on the muscarinic acetylcholine receptors (mAChRs), β-adrenergic receptors (β-ARs) and α_{2A}-ARs in the SH-SY5Y human neuroblastoma and Chinese hamster ovary cells transfected to express α_{2A}-ARs at relatively low numbers (α_{2A}-L).

4.3.1 Characterisation of the mode of α_{2A}-adrenoceptors antagonism by mirtazapine

4.3.1.1 Characterisation of binding to α_{2A}-adrenergic receptors

Reports that mirtazapine antagonises the α_{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 α_{2A}-AR ligands including, UK-14,304 (α_{2A}-AR full-agonist), idazoxan (neutral antagonist) and mianserin (atypical antidepressant with α_{2A}-AR lytic properties) were determined.

Figure 4-2 illustrates competition-binding of mirtazapine, UK-14,304, idazoxan or mianserin against 5 nM [methyl-^{3}H]-yohimbine in the Chinese hamster ovary cells transfected to express α_{2A}-ARs at high numbers (α_{2A}-H cell line).

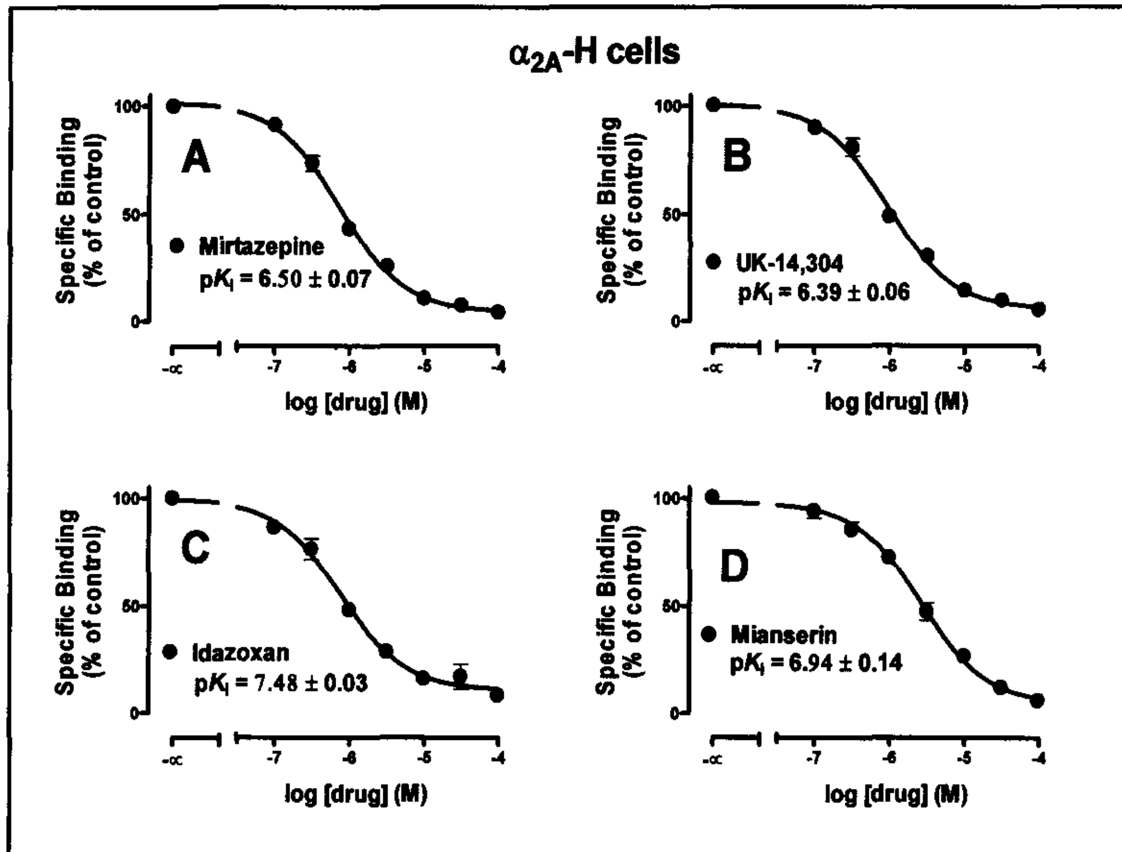

Figure 4-2: Competition binding curves of (A) mirtazapine, (B) UK-14,304,, (C) idazoxan and (D) mianserin against 5 nM [methyl-^{3}H]-yohimbine (0.73 x K_D) in α_{2A}-H cells. The data are presented as mean ± S.E.M and expressed as percentage of control. All data points were obtained from triplicate observation from three independent experiments (*n* = 3).

