The modulating effects of selected antidepressants and related drugs on markers of cellular resilience in cultured human neuroblastoma cells

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Abstract

The neurodegenerative hypothesis of depression postulates that major depression is associated with impaired neuroplasticity, particularly in the hippocampus and prefrontal cortex, and that effectiveness of antidepressants may be partially attributed to the extent to which they exert neuroprotective properties. Therefore the current study investigated the effect of selected antidepressant drugs on the survival of cultured human neuroblastoma (SH-SY5Y) cells, before and after glutamate-induced excitotoxicity, as well as the effects of these drugs on pro- and anti-apoptotic pathways.

The free fraction of plasma protein-bound drugs was determined in Ham's F12 culture medium + 10% foetal bovine serum by means of HPLC analysis. Cultured human SH-SY5Y cells were treated for 24 hours with and without 10 mM glutamate plus a low (pharmacological) or high free concentration of fluoxetine, mirtazapine, tianeptine, imipramine, *myo*-inositol, gabapentin or lithium. Thereafter mitochondrial activity was determined with the MTT cell viability assay. The effects of the drug treatments on genes encoding for pro-apoptotic factors Bcl2-associated X protein (Bax), caspase-3 and caspase-8, and anti-apoptotic factors brain derived neurotrophic factor (BDNF), nuclear factor kappa beta (NF-κβ), protein kinase B (Akt), cyclic adenosine monophosphate response element binding protein (CREB) and define B-cell CLL/lymphoma 2 (Bcl-2), was determined utilising quantitative real-time reverse transcriptase polymerase chain reaction (rt^2-PCR).

Results from HPLC analyses indicate that fluoxetine, mirtazapine, tianeptine and imipramine are moderately bound to plasma albumin in culture medium and subsequently the free drug concentrations were determined. The drug treatments using pharmacological concentrations of lithium, *myo*-inositol, imipramine and gabapentin, but not of fluoxetine, mirtazapine or tianeptine, protected against glutamate induced excitotoxicity, as measured with the standard MTT assay, while no drug-induced changes in cell viability were observed in the absence of glutamate. The MTT assay results also indicated that the high concentration of lithium and gabapentin significantly

increase the viability of the SH-SY5Y cells following glutamate cytotoxicity, while the high concentrations of other drugs failed to alter cell viability. The MTT data were supported by the results from rt^2-PCR, indicating that the low (pharmacological) concentrations of fluoxetine, imipramine, tianeptine and *myo*-inositol significantly increase anti-apoptotic gene expression in human neuroblastoma cells after glutamate induced excitotoxicity, while pro-apoptotic gene expression is significantly decreased with gabapentin, imipramine, lithium, mirtazapine, *myo*-inositol and tianeptine. In conclusion, at pharmacological concentrations all antidepressants displayed neuroprotective effects in the presence of excitotoxicity, but via different mechanisms.

Uittreksel

Volgens die neurodegeneratiewe hipotese van depressie is onderdrukte neuroplastisiteit, veral in brein dele soos die hippokampus en prefrontale korteks, verantwoordelik vir die ontwikkeling van major depressie. Laasgenoemde hipotese verwys ook na die moontlikheid dat antidepressant-effektiwiteit gedeeltelik gesetel kan wees in die graad van neurobeskerming van die antidepressant. As gevolg van hierdie navorsingsdeurbrake is die effek van geselekteerde antidepressante op die oorlewing van neuronale selkulture (SH-SY5Y selle), voor en na die toediening van 10 mM glutamaat, ondersoek. Die geen-uitdrukking van apoptose-induserende en -inhiberende proteïne is ook bepaal na glutamaat- en antidepressant-behandeling.

Hoë-druk-vloeistof-chromatografie (HDVC) analisering is gebruik om die vry fraksie van plasmaproteïn-gebonde middels te bepaal in Ham's F12 medium, wat verryk is met 10% fetale kalfserum. Menslike SH-SY5Y selkulture is vir 24 uur behandel met 'n lae (farmakologiese) of hoë fluoksetien-, mirtazapien-, tianeptien-, mio-inositol-, gabapentien- of litium-konsentrasie, met en sonder die toediening van 10 mM glutamaat. Hierna is die mitochondriale aktiwiteit bepaal deur middel van die standaard MTT-seloorlewingstoets. Die geen uitdrukking van apoptose-induserende (Bax, kaspase-3 en kaspase-8) en inhiberende (BDNF, Akt, CREB, NF-κβ en Bcl-2) faktore is bepaal deur middel van kwantitatiewe intydse inverse transkripsie-polimerase ketting reaksie (it^2-PKR), 24 uur na die toedienning van glutamaat en 'n lae (farmakologiese) antideprerssant-konsentrasie.

Die HDVC-resultate het daarop gedui dat al die studiemiddels matiglik gebind is aan plasmaproteïne in die groei medium, en dat dit wel moontlik is om die vry konsentrasie van die middels te bepaal. Die MTT-toets het aangedui dat die lae (farmakologiese) konsentrasie van litium, mio-inositol, imipramien en gabapentien die studieselle teen glutamaat toksisiteit beskerm het, alhoewel dit nie die geval was vir fluoksetien, mirtazapien en tianeptien nie. Die hoë konsentrasie van litium en gabapentien het ook 'n betekenisvolle vlak van beskerming getoon teen glutamaat seltoksisiteit, soos bereken deur die MTT-toets.

Volgens die kwantitatiewe rt^2-PKR resultate is die lae (farmakologiese) konsentrasie van fluoksetien, imipramien, tianeptien en mio-inostol daartoe instaat om apoptose-inhiberende geenuitdrukking betekenisvol te verhoog in die teenwoordigheid van glutamaat. Die resultate het ook 'n betekenisvolle verlaging in apoptose-induserende geenuitdrukking aangedui, nadat menslike SH-SY5Y selle behandel is met gabapentien, imipramien, litium, mirtazapien, mio-inositol of tianeptien.

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"Appreciation is a wonderful thing: It makes what is excellent in others belong to us as well." ***Voltaire***

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Chapter 1 Introduction

1.1 Dissertation approach and layout

The dissertation is presented in an "article format", implying that the key data is prepared according to the "instructions to the author" (*Appendix C*) of a chosen journal, while the complete study data is given and discussed in *Appendix B*. A final summary and conclusion consolidates all data (as in Appendix B *"Complete results and discussion"*) of the study. The Table of Contents gives an overview of the various chapters and of Appendices, while the following summary will further assist to clarify where to find key elements of the study in the dissertation:

- *Study Objectives:*
  § 1.3

- *Literature background & Problem statement:*
  § 1.2 (Problem Statement), Chapter 2 (Literature Review) & Chapter 3 (Article)

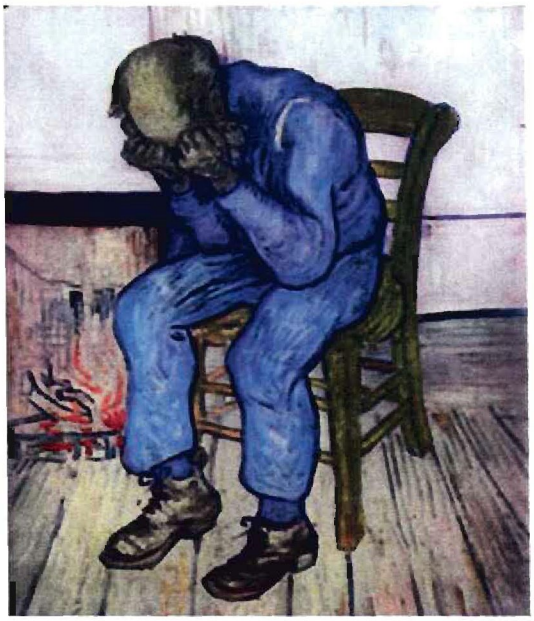
- *Materials & Methods:*
  Chapter 3 (Article) & Appendix A (Additional and Extended Methods)

- *Data & Results:*
  Chapter 3 (Article) & Appendix B (Additional Data and Extended Discussion)

- *Summary, Discussion & Final Conclusions:*
  Chapter 4

1.2 Problem statement

Depression – a serious and disabling disorder:



On the threshold of insanity, painted by Vincent Willem van Gogh in 1890. This piece of art is seen by some as an illustration of the hopelessness and despair experienced in depression. Van Gogh suffered from depression and this portrait was painted by him a few months before he committed suicide.

Depression is the second most ubiquitous disorder in the Western world, after cardiovascular disorders, with a prevalence of 9-18% (Schloss & Henn, 2004). In the United States of America an estimated 2 to 5% of the population suffers from major depression, and another 1 to 2% from bipolar disorder (Akiskal, 2000). The incidence of depression in South Africa is also vastly increasing, especially under patients suffering from HIV (Janssen *et al.*, 1989; Ostrow *et al.*, 1989; Judd & Mijch, 1996). The high incidence of violence and crime in this country is also believed to be predisposing factors for depression development (Van Winkle, 2000; Nixon *et al.*, 2004; Bandelow *et al.*, 2005). Therefore, depression is believed to be among the most common causes of morbidity, disability and suicide around the world (Greden, 2001; Schloss & Henn, 2004). The fact that current drug treatments are plagued by unfavourable side-effect profiles, delayed onsets of action and high incidence of treatment resistance, created the need for superior drug development and a better understanding of the underlying intra-cellular mechanisms of depression (Greden, 2001; Manji & Duman, 2001; Duarte *et al.*, 2006).