Results of competition-binding studies illustrated in Figure 4-2**A** and 2**B** shows that mirtazapine and UK-14,304 have similar affinity values at the porcine α_{2A}-ARs. The pK_i values were determined as pK_i = 6.39 ± 0.06 for UK 14,304 and 6.50 ± 0.07 for mirtazapine and do not differ statistically significantly. Figure 4.1 **(C & D)** shows the affinity values of other α_{2A}-ARs ligands used in this study, i.e. idazoxan, (pK_i = 7.48 ± 0.03) and mianserin (pK_i = 6.94 ± 0.14). From these results it can be seen that mirtazapine binds to the α_{2A}-ARs with an affinity similar to that of the full agonist UK-14,304 and α_{2A}-ARs antagonist mianserin.

For the characterisation of the mode of α_{2A}-ARs action by mirtazapine, any possible partial agonism was investigated in the α_{2A}-H cells, while the possibility of inverse agonism was investigated in the α_{2A}-CAM cells.

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

The α_{2A}-H cell line is known to express the α_{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 [^{3}H]-cAMP accumulation). Figure 4-3 depicts the concentration-effect curves of the classical α_{2A}-ARs full agonist UK-14,304 and atypical antidepressant mirtazapine measuring the whole-cells [^{3}H]-cAMP accumulation in the α_{2A}-H and Neo cells.

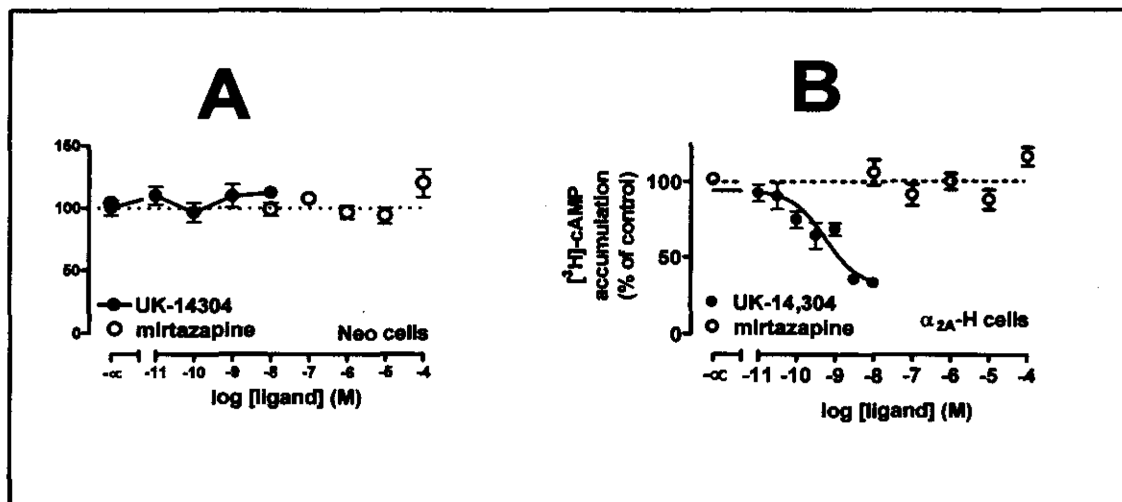

Figure 4-3: Concentration-effect curves of mirtazapine and UK-14,304, measuring the whole-cell [^{3}H]-cAMP accumulation in (A) Neo cells (B) and α_{2A}-H cells. The data are represented as mean ± S.E.M and expressed as percentage of control. The data represent triplicate observations from three independent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

In Figure 4-3**A** it can be seen that both α_{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 α_{2A}-AR). This would confirm that any observed responses in the transfected α_{2A}-H cells result from interaction with the α_{2A}-ARs.

In Figure 4-3**B** it is evident that the α_{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. $EC_{50} \approx 0.001 \times K_i$), suggesting a large proportion of spare receptors in

the α_{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 α_{2A}-ARs even at the concentration of 0.1 mM (± 330 x K_i). These results suggest that mirtazapine lacks any weak partial agonist effect at α_{2A}-ARs and that it may either act as a neutral antagonist or as an inverse agonist at α_{2A}-ARs.

4.3.1.3. Inverse efficacy at the α_{2A}-CAM cells

Figure 4-4 depicts DRCs of idazoxan, yohimbine, mianserin, mirtazapine and UK 14,304 measuring the whole-cell [^{3}H]-cAMP accumulation on the CAM α_{2A}-H cells.

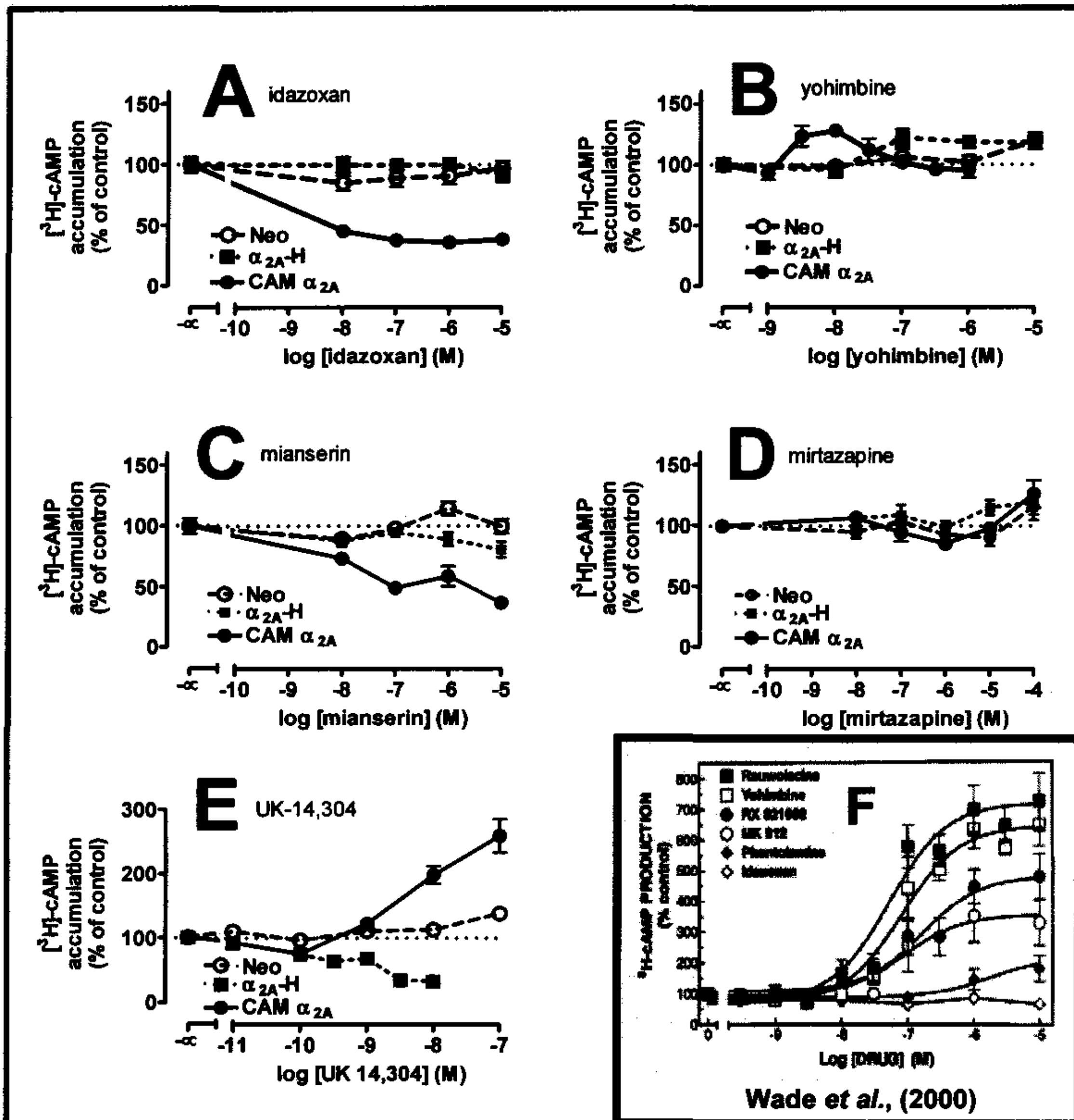

Figure 4-4: Ligand activity at α_{2A}-ARs. Concentration-effect curves of (A) idazoxan, (B) yohimbine, (C) mianserin, (D) mirtazapine and (E) UK-14,304 in the neo, α_{2A}-H and α_{2A}-CAM cells, measuring the whole-cell [^{3}H]-cAMP accumulation. The data are represented as mean ± S.E.M and expressed 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 α_{2A}-CAM cells for comparison, as obtained by Wade *et al.* (2000).

Figure 4.4**A** 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)[1], which would resemble normal agonism at α_{2A}-ARs. These results are different from what was found by Wade *et al.* (2000) (see Figure 4-4**F**). No effects were observed in control neo cells or in α_{2A}-H cells containing the wild-type α_{2A}-AR. It can be seen in Figure 4-4**B** 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 α_{2A}-CAM cells, which would indicate inverse agonism at α_{2A}-ARs, as was found by Wade *et al.* (2000) (see Figure 4-4**F**). 