Hypotheses of Depression:

Previous hypotheses were based on aberrant concentrations of monoamines (i.e. serotonin, noradrenalin and dopamine) in the synaptic cleft, and assumed that antidepressants exert their primary bio-chemical effects by readjusting the intrasynaptic concentrations of these neurotransmitters (Duman *et al.*, 1999; Manji *et al.*, 2000). However, while antidepressant drugs evoke an almost immediate increase in intrasynaptic monoamines, the onset of antidepressant action only begins to appear 3-4 weeks later (Harvey, 1997). This has lead investigators to consider that the immediate

action of the antidepressant on synaptic monoamines acts to switch on certain sub-cellular signalling pathways that initiate time-dependent neural adaptive changes that are ultimately responsible for initiating the antidepressant effects (Harvey, 1997; Duman *et al.*, 1997; Manji *et al.*, 2000). In line with this thinking, recent neuro-imaging studies revealed selective structural changes as well as volume reductions in certain limbic and non-limbic brain regions in several stress-related neuropsychiatric illnesses, such as major depressive disorder (Manji *et al.*, 2003; Sheline *et al.*, 2003; Fuchs *et al.*, 2004), and which are reversible following chronic antidepressant administration (Magarinos *et al.*, 1999). These findings lead to the formulation of the neurodegenerative hypothesis of depression (Duman *et al.*, 1999; Manji *et al.*, 2000; Sapolsky, 2000).

Depression & Neuroplasticity:

The hippocampus, one of the major brain areas implicated in depression, has shown up to 20% reduction in volume in neuro-imaging studies of depressed patients (Bremner, 1999; Sapolsky, 2000). The high vulnerability of this brain region stimulated considerable research into the underlying causes of the aforementioned anatomical changes. From this emerged a central dogma centred around glutamate being the predominant neurotransmitter in the hippocampus (Sapolsky, 2000).

An increase in glutamate neurotransmission, as has been noted during depression (Stewart & Reid, 2002; Krystal *et al.*, 2002), causes an excessive influx of calcium ions (Ca^{2+}) into the neuron which ultimately leads to neuronal demise (Sapolsky, 2000).

Several prototype and experimental anti-depressants have illustrated prevention and/or reversal of hippocampal atrophy (Fuchs *et al.*, 2004). These include tianeptine (affects structural plasticity in the hippocampus and is an effective anti-depressant (Kuroda & McEwen, 1998; Plaisant *et al.*, 2003; Alfonso *et al.*, 2006)); fluoxetine (down-regulates neurodegenerative enzymes and is anti-apoptotic (Song *et al.*, 2006)); lithium (neuroprotective (Nonaka & Chuang, 1998; Moore *et al.*, 2000b; Manji *et al.*, 2001) and anti-apoptotic (Fukumoto *et al.*, 2001; Bush & Hyson, 2006; Dhikav & Anand, 2007)); imipramine (increases hippocampal neurogenesis and is neuroprotective (Xia *et al.*, 1999; Van Hoomissen *et al.*, 2003; Song *et al.*, 2006)); mirtazapine (decreases corticosterone-induced gene transcription and is neuroprotective (Dhikav & Anand, 2007)). Although some data are available on the suspected protective and antidepressant properties of these drugs, their mechanisms of action are still poorly understood (Paykel, 2001). Thus, determination of the intra-cellular mechanisms of

these drugs might result in the discovery of new antidepressant targets as well as new and advanced treatment regimes (Dhikav & Anand, 2007).

1.3 Study objective

The objective of the current study was to determine the effect of diverse types of anti-depressive drugs (viz. fluoxetine, gabapentin, imipramine, lithium, mirtazapine, *myo*-inositol and tianeptine) on neuroplasticity during neuronal stress (i.e. glutamate-induced excitotoxicity), measuring mitochondrial activity and the expression of genes and proteins involved in neuronal survival and growth.

1.4 Study layout

In order to induce excitotoxic effects in human SH-SY5Y cells, an optimum glutamate co-treatment concentration had to be determined. To achieve this, SH-SY5Y cells were pre-treated with 2 mM, 5 mM, 10 mM and 15 mM of glutamate respectively, for 24 hours. The extent of the excitotoxicity was measured via the standard MTT cell viability assay as described by T. Mosmann in 1983 (Mosmann, 1983).

For the purpose of this study SH-SY5Y cells were pre-treated in Ham's F12 medium enriched with 10% foetal bovine serum (FBS). Bovine serum albumin was added to the Ham's F12 medium in order to prevent acute stress on the cultured cells due to serum deprivation. Due to the highly plasma bound nature of some of the study antidepressants, the free fraction (effective dose) of the plasma albumin bound drugs (fluoxetine, imipramine, mirtazapine and tianeptine) had to be determined in the above-mentioned Ham's F12 medium mixture. Moreover, being able to pre-treat the SH-SY5Y cells with pharmacological concentrations (peak plasma concentrations) of drugs, in a more "physiologically correct" infironment (in the presence of serum albumin), renders the results obtained in the *in vitro* study more applicable and comparable to an *in vivo* situation.

In order to obtain the free concentration of these drugs, two similar concentration ranges (4 concentrations per range) of the implicated drugs were spiked into Ham's F12 medium ± 10% FBS. The concentration range samples, containing FBS, were centrifuged in microcentrifuge tubes (30 kDa pore size) to remove the FBS along with the bound fraction of the study drug. The samples of both the concentration ranges

were then analysed by HPLC and the areas under the curves used to calculate the percentage free study drug in Ham's F12 medium + 10% FBS.

Human SH-SY5Y cells were pre-treated for 24 hours with the pre-determined 10 mM glutamate along with a pharmacological study drug concentration. Hereafter the effect of this pre-treatment regime was determined on mitochondrial activity (cell viability) by means of the previously-mentioned standard MTT assay, and on the expression of selected genes responsible for the regulation of neuronal survival via rt^2-PCR. These include the anti-apoptotic genes BDNF, CREB, Akt, Bcl-2 and NF-κβ as well as the pro-apoptotic genes Bax, Caspase-3 and Caspase-8.

Chapter 2 Literature overview

The current study deals with the neurodegenerative hypothesis of depression and the possible neuroprotective properties of novel and prototype antidepressants. This chapter will therefore provide a review on the brain regions implicated in depression as well as some features concerning the prevalence, common symptoms and classification of depression. The different hypotheses of depression and the scientific basis thereof will be discussed followed by a more extensive overview of the neurodegenerative hypothesis of depression. In this chapter different antidepressants as a therapeutic approach to treating depression, and their proposed neuroprotective mechanisms of action, will be discussed. Finally, this chapter also focuses on the possible role of pro- and anti-apoptotic proteins in depression and antidepressant efficacy.

2.1 The brain

Apart from its role in higher cognitive functions and emotions, the brain is one of the most important vital organs of the human body (Sherwood, 2001b). This, in particular, is due to its central role in unison with the peripheral nervous system to control almost all bodily functions.

Different parts of the brain can be distinguished and regions can be identified and classified anatomically or according to function. Anatomically the brain is divided into three main parts, namely the forebrain, midbrain and hindbrain (Sherwood, 2001b). The forebrain can be subdivided into the cerebrum, thalamus and hypothalamus, the midbrain into the tectum and tegmentum, and the hindbrain into the cerebellum, pons and medulla. Of particular importance for the current study, is the functionally defined limbic system, since many studies suggest that compromised brain plasticity, particularly in the limbic areas, may be associated with the pathophysiology of depression (Sheline, 1996; Bremner *et al.*, 2000; Duman, 2002; Manji *et al.*, 2003).

2.1.1 The limbic system

The limbic system is not an anatomically defined structure, but refers to interconnected neuronal pathways comprising of a ring of structures that surround the brain stem (Sherwood, 2001b). This complex ring of structures lies on both sides of the brain underneath the thalamus, just under the cerebrum (Boeree, 2002). The limbic system includes the hypothalamus, hippocampus, amygdala as well as several other adjacent brain areas which are intimately connected to this system (Boeree, 2002). These areas include the cingulate gyrus, fornicate gyrus, mammillary body, nucleus accumbens, orbitofrontal cortex, parahippocampal gyrus and the thalamus. The limbic system plays a key role in human emotion, motivation, learning, memory, emotional association with memory, neuroendocrine regulation and contextual fear conditioning (Sherwood, 2001b; Boeree, 2002). The key structures of the limbic system are depicted in the partially transparent view of the brain in ***Figure 2-1*** (Sherwood, 2001b).