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 α_{2A}-H cells containing the wild-type α_{2A}-AR. These results, as illustrated in Fig. 4-4**A** and Fig. 4-4**B**, suggest reduced signalling by the CAM α_{2A}-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 α_{2A}-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 α_{2A}-ARs. However, its concentration-effect curve is compared with that of idazoxan (Fig. 4-4**A**, 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 α_{2A}-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 α_{2A}-H cells containing the wild-type α_{2A}-AR. As illustrated in Figure 4-4**D**, 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 α_{2A}-H cells containing the wild-type α_{2A}-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.

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[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 α_{2A}-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$_x$ production and the total cellular *myo*-[2-^{3}H]-inositol uptake during the radio-labelling of the SH-SY5Y neuroblastoma cells.

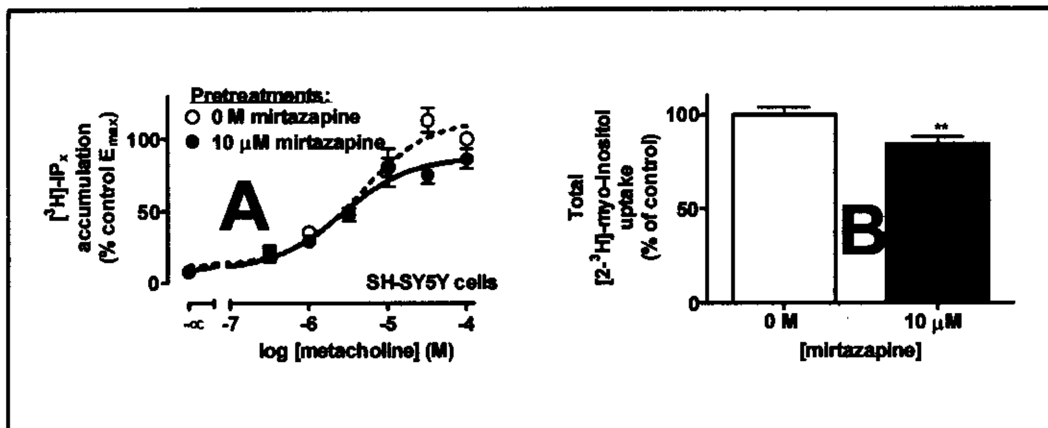

Figure 4-5: The modulatory effects of 24-hour pre-treatment with 0 M or 10 μM mirtazapine on the mAChR function. (A) DRCs of metacholine-induced inositol multiphosphates (IP$_x$) accumulation on the mAChRs in the SH-SY5Y cells, with or without pre-treatment with 10 μM mirtazapine. (B) Total cellular *myo*-[2-^{3}H]-inositol uptake during the radio-labelling of the SH-SY5Y cells. The data are represented as mean ± S.E.M and expressed as percentage of control E_{max}. The data represent triplicate observations from four independent experiments (n = 4).

In Figure 4-5**A** 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_{max} and EC_{50} values not statistically significantly different). As depicted in Fig 4-5**B**, there is a significant decrease in total [2-^{3}H]-*myo*-Inositol uptake following 24-hour pre-treatment with mirtazapine from 100.0 ± 3.655 to 84.20 ± 3.748 (P = 0.008). This decrease in total [2-^{3}H]-*myo*-Inositol uptake might explain the slight decrease in [^{3}H]-IP$_x$ accumulation following 10 μM 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 [^{3}H]-cAMP production and the total cellular [2-^{3}H]-adenine uptake during the radio-labelling of the SH-SY5Y neuroblastoma cells.

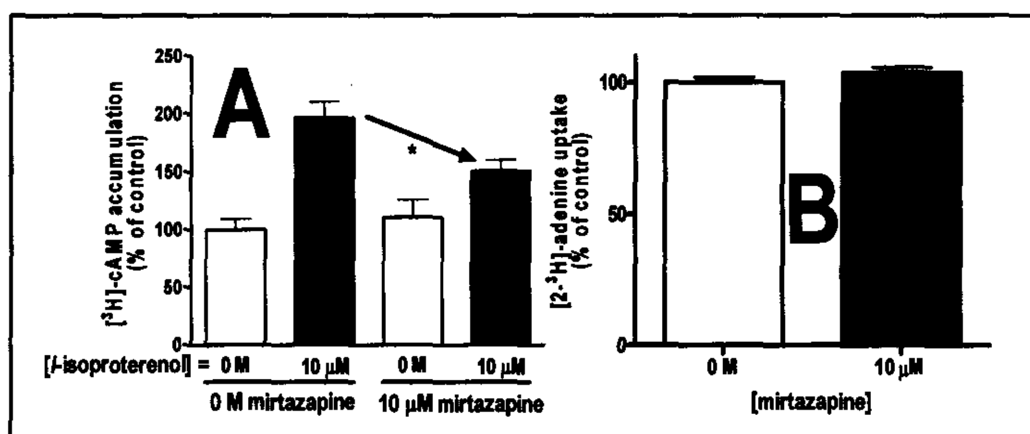

Figure 4-6: (A) single maximum concentration effects (black bars) of the β-ARs full agonist *l*-isoproterenol) (10 μM) after 24-hour pre-treatment with either 0 or 10 μM mirtazapine in SH-SY5Y human neuroblastoma cells, measuring the whole-cell [^{3}H]-cAMP accumulation. (B) Total [2-^{3}H]-adenine uptake during the radio-labelling of the SH-SY5Y cells. The data represent triplicate observations from four independent experiments (n = 4).

In Figure 4-6**A** 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 4-6**B**, it is evident that mirtazapine pre-treatment has no effect of the total Total [2-^{3}H]-adenine uptake during radio-labelling, thus suggesting that the reduced cAMP seen in Figure 4-7**A** 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 α_{2A}-adrenoceptors