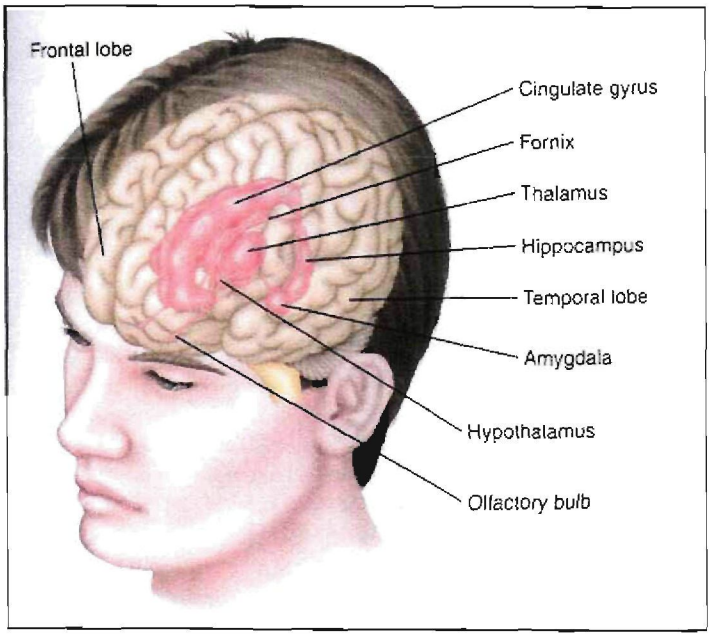


Figure 2-1: *A partially transparent view of the brain revealing the ring of structures that forms part of the limbic system.*

It has been postulated that the limbic structures, especially the hippocampus, is crucially involved in the aetiology of a variety of neuropsychiatric diseases, such as depression (Sheline, 1996; Bremner *et al.*, 2000). Recent research indicates that the hippocampus and amygdala exhibit pathological changes during the course of depression (Bremner *et al.*, 2000; Sapolsky, 2000; Mervaala *et al.*, 2000). Imaging studies of depressed patients indicate impairments in blood flow and a decreased volume of limbic structures

(Drevets, 2000; Manji *et al.*, 2003). A study comparing the hippocampal volumes of non-depressed and depressed patients was conducted by R.M. Sapolsky in the year 2000. The latter study results indicated a hippocampal volume reduction of up to 20% in the depressed group, which increased according to the duration and severity of the depression (Sapolsky, 2000). During depression, the most frequently observed structural changes in the brain include dendritic remodelling of the hippocampal CA3 pyramidal cells and suppression of adult neurogenesis in the dentate gyrus (Smith *et al.*, 1995; Conrad *et al.*, 1999; Fuchs *et al.*, 2004). To date little is known about the apoptotic events that occur in these brain areas, although there is some evidence for apoptosis in depression (Lucassen *et al.*, 2001). It is suggested that stress-induced, lasting decreases in the dentate gyrus cellular turnover could be an important factor in precipitating episodes of depression and might be implicated in the hippocampal volume reduction (Sheline, 1996; Brernner, 1999; Sheline *et al.*, 2003). The latter findings were confirmed during a structural magnetic resonance imaging study performed on the brains of depressed patients which also showed a reduction in hippocampal and amygdala volumes as well as the volumes of gray and white matter of the prefrontal cortex (Drevets, 2000; Mervaala *et al.*, 2000; Moore *et al.*, 2000b). Previous studies also demonstrated that the hippocampal volume loss correlated with the duration of depression and that the volume loss is reversible with antidepressant treatment (Nibuya *et al.*, 1995; Manji *et al.*, 2001; McEwen, 2002). Furthermore, these researchers showed that hippocampal atrophy in major depression worsened with repeated depressed episodes. The above-mentioned findings along with the evidence that hippocampal volume is not reduced after the first episode of major depression, suggest that the hippocampal atrophy is the result of the illness rather than the cause (Manji *et al.*, 2003; Swaab *et al.*, 2005). Therefore it is suggested that therapeutic interventions must act at least in part by augmenting neuronal plasticity or neurogenesis in the brain areas implicated in depression.

2.1.2 The Hippocampus

As mentioned earlier, the hippocampus is vital for learning and spatial memory and is also a prime regulator of the hypothalamus-pituitary adrenal (HPA) axis (Eichenbaum *et al.*, 1996; Dhikav & Anand, 2007). The HPA-axis is the biological system mainly responsible for the regulation of stress response in mammals (Swaab *et al.*, 2005). Cortisol, a glucocorticoid, is the main hormone released in response to stress and its

secretion is regulated by the HPA-axis (O'Brien *et al.*, 1994; Sapolsky, 2000; Fuchs *et al.*, 2004; Thompson *et al.*, 2007). The hippocampus contains the highest number of glucocorticoid receptors and undergoes atrophy due to their over stimulation in several common psychiatric and neurological conditions, as well as during severe or constant exposure to stress (Sapolsky, 2000; Dhikav & Anand, 2007). The reasons for the high vulnerability of the hippocampus to undergo atrophy under stressful conditions are unclear, although a number of identified neurochemical changes may at least be partially responsible for the latter phenomenon (Manji *et al.*, 2003; Sheline *et al.*, 2003). The above-mentioned neurochemical changes include altered glucocorticoid, serotonin, excitatory amino acid (e.g. glutamate) and transporter molecule synthesis (Sapolsky, 2000). Previous research also established that glutamate is the predominant neurotransmitter in the hippocampus (Sapolsky, 2000; Trist, 2000; Arundine & Tymianski, 2004). Therefore hippocampal atrophy may in part be attributed to an increase in glutamate neurotransmission, as is noted during depression, which causes an excessive influx of calcium (Ca^{2+}) into the neuron and ultimately leads to neuronal demise (Sapolsky, 2000).

2.2 Depression

Clinical depression manifests as a depressive disorder which involves the body, mood and thoughts (Barlow & Durand, 2002). A distinction can be made between different forms of depressive disorders, according to their symptoms and severity (Judd, 1997; Solomon *et al.*, 2000). The underlying biochemical events in depression are still unclear and largely unknown. A dysregulation of the CNS involving the neurotransmitters norepinephrine (*l*-NE), serotonin (5-HT) and dopamine (DA) has been suggested as causative factors (Coppen, 1967; Duman *et al.*, 1999; Manji & Duman, 2001). Resent research has also implicated factors such as pro-inflammatory cytokines, alterations of the HPA-axis and decreased neuroplasticity in depressive disorders (Holsboer *et al.*, 1985; Meltzer *et al.*, 1987; Swaab *et al.*, 2005; Dhikav & Anand, 2007).

The mainstream of research done in the field of depression has been focussed on the *l*-NE and 5-HT systems (Owens *et al.*, 1997). Currently the most effective treatment of major and related depression is considered to be an increase in 5-HT neurotransmission (inhibition of 5-HT reuptake); although an increase in *l*-NE neurotransmission may be necessary as well (Owens *et al.*, 1997; Duman *et al.*, 1999;

Manji *et al.*, 2000; Manji & Duman, 2001). Although these current treatments have proven to be relatively effective in the treatment of most cases of depression, they are often plagued by an unfavourable side-effect profile, delayed onset of action and high incidence of treatment resistance (Greden, 2001; Manji *et al.*, 2003).

2.2.1 Types of depression

2.2.1.1 Major depressive disorder

The most commonly diagnosed and most severe depression is called major depressive disorder (Barlow & Durand, 2002; Schloss & Henn, 2004). The DSM-IV (2000) criteria for major depression indicate an extremely depressed mood state that lasts for at least 2 weeks and which includes cognitive symptoms (such as feelings of worthlessness and indecisiveness) and disturbed physical functions (such as altered sleeping patterns, significant change in appetite and weight, or a very notable loss in energy) (American Psychiatry Association, 2000a). These symptoms may persist to the point that even the slightest activity or movement requires an overwhelming effort (Barlow & Durand, 2002; Schloss & Henn, 2004). Major depressive episodes are commonly accompanied by a noticeable decrease in interest and the ability to experience any pleasure from life (Buchwald & Rudick-Davis, 1993). In some cases of major depressive disorder the morbid mood is so intense that the patient is unable to experience normal emotions such as joy, grief and happiness. In such cases it is seen as a sign of improvement if the patients regain their ability to cry (Buchwald & Rudick-Davis, 1993; Barlow & Durand, 2002). In the event of a major depressive disorder being left untreated, a single depressive episode may persevere for approximately 9 months (Eaton *et al.*, 1997; Barlow & Durand, 2002).

2.2.1.2 Dysthymic disorder

Dysthymic disorder is defined as a persistent depressed mood that continues for at least 2 years, during which the patient is not without symptoms for more than 2 months at a time (Barlow & Durand, 2002).

Dysthymic disorder and major depressive disorder differ only in severity, chronicity and the number of symptoms, which are fewer and milder, but last longer in the case of dysthymic disorder (Rush, 1993; Klein *et al.*, 2000). Although the symptoms of

dysthymic disorder are somewhat milder than those of major depressive disorder, they may be present for 20 years or longer (Klein *et al.*, 2000; Barlow & Durand, 2002). The long-term, chronic symptoms of dysthymic disorder do not disable, but prevent a person from functioning or feeling well. Most patients suffering from this type of depressive disorder eventually experience a major depressive episode (Klein *et al.*, 2000).

2.2.1.3 Double depression

Individuals who suffer from both major depressive episodes and dysthymic disorder are said to have "double depression" (Barlow & Durand, 2002). Typically, dysthymic disorder develops first which is then followed by one or more major depressive episodes (Eaton *et al.*, 1997; Klein *et al.*, 2000). Patients who recover from the superimposed major depressive episode have a very high tendency of relapse and recurrence (Barlow & Durand, 2002).

2.2.1.4 Bipolar disorder

This type of depression is also known as manic-depressive illness (Barlow & Durand, 2002). Bipolar I disorder (severe bipolar disorder) is a very recurrent disorder and affects between 0.4 and 1.6% of the population (Akiskal & Pinto, 1999; American Psychiatry Association, 2000b). The key feature of bipolar I disorder is mood episodes that include at least one manic episode (severe high) to alternate with major depressive episodes in an unending emotional roller-coaster ride (Depue *et al.*, 1981; Akiskal & Pinto, 1999; Barlow & Durand, 2002). Bipolar II disorder (mild bipolar disorder) indicates a recurrent course of major depressive episodes and at least one hypo-manic episode. Sometimes the mood switches of bipolar disorders are dramatic and rapid, but most often they are gradual (Akiskal & Pinto, 1999). When in the depressed cycle, an individual may have any or all of the symptoms of a depressive disorder. When in the manic cycle, the individual may be overactive, over-talkative and extremely energetic. Mania often affects the judgement, thinking ability and social behaviour of the patient in such a way that it commonly causes serious problems and embarrassment (Depue *et al.*, 1981; Akiskal & Pinto, 1999). Mania, if left untreated, may worsen to a psychotic state. The lifetime prevalence of bipolar disorder types I and II are estimated to be as high as 5% of the population (Dunner, 2003).