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

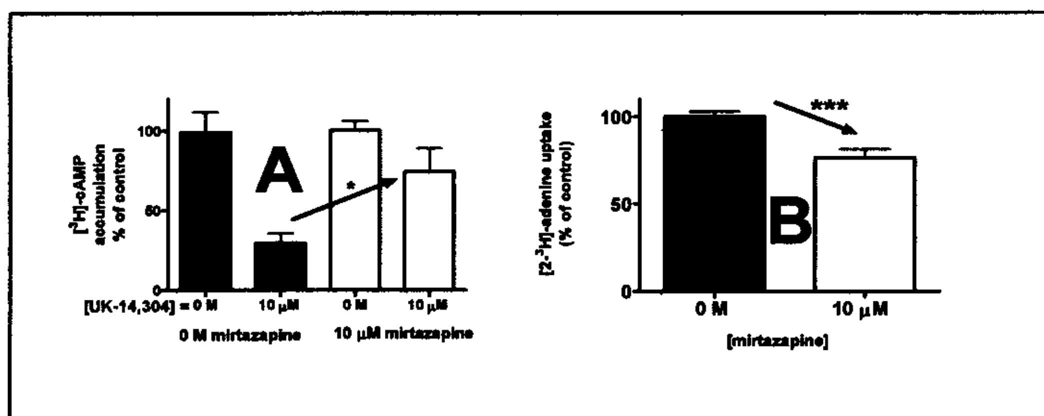

Figure 4-7: The modulatory effects of 24-hour pre-treatment with 10 μM mirtazapine on the α_{2A}-AR function. (A) Concentration-effect curves of the full agonist UK-14,304-induced decrease in [^{3}H]-cAMP accumulation on the α_{2A}-ARs in the α_{2A}-L cells with either 0 M or 10 μM mirtazapine pre-treatment. (B) Total cellular [2-^{3}H]-adenine uptake during the radio-labelling of the α_{2A}-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 α_{2A}-L cells with mirtazapine significantly reduces the full-agonist UK-14,304-induced reduction in [^{3}H]-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 4-7**B**) it can be seen that there is a decrease in cellular [2-^{3}H]-adenine uptake following 10 μM mirtazapine pre-treatment on the

α_{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 4-7**B** complicates the interpretion of the results in **A**, since it is not clear whether the reduction in the agonist-induces 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 α_{2A}-AR function.

However in previous studies it was shown that desipramine and selective *l*-NA reuptake inhibitors induce α_{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 α_{2A}-ARs in α_{2A}-H and α_{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 α_{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 β-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 α_{2A}-ARs function.

Summary, conclusions & recommendations

Chapter 5

5.1 SUMMARY

The current study had two main objectives. The first objective entailed the characterisation of the α_{2A}-adrenergic receptor (α_{2A}-AR) antagonism by mirtazapine. For this purpose Chinese hamster ovary cells (CHO-K1) expressing the porcine α_{2A}-ARs at high numbers (α_{2A}-H), the constitutively active mutant of α_{2A}-ARs (α_{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 (β-AR) functions, using SH-SY5Y human neuroblastoma cells.

Control experiments were performed to determine the α_{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 5-1: α_{2A}-ARs concentrations in CHO-K1 cell lines

[illegible]	[illegible]	[illegible]	[illegible]
α_{2A}-H	46.18 ± 5.23	6.72 ± 2.05	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 α_{2A}-H cells.
α_{2A}-CAM	25.37 ± 3.59	2.90 ± 1.22	The B_{max} value is relatively high, corresponding with the value reported by Wade *et al.* (2000).
α_{2A}-L	-	-	The B_{max} & K_D values could not be determined, since the non-specific binding was proportionally too large compared to total binding.

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

Table 5-2: The affinity values at the α_{2A}-ARs

[illegible]	[illegible]	[illegible]
Mirtazapine (atypical antidepressant)	6.50 ± 0.07	All the values are in the low micro molar concentration range, suggesting intermediate affinity for the wild-type α_{2A}-AR
UK-14,304 (full-agonist)	6.39 ± 0.06	
Idazoxan (neutral antagonist)	7.48 ± 0.03	
Mianserin (atypical antidepressant)	6.94 ± 0.14	

The α_{2A}-H cell line was used to investigate whether mirtazapine possesses any partial agonist properties at the α_{2A}-AR. Since this cell line is known to express the α_{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 α_{2A}-ARs.

The α_{2A}-CAM cell line was used to investigate whether mirtazapine possesses any inverse agonism at the α_{2A}-AR by constructing concentration-effect curves, measuring whole-cell [^{3}H]-cAMP accumulation. However, the CAM α_{2A}-ARs in the α_{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 α_{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 α_{2A}-H cells containing the wild-type α_{2A}-ARs, confirming that the activity observed in α_{2A}-CAM cells could be interpreted as resulting from interaction with CAM α_{2A}-ARs.

The effect of mirtazapine pre-treatment on mAChR, β-AR and α_{2A}-AR functions was also investigated, using the SH-SY5Y neuroblastoma cells (for mAChRs, β-ARs) and α_{2A}-L cells (for wild-type α_{2A}-AR). The cells were pre-treated with either 0 M or 10 μM 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 (β-AR

agonist = *l*-isoproterenol and α_{2A}-AR agonist = UK-14,304). In addition, the effect of mirtazapine pre-treatment on the total *myo*-[2-^{3}H]-inositol or [2-^{3}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, β-AR and α_{2A}-AR functions

[illegible]	[illegible]	[illegible]	[illegible]
mAChR	No change	Decreased	Unlike fluoxetine and imipramine (Brink *et al.*, 2004), mirtazapine has no effect on the mAChR function
β-AR	Decreased	No change	As commonly associated with chronic antidepressant treatment, mirtazapine reduces β-ARs function
α_{2A}-AR	Decreased	Decreased	Inconclusive, although mirtazapine apparently reduces α_{2A}-AR function

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 α_{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 α_{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-α_{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 (α_2-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 α_2-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 α_2-ARs, thus enhancing the activity at both serotonergic and noradrenergic nerve terminals. It has also been shown that chronic antidepressant therapy down-regulates α_2-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 α_2-AR function. Whether an inverse agonist would regulate receptor trafficking differently from a neutral antagonist at α_2-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 $5HT_{2A}$-Rs, as an example, that both agonists and antagonists paradoxically cause $5HT_{2A}$-R down-regulation (Gray & Roth, 2001). This phenomenon at α_2-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 B_{max} 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 α_2-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 of 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 α_{2A}-ARs were inconclusive, since the α_{2A}-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 α_{2A}-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-HT_{2A}-R or dopaminergic-receptor function.

In vivo animal behavioural studies should also be conducted to investigate the role of α_{2A}-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 (α_{2A}-AR inverse agonist) or idazoxan (α_{2A}-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.

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Chapter 4

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Abbreviations

Appendix: A

5-HT	serotonin
α_{2A}-**ARs**	Type-2A α-adrenergic receptors
α_{2A}-**H**	Chinese hamster ovary cells transfected to express Type-2A α-adrenergic receptors in high numbers
α_{2A}-**L**	Chinese hamster ovary cells transfected to express Type-2A α-adrenergic receptors in low numbers
AC	Adenylyl cyclase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic monophophate
CHO-K1	Chinese hamster ovary cells
CYP	Cytochrome P450 enzyme system
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DSM-IV	Diagnosis and Statistical Manual of Mental Disorders
ECT	Electroconvulsive shock therapy
EMEM	Minimum essential medium (Earle's base)
FBS	Fetal bovine serum

GABA	γ-aminobutyric acid
GPCRs	G-protein-coupled receptors
GTP	Guanosine triphosphate
HEPES	N-(-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid
IBMX	3-isobutyl-1-methylxanthine
IP_3	Inositol-1,4,5-trisphosphate
LiCl	Lithium Chloride
mACh-Rs	Muscarinic acetylcholine receptors
MAOIs	Monoamine oxidase inhibitors
NA	Noradrenaline
NARI	Selective Noradrenaline Reuptake Inhibitors
NaSSA	Noradrenaline and specific serotonergic antidepressant
PBS	Phosphate buffered saline
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein-kinase C
S.E.M.	Standard error on the mean
SNRI	Serotonin and Noradrenaline reuptake inhibitors
SSRIs	Selective serotonin reuptake inhibitors
TCA	Trichloroacetic acid
TCAs	Tricyclic antidepressants
TH	Tyrosine hydroxilase
Tris	2-amino-2-hydroxymethyl-propan-1,3,-diol

Congress Presentation

Appendix: B

KHOZA, K*, BRINK, C.B., & HARVEY, B.H. 2004. **Characterization of the α_{2A}-lytic effects of mirtazapine and its effects on muscarinic acetylcholine and β-adrenergic receptor functions.** (Paper presented as podium presentation at the 38th South African Pharmacology Congress, held at Bloemfontein, South Africa, 24-27 October 2004.) www.sapharmacol.co.za