2.2.2 Causes of depression

As mentioned previously, the neurobiological basis of depressive disorder is still illusive. Over the past few years different hypotheses have been postulated in an attempt to elucidate this complex disorder. The best known of these is the monoamine hypothesis of depression (Duman *et al.*, 1999; Manji & Duman, 2001; Katzung, 2004), while the cholinergic hypersensitivity hypothesis (Rubin *et al.*, 1999; Chau *et al.*, 2001), HPA-axis malfunction hypothesis (Appelhof *et al.*, 2006; Johnson *et al.*, 2006; Skynner *et al.*, 2006) and immunological hypothesis (Raju, 1998; Myint & Kim, 2003; Myint *et al.*, 2007) of depression are also recognised, but less promoted. More recently, there has been interest in the fact that major and prolonged depression appears to involve morphological and biochemical changes in the brain that compromise neuroplasticity, forming the basis of the neurodegenerative hypothesis of depression (Duman *et al.*, 1999; Bremner *et al.*, 2000; Manji *et al.*, 2000; Manji & Duman, 2001). The above-mentioned hypotheses and their suspected role in depression are discussed below.

2.2.2.1 Monoamine hypothesis

In the 1950s, soon after the introduction of reserpine, it became apparent that this drug is able to induce depression in patients. In the next few years researchers discovered that the main mechanism of action of reserpine is to deplete monoamine neurotransmitter stores (Coppen, 1967; Katzung, 2004). This discovery gave birth to one of the oldest and best known hypotheses of depression namely the monoamine hypothesis (Sapolsky, 2000; Fuchs *et al.*, 2004; Katzung, 2004). The monoamine hypothesis of depression postulates that depression is caused by defective monoaminergic activity in the brain, for example low levels of monoamine neurotransmitters (Coppen, 1967; Sapolsky, 2000; Baldessarini & Tarazi, 2001). These monoamines include serotonin (5-HT), *l*-norepinephrine (NE) and dopamine (DA) (Sapolsky, 2000; Lambert *et al.*, 2000; Katzung, 2004). Increased DA levels were observed in the brains of suicide victims (carbon monoxide poisoning), however researchers are unsure whether this change was caused by depression or hypoxia (Arranz *et al.*, 1997). A major question arose regarding this hypothesis due to the fact that the pharmacological actions of the classic antidepressant (monoamine oxidase inhibitors, selective serotonin re-uptake inhibitors and tricyclic antidepressants) are prompt, but the clinical effects require weeks to manifest (Baldessarini, 1989; Katzung,

2004). These findings lead to the formulation of newer and more complex depression hypotheses, as will be discussed in the following sections.

Although the monoamine hypothesis are now regarded as far too simplistic to explain antidepressant efficacy, the classic antidepressant treatments are still classified as having their primary actions on the metabolism, reuptake, or selective receptor antagonism of 5-HT, *l*-NE, or both (Katzung, 2004).

2.2.2.2 Cholinergic super sensitivity hypothesis of depression

The cholinergic hypothesis of depression postulates that depression results from super sensitivity of the cholinergic system in the central nervous system. For example, an over stimulation of muscarinic (Daws & Overstreet, 1999; Chau *et al.*, 2001) or nicotinic acetylcholine receptors (Shytle *et al.*, 2002a) have been associated with depression, while antagonism at these receptors has been associated with antidepressive effects. The antidepressants fluoxetine and imipramine, as well as the experimental antidepressant *myo*-inositol, have been shown to modulate muscarinic acetylcholine receptor signalling (Brink *et al.*, 2004), while mecamylamine, a potent nicotinic acetylcholine receptor antagonist, has also been shown to reduce depressive symptoms in patients with major depression (Shytle *et al.*, 2002a; Shytle *et al.*, 2002b). Consequently, it has been proposed that the anticholinergic activity of antidepressant drugs may contribute to their clinical efficacy (Shytle *et al.*, 2002b; Brink *et al.*, 2004). Thus, there is some strong evidence that the cholinergic system may be involved in, and contribute to, the aetiology of depression and its treatment.

2.2.2.3 Hypothalamic-pituitary-adrenal axis hyperactivity hypothesis

Over the last four decades a vast amount of research has been accumulated regarding the role of the hypothalamic-pituitary-adrenal (HPA) axis in the development of depression (Hatzinger, 2000; Duval *et al.*, 2006). Several changes in HPA-axis function have been noted in patients suffering from major depression including hypercortisolism, non-suppression of serum cortisol in the dexamethasone (DEX) suppression test (test for HPA-axis over activity based on the ability of the synthetic glucocorticoid DEX to reduce HPA-activity in normal test subjects) (Carroll *et al.*, 1976; Johnson *et al.*, 2006) as well as an increased release of adrenocorticotrophic hormone (ACTH) and cortisol in response to corticotrophin releasing hormone after DEX administration (Holsboer & Barden, 1996). Some researchers suspect that the non-suppression of cortisol in the

DEX suppression test is due to impaired negative feedback to the pituitary corticotroph resulting in endogenous HPA-axis hyperactivity (Swaab *et al.*, 2005). The hippocampus posses a large amount of both mineralocorticoid and glucocorticoid receptors and is therefore very susceptible to stress, and subsequent high levels of cortisol cause damage in this brain region (Lee *et al.*, 2002). Cortisol induced hippocampal damage include dendritic remodelling of CA3 pyramidal neurons, neuronal loss, reduced dendritic arborisation and decreased adult neurogenesis (Sapolsky *et al.*, 1991; Sapolsky, 2000; Lee *et al.*, 2002). These hippocampal changes were found to be linked to deficits in memory and the development of psychiatric and cognitive symptoms in synthetic glucocorticoid treated rats (Landfield *et al.*, 1981a; Landfield *et al.*, 1981b; Swaab *et al.*, 2005). Furthermore, elevated levels of glutamatergic neurotransmission can be associated with increased glucocorticoid levels, and the increased stimulation of the N-methyl-D-aspartate (NMDA) receptor by glutamate might be responsible for the deleterious effect of glucocorticoids on the brain (McEwen, 1999; McEwen, 2000; Popoli *et al.*, 2002). Several existing antidepressants are able to regulate cortisol secretion (clomipramine and fluoxetine) (Pariante *et al.*, 2003a; Pariante *et al.*, 2003b), and novel antidepressants that inhibit cortisol secretion (mifeprestone, metyrapone and hydrocortisone) (O'Dwyer *et al.*, 1995; Young *et al.*, 2004) have shown promise in clinical trails. Cortisol is present in much higher levels in women (Swaab *et al.*, 2005), as is the risk of developing major depression (Moldin *et al.*, 1991; Weissman *et al.*, 1996; Levinson, 2006), which implicates cortisol and the HPA-axis in the pathophysiology of this disorder.

2.2.2.4 Immunological hypothesis of depression

The relationship between psychiatric illness and the immune system was first observed in 1927 by Wagner-Jauregg (Raju, 1998). Since, a substantial amount of research has been directed at determining the relationship between the immune system and major depression (Connor & Leonard, 1998; Song *et al.*, 1999; Maes, 2001). There is a growing body of circumstantial evidence that major depression is associated with dysregulation of immune mediators such as an inappropriate rise in IL-1β, IL-6, soluble IL-6R, IL-2 and soluble IL-2R (Maes *et al.*, 1993; Sluzewska *et al.*, 1996; Maes *et al.*, 1997). It is currently well documented in the literature that pro-inflammatory cytokines such as interferon-α could induce depression (Beratis *et al.*, 2005; Wichers *et al.*, 2005a), and there is a significant body of evidence that the levels of pro-inflammatory

cytokines, prostaglandin E2 and negative immuno-regulatory cytokines are significantly higher in depressed patients than in non-depressed controls (Beratis *et al.*, 2005; Myint *et al.*, 2005; Wichers *et al.*, 2005a). Pro-inflammatory cytokines such as interleukin-1beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNFα) are also associated with a synergistic induction of neurological and psychiatric manifestations (Plata-Salaman, 1998). The so-called anti-inflammatory cytokines are able to antagonise the actions of the pro-inflammatory cytokines. These include interleukin-1Ra (IL-1Ra), interleukin-4 (IL-4), interleukin-10 (IL-10) and tumour growth factor-beta1 (TGFβ1) (Myint & Kim, 2003). Most of the above-mentioned pro- and anti-inflammatory cytokines can be synthesised and released within the nervous system (Kronfol & Remick, 2000). Although most cytokines in the brain are secreted by astrocytes and microglia, some evidence suggests that, under certain conditions, neurons can also produce cytokines (Freidin *et al.*, 1992). IL-1 has been found in several brain regions, including the hippocampus and specific hypothalamic structures, such as the paraventricular nucleus and arcuate nucleus (Breder *et al.*, 1988). When a human astroglial cell line is stimulated by IL-1 it has been shown to produce colony stimulating factor, TNFα, additional IL-1 and IL-6 (Tweardy *et al.*, 1990; Bethea *et al.*, 1992a; Bethea *et al.*, 1992b). Previous studies have proven that antidepressants are able to decrease the interferon-gamma (INFγ) to IL-10 ratio. The latter observation may be partly due to an increase in anti-inflammatory cytokine IL-10 (Kubera *et al.*, 2001). In addition, the pro-inflammatory cytokine, IL-12, is found to be suppressed by anti-depressive drugs (Kim *et al.*, 2002).

Recently, pro-inflammatory cytokines have been found to display profound effects on the metabolism of brain 5-HT, DA and *l*-NE (Dunn *et al.*, 1999). During clinical trails a significant decrease in serum tryptophan (the precursor of 5-HT) concentrations were observed in subjects receiving IL-2 and IFNα (Brown *et al.*, 1989; Brown *et al.*, 1991). Cytokine-induced tryptophan depletion is brought about by two mechanisms. Firstly, increased stimulation of the brain by pro-inflammatory cytokines causes behavioural changes, such as altered sleep patterns and decreased appetite (may even lead to anorexia). Since tryptophan levels are strongly regulated by dietary intake (Smith *et al.*, 1997), a cytokine-induced reduction in appetite may drastically reduce tryptophan levels (Plata-Salaman & Ilyin, 1997; Reichenberg *et al.*, 2001)\. Secondly, pro-inflammatory cytokines induce tryptophan depletion by enhancing the activity of indoleamine-2,3-dioxygenase (IDO), the first enzyme in the kynurenine pathway (Carlin *et al.*, 1987;

Carlin *et al.*, 1989; Mellor & Munn, 1999). IDO converts tryptophan to kynurenine and is also responsible for the catabolism of 5-HT, melatonin, *l*-hydroxytryptophan and *l*-5-hydroxytryptophan (Hirata & Hayaishi, 1971). IDO is widely distributed in the intestinal tissues, lungs, placenta and brain (Heyes *et al.*, 1993; Mellor & Munn, 1999). It has also been observed that IFNγ, TNFα, IL-1 and IL-2 can increase the activity of IDO, whereas IL-4 (anti-inflammatory cytokine) inhibits IDO (Musso *et al.*, 1994; Daubener & MacKenzie, 1999; Babcock & Carlin, 2000; Currier *et al.*, 2000; Wichers *et al.*, 2005b).

Kynurenine, formed from the metabolism of tryptophan, is metabolised to kynurenic acid, quinolinic acid and anthranilic acid by kynurenine aminotransferase, kynurenine 3-hydroxylase and kynureninase, respectively (Perkins & Stone, 1982; Bender & McCreanor, 1982). Both kynurenine 3-hydroxylase and kynureninase are activated by IFNγ and TNFα (Chiarugi *et al.*, 2001). Anthranilic acid is metabolised to quinolinic acid, an excitotoxic NMDA receptor agonist (Schwarcz *et al.*, 1983; Chiarugi *et al.*, 2001). Kynurenic acid, another product formed through the metabolism of kynurenine, is an antagonist on all three ionotropic excitatory amino acid receptors (NMDA, AMPA/kainate, and high affinity kainate receptors) (Perkins & Stone, 1982; Siegel *et al.*, 1995). The protective effect of kynurenic acid against the excitotoxic effect of quinolinic acid has been detected in neuronal cell cultures (Kim & Choi, 1987). However, a study on the blood concentration of quinolinic acid in patients suffering from different major psychiatric and neuro-degenerative disorders failed to indicate an elevated quinolinic acid concentration (Heyes *et al.*, 1992). An association has been proposed to exist between a misbalance of kynurenic acid and quinolinic acid brain levels, and the general neuroprotective-neurodegenerative balance in the brain of patients with chronic depression (Myint & Kim, 2003). Moreover, recent animal and human studies have shown that psychological stressors can activate the inflammatory response system (Maes, 1999; Leonard & Song, 1999; Maes, 2001)

All the above-mentioned products are formed as a result of cytokine-serotonin interaction via the IDO enzyme, which plays a pivotal role in the depletion of the 5-HT precursor tryptophan in depression (Myint & Kim, 2003).

2.2.2.5 Neurodegenerative hypothesis of depression

The most recent depression hypothesis is the neurodegenerative hypothesis. This hypothesis proposes that major depression is a consequence of the imbalance between neuroprotective and neurodegenerative processes/substances in the brain (Myint *et al.*, 2007), and unifies previous hypotheses of depression (***Figure 2-2***).

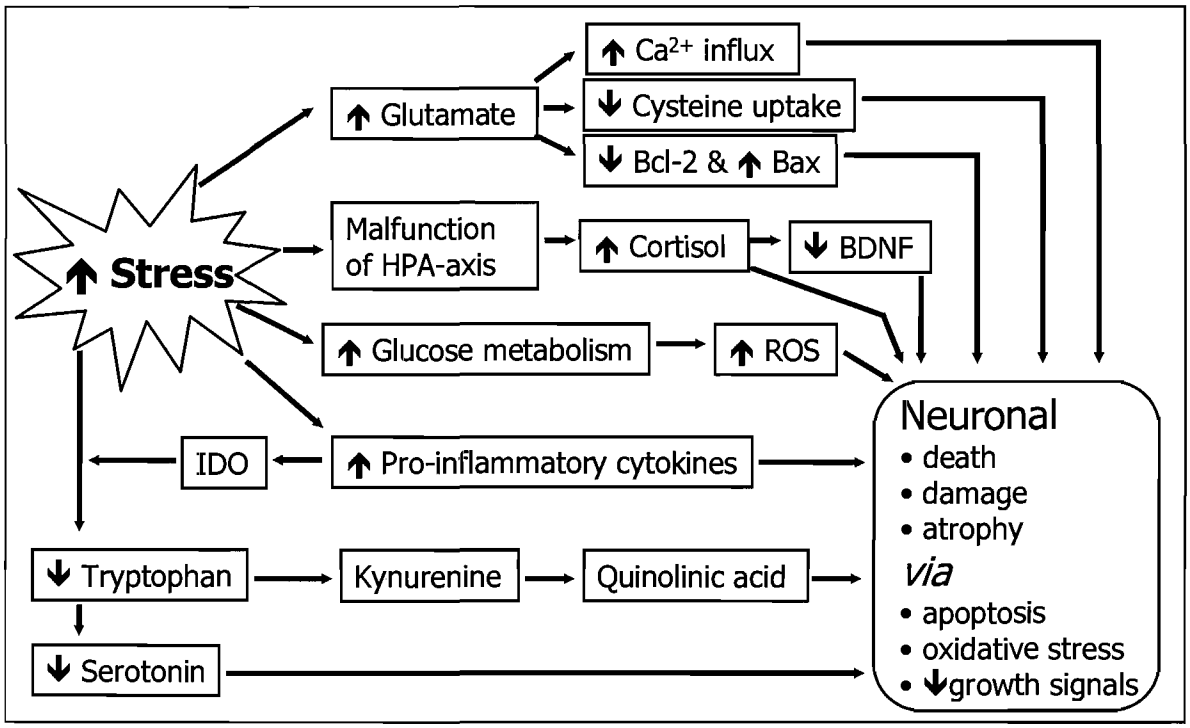


Figure 2-2: *Intertwinement of the suspected pathways and mediators in the development of depression as explained from a neurodegenerative perspective.*

The mainstream of research regarding this hypothesis has been focussed on the reduction of neurotrophic factors such as brain derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response element binding protein (CREB) in the brain of depressed patients (Smith *et al.*, 1995; Tao *et al.*, 1998; Conti *et al.*, 2002). Studies indicated that prolonged and acute stress is able to significantly reduce the expression of BDNF in the hippocampal pyramidal cells and dentate gyrus of rodents (Smith *et al.*, 1995). A reduction in the neurotrophic transcription factor CREB has also been noted during depression (Walton & Dragunow, 2000; Koch *et al.*, 2003). CREB is responsible for the transcription of BDNF expression *in vivo* and *in vitro* (Tao *et al.*, 1998; Conti *et al.*, 2002). In *in vitro* animal and human studies on mechanisms of antidepressant action have indicated that chronic administration of almost all antidepressant drug types are able to increase CREB and BDNF expression (Nibuya *et al.*, 1996; Thome *et al.*, 2000). A substantial number of antidepressants can also prevent a stress-induced

decrease in the expression of CREB and BDNF (Chen *et al.*, 2001a). The *in vivo* administration of BDNF (Shirayama *et al.*, 2002), as well as the stimulation of the expression of CREB by injecting a CREB encoding virus into the hippocampal dentate gyrus of rodents, both produced antidepressant-like effects in the study animals (Chen *et al.*, 2001a).

While a decrease in the expression of CREB and BDNF and stress is the main focus of the neurodegenerative hypothesis, the malfunction of the HPA-axis, increased pro-inflammatory cytokines and increased glutamate neurotransmission are also believed to be involved, and can be linked to the expression of CREB and BDNF. Except for the fact that HPA-axis malfunction increases cortisol levels (which is mainly responsible for the decreased BDNF and CREB levels in depression (Smith *et al.*, 1995; Tao *et al.*, 1998; Conti *et al.*, 2002)), increased cortisol can also damage neurons by inducing oxidative stress (Himi *et al.*, 2003). High cortisol levels increases brain activity by directly influencing neurotransmission (Smith *et al.*, 1995; McEwen, 1999; McEwen, 2000). This increases the demand of energy in the brain, leading to increased cellular metabolism of glucose and the formation of free radicals as by-products (Coyle & Puttfarcken, 1993; Hensley *et al.*, 2000; Floyd & Hensley, 2002). The amount of free radicals then exceeds the anti-oxidant defence capacity of the brain, and damages the neurons through oxidative stress (Halliwell & Cross, 1994; Halliwell, 1994; Marnett *et al.*, 2003). Increased cortisol also leads to increased glutamate neurotransmission (Reagan *et al.*, 2004). Excessive glutamate (endogenous NMDA receptor agonist) can induce neuronal dysfunction, cell damage or even cell death by causing excessive Ca^{2+} influx through NMDA receptor channels (Sapolsky, 2000). Elevated levels of extracellular glutamate inhibits cysteine uptake, resulting in the induction of oxidative stress and neuronal damage (Coyle & Puttfarcken, 1993). The balance between the intracellular anti-apoptotic protein B-cell CLL/lymphoma 2 (Bcl-2) and the pro-apoptotic protein Bcl2-associated X protein (Bax) can also be disturbed by high glutamate levels. This disturbance causes the amount of Bcl-2 to decrease and Bax to increase, which ultimately leads to neuronal death via apoptosis (Tsujimoto, 1998; Chao & Korsmeyer, 1998; Xia *et al.*, 1999).

Chronic stress may also cause an inappropriate rise in pro-inflammatory cytokines (Connor & Leonard, 1998; Maes, 1999). Pro-inflammatory cytokines enhance the activity of the IDO enzyme leading to an excessive formation of quinolinic acid, where

the latter is a neurotoxic metabolite with an agonistic action at NMDA receptors (Bender & McCreanor, 1982; Chiarugi *et al.*, 2001). Pro-inflammatory cytokines can also bind to cellular receptors and activate proteins that mediate cellular demise (Boatright *et al.*, 2003; Song *et al.*, 2003).

While the effect of antidepressants on neuroprotective and neurotrophic factors is not immediate, taking some time to readjust the down regulated neuroprotective and neurotrophic factors to their "normal" expression levels, this hypothesis can also explain the delayed onset of action of antidepressants (Duman *et al.*, 1999; McEwen, 1999; Kronfol & Remick, 2000; McEwen, 2000; Levinson, 2006; Kosten *et al.*, 2007).

2.2.3 The role of glutamate in depression

Glutamate (L-glutamic acid) is an excitatory amino acid and is present in high concentrations throughout the CNS (Trist, 2000; Blaabjerg *et al.*, 2003; Greenwood & Connolly, 2007). It has been shown in previous studies that most neurons are strongly excited by this amino acid (Sapolsky, 2000; Rauen & Wiessner, 2000; Greenwood & Connolly, 2007). Glutamate causes excitation by stimulation or activation of metabotropic and ionotropic receptors (Sapolsky, 2000). The ionotropic receptors can be classified as receptors which directly gate cation-selective channels. The latter receptor type can also be further divided into three subtypes, based on the selective agonists that were first used for their identification (Trist, 2000). These receptor subtypes include kainite receptors, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors and NMDA receptors (Bolanos *et al.*, 1998; Trist, 2000; Blaabjerg *et al.*, 2003).

In the hippocampus, memory formation involves excitatory glutamatergic neurons, especially of the NMDA subtype (Riedel *et al.*, 2003). This receptor subtype is highly permeable to calcium ions and has slow gating kinetics (Trist, 2000). These characteristics enable the NMDA receptor to mediate plastic changes in the brain, such as learning and memory (McLeod *et al.*, 2001).

Although the NMDA receptor plays an important role in learning and memory, excessive stimulation of this receptor by an agonist such as glutamate can induce neuronal dysfunction, cell damage or even cell death (McLeod *et al.*, 2001; Arundine & Tymianski, 2004). Calcium ions have been shown to regulate the synthesis and release

of neurotransmitters, neuronal excitability, cytoskeletal remodelling, and long-term neuroplastic events (Galeotti *et al.*, 2006).

Neuronal death, due to excessive glutamate stimulation, is termed excitotoxicity (Blaabjerg *et al.*, 2003). Glutamate receptor-mediated excitotoxicity may result from acute insults on the neurons such as oxidative injury and trauma (Garcia de Arriba *et al.*, 2006). An increase in glutamate transmission is also noted is some psychological disorders such as depression (Sapolsky, 1996). During this type of neurotoxicity the excessive NMDA receptor stimulation leads to a major influx of calcium ions into the cell followed by a cascade of neurotoxic events which ultimately leads to apoptosis of the implicated neuron (Trist, 2000; McEwen & Magarinos, 2001; Arundine & Tymianski, 2004).

NMDA receptor overstimulation by glutamate can also lead to apoptotic cell death by inhibiting cysteine uptake (McEwen & Magarinos, 2001). Cysteine is a critical component for the synthesis of intracellular glutathione. Because of the redox instability of extracellular cysteine, this substance is primarily present in an oxidized state. Cysteine is taken up by cells via the cysteine/glutamate transporter system. Thus, elevated levels of extracellular glutamate competitively inhibit cysteine transport, which leads to depletion of intracellular glutathione. Depletion of intracellular glutathione results in decreased cellular antioxidant capacity, which leads to the accumulation of ROS and ultimately apoptotic cell death (Shih *et al.*, 2006).

NMDA receptor activation also activates the nitric oxide (NO) pathway which has been implicated in the neurobiology of depression (McLeod *et al.*, 2001; Harvey *et al.*, 2006) as well as in the mechanism of action of antidepressant agents (Wegener *et al.*, 2003). NO is released following chronic stress (Harvey *et al.*, 2004), has been strongly implicated in neurodegenerative pathways (McLeod *et al.*, 2001), and is implicated in the degenerative pathology of depression (Harvey *et al.*, 2003; Harvey *et al.*, 2006).

2.3 Apoptosis

The term apoptosis was first described by Kerr and Wyllie in 1972, to delineate a morphologically distinctive mode of cell death responsible for cell loss within living tissues (Kerr *et al.*, 1972). In other words, apoptosis is defined as "normal" or "programmed" cell death, which is the process whereby unwanted or useless cells are

eliminated during development and other biological processes (Thompson, 1995; Sherwood, 2001a; Delhalle *et al.*, 2003; Shi, 2004). This process of cell death is energy dependent and can be initiated by a variety of intra- or extracellular stimuli (Kerr *et al.*, 1972).

Necrosis, on the other hand, is defined as accidental cell death via physiological processes caused by exposure to a serious physical or chemical insult (Schwartzman & Cidlowski, 1993). This means the necrotic cells are not actively involved in the initiation of their own demise. Thus, in contrast with the carefully orchestrated process of apoptosis, necrosis can be seen as an unorganised type of cellular demise.

The process of apoptosis involves a dynamic interplay of several molecules with up- and down-regulatory properties, although it is unlikely that activation or inactivation of a single component will alter the ultimate fate of the cell.

Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen, 1993; Fernandez-Flores *et al.*, 2002). The stereotypical pattern of morphological events which has been described in the majority of apoptotic cells includes cell shrinkage, membrane blebbing, nuclear condensation and fragmentation, and finally the formation of sealed membrane-bound vesicles called apoptotic bodies (Kerr *et al.*, 1972; Waibel *et al.*, 2007). These apoptotic bodies ensure that membrane integrity is maintained prior to phagocytosis. Changes in several cell surface molecules also ensure that apoptotic cells are immediately recognised and phagocytosed by their neighbouring cells (Savill *et al.*, 1989). The result is that many cells can be deleted from tissues in a relatively short time and therefore there is little remains in conventional microscopic sections (Wyllie, 1997). Due to this efficient mechanism by which apoptotic cells are removed, no inflammatory response is elicited (Savill *et al.*, 1989). The above-mentioned cellular process is in stark contrast with the swelling membrane lyses and inevitable inflammatory response observed during necrosis (Krahenbuhl & Tschopp, 1991).

The following flow chart depicts the morphological changes of the cell from early to late stage apoptosis, as can be detected by electron microscopy.

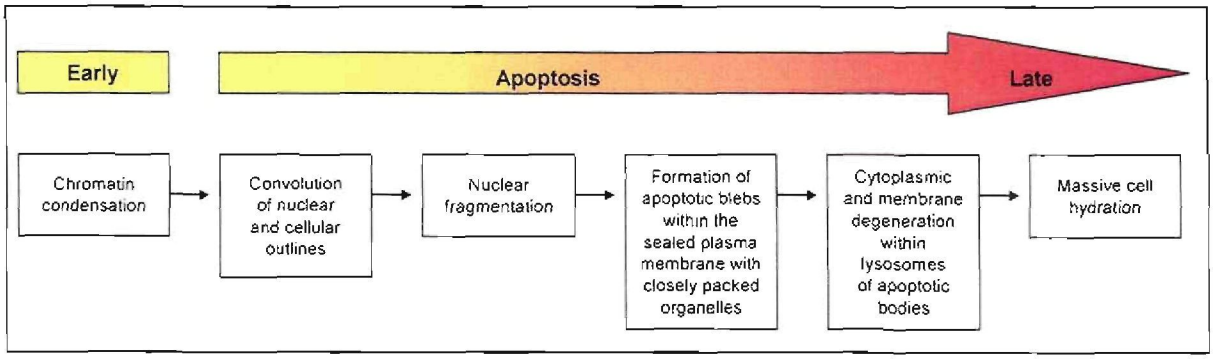


Figure 2-3: *The intracellular alterations as can be observed via electron microscopy from early to late stage apoptosis*

Recent studies question the role of apoptosis as a key contributor to many degenerative illnesses such as Alzheimer's disease, Parkinson's disease and recently also major depression (Sheline, 1996; Bremner *et al.*, 2000; Sapolsky, 2000; Culmsee & Mattson, 2005). Thus, over the past 30 years extensive examination has been done to enhance the screening methods for apoptosis. Therefore, rt^2-PCR apoptosis screening is included into this study in an attempt to determine the role of programmed cell death in the protective ability of the study therapeutics.

2.3.1 Apoptotic pathways

Scientists now recognise that most, if not all, physiological cell deaths occur by means of apoptosis (Wyllie, 1997). Consequently, in the last few years, interest in apoptosis has increased greatly. Great progress has been made in the understanding of the basic mechanisms of apoptosis and the genes involved, such as apoptosis triggered by intracellular signals; death activators such as TNF-α; lymphotoxin and Fas ligand (FasL) binding to cell surface receptors; reactive oxygen species (ROS); factors such as xenobiotics, growth factor withdrawal, ionizing radiation, viral infection and pro-inflammatory cytokines (Halliwell & Cross, 1994; Delhalle *et al.*, 2003; Borner, 2003).

Although all of the above-mentioned factors are able to induce apoptosis, the execution step usually involves one of two signalling pathways (Kluck *et al.*, 1997; Delhalle *et al.*, 2003), namely the:

- death receptor-mediated pathway (extrinsic pathway), or the
- mitochondria-driven pathway (intrinsic pathway).

Both of the above-mentioned pathways make use of the activation of cysteinyl-aspertate-specific proteinases (caspases), which are mainly responsible for the demise

of the cell undergoing apoptosis (Cryns & Yuan, 1998; Ashkenazi & Dixit, 1998; Desagher & Martinou, 2000; Degterev *et al.*, 2003; Boatright *et al.*, 2003).

2.3.1.1 The death receptor-mediated and mitochondria-driven pathways

When the process of apoptosis is activated through the death receptor-mediated pathway it is also referred to as Type 1 apoptosis (Ashkenazi & Dixit, 1998; Kronke & Adam-Klages, 2002). This pathway can be activated via the binding of the TNF ligand to the TNF-receptor 1, TRAIL ligand (TNF-related apoptosis inducing ligand) to the DR4 and DR5 receptors, or FasL ligand to the Fas receptors (Ashkenazi & Dixit, 1998). The 'death receptors' (***Figure 2-4***) can initiate apoptosis by activating a signal transduction cascade, that leads to caspase-dependant apoptosis (Boatright *et al.*, 2003; Song *et al.*, 2003).

The mitochondria-driven pathway (***Figure 2-4***) is initiated by intrinsic apoptotic signals received by the mitochondria, typically via the activity of Bcl-2 family members (Borner, 2003; Deshpande & Kehrer, 2006). These act as sensors, leading to the release of various proteins (including cytochrome c, Smac and apoptosis inducing factors) from the mitochondrial intermembrane space (Kluck *et al.*, 1997; Du *et al.*, 2000; Wang, 2001).

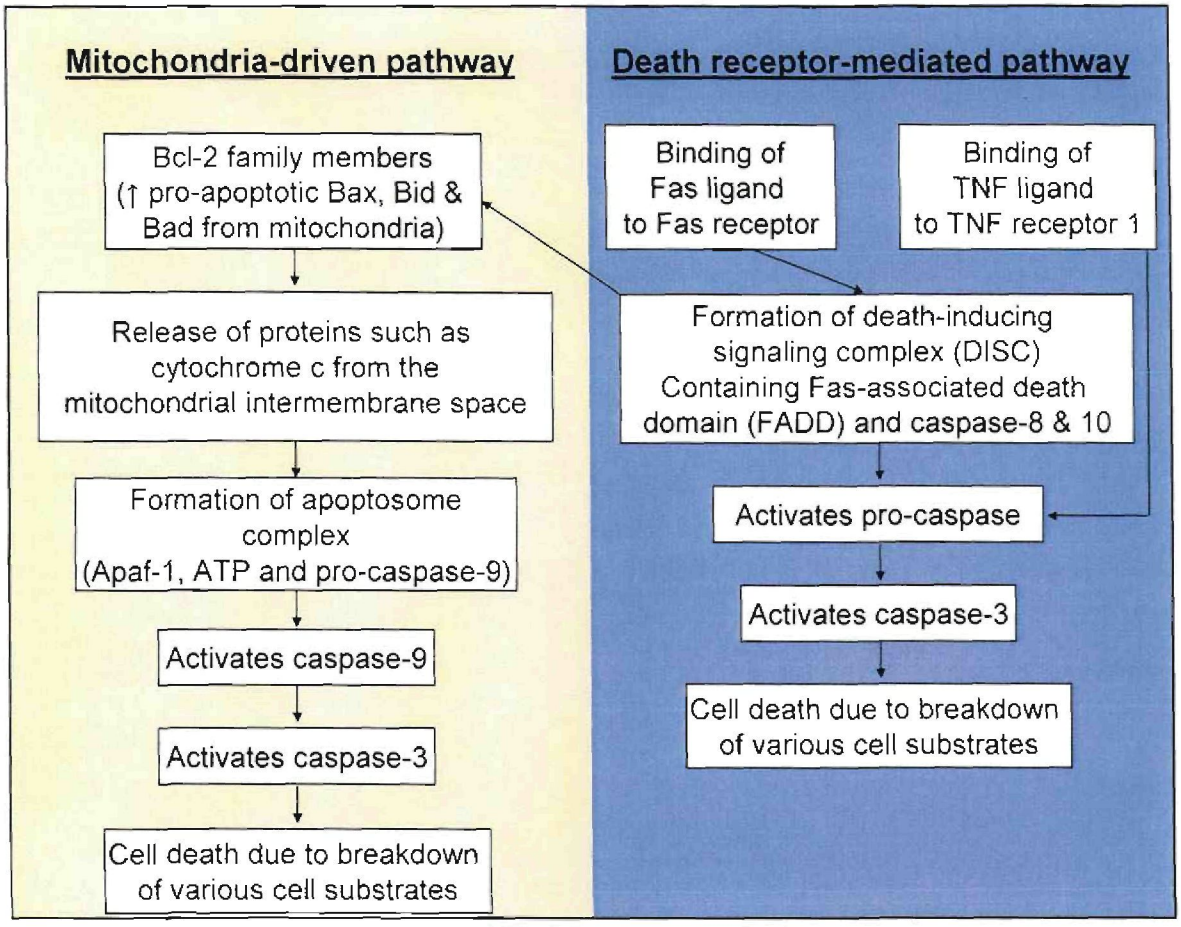


Figure 2-4: *The two signalling pathways by which a cell can be stimulated to undergo apoptosis.*

The death receptor pathway *can be initiated by the binding of the cytokine TNF, or the transmembrane protein Fas ligand, to their respective receptors. The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to the activation of caspases (apoptotic enzymes). The interaction between Fas and FasL results in the formation of a death-inducing signalling complex (DISC), which contains the Fas-associated death domain (FADD) protein, as well as caspases 8 and 10 (Boatright et al., 2003; Song et al., 2003). This leads to the activation of the pro-caspases, which then activates the effector caspases (e.g. caspase-3). The activated effector caspases are then responsible for the breakdown of various substrates in the cell, resulting in the typical morphological features of apoptosis (Budihardjo et al., 1999). Another possibility is that the DISC elicits a feed-back loop, causing an increased release of pro-apoptotic factors (Bax, Bid and Bad) from the mitochondria, and an amplification of caspase-8 activation.* ***The mitochondria-driven pathway*** *is initiated by intrinsic apoptotic signals received by the mitochondria, typically via the activity of Bcl-2 family members (Borner, 2003). These act as sensors, leading to the release of various proteins (including cytochrome c) from the mitochondrial intermembrane space (Kluck et al., 1997; Du et al., 2000). Cytochrome c release causes the recruitment of the apoptosome complex (Apaf-1, ATP and pro-caspase-9) causing the activation of caspase-9 (Zou et al., 1997; Saleh et al., 1999). The activated caspase-9 then cleaves and activates the effector caspase, caspase-3, which is ultimately responsible for cellular apoptosis.*

2.3.2 Specific mediators of apoptosis and their relevance to depression

There is a growing body of evidence indicating that the pathological changes observed in the hippocampus of depressed patients can be attributed to altered pro- and anti-apoptotic gene expressions (Duman *et al.*, 1997; Bremner *et al.*, 2000). Indeed, Lucassen and colleagues (2001) have provided some evidence for a role for apoptosis in depression (Lucassen *et al.*, 2001). The key apoptotic elements that have been identified to be involved in depression are briefly discussed below.

2.3.2.1 Nuclear factor-kappa beta

Background:

Nuclear factor-kappa beta (NF-κβ) is a family of dimeric transcription factors present in most cell types of vertebrates as homo- and heterodimers of five structurally related NF-κβ proteins (Baldwin, Jr., 1996; Ghosh *et al.*, 1998; Andela *et al.*, 2005). As in other organ systems, functional NF-κβ complexes are present in essentially all cell types of the nervous system, including neurons, astrocytes, microglia and oligodendrocytes (Mattson & Meffert, 2006). The most commonly expressed sub-units in the nervous system include p50, p65 and inhibitory κβα (I κβα) protein as well as the recently described neuronal κβ factor (Moerman *et al.*, 1999; Mattson & Camandola, 2001). NF-κβ is widely known for its ubiquitous roles in inflammation and immune responses, as well as for its control of cell division and apoptosis (Baeuerle & Baltimore, 1996; Baldwin, Jr., 1996). These roles are apparent in the nervous system, but neurons and their neighbouring cells employ the NF-κβ pathway for distinctive functions as well, ranging from development to the coordination of cellular responses to injury of the nervous system and to brain-specific processes such as the synaptic signalling that underlies learning and memory (Mattson & Camandola, 2001; Kaltschmidt *et al.*, 2002; Andela *et al.*, 2005).

Mechanism of action:

In un-stimulated neuronal cells, NF-κβ (with the prototypical p65 and p50 sub-unit) is sequestered in the cytoplasm in an inactive form via interaction with the akrynin repeats of the inhibitory κβα protein (Iκβα) (Beg *et al.*, 1993; Ghosh *et al.*, 1998; Gerondakis *et al.*, 1999; Mattson & Camandola, 2001). The Iκβα proteins are able to bind to, and

mask the nuclear localization signal (NLS) of NF-κβ (Henkel *et al.*, 1993) . Upon stimulation with various agonists such as bacterial products, pro-inflammatory cytokines (TNF-α), viral proteins, glutamate, reactive oxygen species (such as H_2O_2) and radiation, Iκβα is rapidly phosphorylated to expose the NLS of NF-κβ (Mattson & Camandola, 2001; Andela *et al.*, 2005). The latter occurrence causes a rapid translocation of NF-κβ to the nucleus, where it binds to target decanucleotide sequences (Karin, 1999). This binding step causes the activation of several genes that promote neuronal survival, including those encoding manganese superoxide dismutase (Mn-SOD; a mitochondrial antioxidant enzyme that has been shown to be neuroprotective), inhibitor of apoptosis proteins (IAPs), pro-inflammatory cytokines, adhesion molecules and nerve growth factors (NGF) and anti-apoptotic genes (Bcl-2) (Carter *et al.*, 1996; Mattson & Camandola, 2001). After the NF-κβ-dependant nuclear alterations have been executed, Iκβα enters the nucleus and enhances NF-κβ removal from DNA and takes it back to the cytoplasm, thus restoring the cytoplasmic pool of NF-κβ (Arenzana-Seisdedos *et al.*, 1995).

Relevance to depression:

Many signal transduction molecules and pathways that regulate neuronal survival and growth during nervous system development appear to be involved in various neurodegenerative conditions (Mattson & Camandola, 2001; Kaltschmidt *et al.*, 2002). During nervous system development NF-κβ is activated in growing neurons by means of neurotrophic factor stimulation, which then results in the expression of genes involved in cell differentiation and survival (Mattson, 2005; Andela *et al.*, 2005). In contrast with this, NF-κβ is activated in the synapses of mature nervous systems in response to excitatory neurotransmission (especially in the hippocampus) and may play a pivotal role in processes such as learning and memory (Mattson & Camandola, 2001; Mattson, 2005; Mattson & Meffert, 2006) and in the response to stress. The former finding may be a reason for the relatively high activity of NF-κβ in brain tissue compared to other tissues.

During depression an increase in excitatory (glutamate) neurotransmission is observed in the hippocampus which overloads the protective capacity of NF-κβ and causes pathological changes in this brain region (Harrison, 2002; Fuchs *et al.*, 2004). A study conducted on cultured rat hippocampal neurons provided evidence that NF-κβ activation can modulate the sensitivity of neurons to glutamate stimulation, possibly by

affecting the expression of regulation of the NMDA and/or AMPA receptors (Furukawa & Mattson, 1998). Therefore, the neuroprotective mechanism of antidepressant therapeutics might involve NF-κβ gene up-regulation which might desensitise neurons to the damaging effects of excitotoxic glutamate stimulation (Furukawa & Mattson, 1998; Mattson, 2005).

2.3.2.2 Cyclic adenosine monophosphate response element binding protein (CREB)

Background:

CREB is a 43-kDa nuclear transcription factor which is a member of the beta leucine zipper family of transcription factors (Finkbeiner, 2000). Other members of this family of transcription factors are CREM (cAMP response modulatory protein) and activating transcription factor 1 (ATF1) (Molina *et al.*, 1993; Mayr & Montminy, 2001). All of the above-mentioned transcription factors regulate the expression of a wide variety of genes that contain cAMP-response elements (CREs) within their promoters (Molina *et al.*, 1993; Stehle *et al.*, 1993; Dawson & Ginty, 2002). CREB has been shown to have important functions in differentiation of numerous target tissues and cell types, including adipocytes, vascular smooth muscle cells, neurons and cardiac myocytes as well as being a pivotal regulator of apoptosis (Finkbeiner, 2000; Reusch & Klemm, 2003)..

Mechanism of action:

CREB regulates the expression of genes through the coordinated action of transcriptional co-activators, such as the CREB-binding protein (CBP) (Bonni *et al.*, 1999). The CREB family of transcription factors are activated by phosphorylation (Dawson & Ginty, 2002). The activated transcription factors have critical roles in the response to many signal transduction cascades activated by substances such as hormones, growth factors, bursts of synaptic activity and other cellular stimuli. Intracellular signalling pathways, including Ras/MAPK, Ca^{2+}/CaMK, P13K/Akt and cAMP/PKA, are known to promote CREB-dependant transcription (Bonni *et al.*, 1999). CREB is also responsible for the regulation of BDNF expression in response to antidepressant treatment (Nibuya *et al.*, 1996; Dias *et al.*, 2003).

Relevance to depression:

Recent studies determined that CREB plays a critical role in neuronal plasticity and long-term memory in the nervous system, and that hippocampal neurons are particularly

vulnerable to CREB deficiency (Dawson & Ginty, 2002). During depression a significant decrease in CREB expression can be noted in the hippocampus as determined via post-mortem studies (Smith *et al.*, 1995; Nibuya *et al.*, 1995). Because of the sensitivity of the hippocampus to decreased CREB expression, a high apoptotic tendency develops in this brain region in response to CREB down-regulation (Dawson & Ginty, 2002). Additionally, CREB expression seems to be enhanced by the administration of several antidepressants (Nibuya *et al.*, 1996; Dias *et al.*, 2003). The latter increase in CREB is observed with chronic but not acute antidepressant treatment, which causes an enhancement in monoaminergic activation of G-protein-coupled receptors, which leads to an up-regulation of adenylyl cyclase and cAMP. Protein kinase A then mediates the phosphorylation and activation of CREB, activated CREB then proceeds to enhance the expression of the neurotrophic factor BDNF (Schloss & Henn, 2004). These findings are supported by a study conducted by Koch and colleagues in which it was found that antidepressants are able to increase CREB-phosphorylation without the induction of apoptosis depending on the concentration and duration of the treatment. They assumed that antidepressant treatment induces CREB-phosphorylation via signal transduction pathways that are different from those inducing apoptosis (Koch *et al.*, 2003). These findings lead to the hypothesis that the transcription factors of neurotrophins, such as CREB, might play a key role in the symptomatology and treatment of depression (Duman, 2002; Castren, 2004).

2.3.2.3 Caspases

Background:

The caspases are a group of proteases with a crucial cysteine residue that cleaves other proteins and are considered essential in apoptosis, while some caspases are also required for the maturation of cytokines in the immune system (Cryns & Yuan, 1998; Thornberry & Lazebnik, 1998; Degterev *et al.*, 2003). These enzymes cleave protein molecules after an aspartic residue, which is unusual among proteases. The name "caspases" are derived from the enzymes' characteristic molecular function (cysteine-aspartic-acid-proteases) (Fernandez-Flores *et al.*, 2002).

There are two types of caspases, namely the initiator caspases and the effector caspases. Initiator caspases (e.g. caspase-8 and caspase-9) are responsible for the cleaving of pro-forms of effector caspases, thereby activating them (Budihardjo *et al.*,

1999). The effector caspases (e.g. caspase-3 and caspase-7) in turn cleave other protein substrates within the cell, resulting in the apoptotic process (Tang *et al.*, 2000; Kulik *et al.*, 2001).

Mechanism of action ('The caspase cascade'):

The caspase family is regulated at a post-translational level, ensuring their rapid activation in response to appropriate stimuli. As mentioned previously, the caspases are synthesised as inactive pro-enzymes, which contain a prodomain consisting of a small and a large sub-unit (Lazebnik *et al.*, 1995). The latter domains enable the caspases to interact with other molecules that regulate their activation. These molecules respond to stimuli and thereafter cause clustering of the initiator caspases, allowing them to auto-activate (i.e. cleave each other to produce the active enzyme) and proceed with the activation of the effector caspases (Shi, 2004).

The caspase cascade can be activated by Granzyme B, a substance released by cytotoxic T lymphocytes. Granzyme B is known to activate caspase-3,-7,-8 and -10 (Schimmer, 2004). Stimulation of death receptors such as FAS, TRAIL and TNF, can also activate caspase-8 and -10 (Kronke & Adam-Klages, 2002). Caspase-9 can also be activated by a apoptosome, which is regulated by cytochrome c and the Bcl-2 family (Adams & Cory, 2002). A positive feedback system ensures that the cell undergoes apoptosis after the cascade was initiated. For example, apoptosome-activated caspase-9 cleaves and activates caspase-3 (Adams & Cory, 2002). Besides cleaving its target proteins, the activated caspase-3 will also cleave more caspase-9 which in turn will activate more caspase-3, thus a positive feedback system is created (Budihardjo *et al.*, 1999).

Some of the final targets of the caspases include nuclear lamins (Lazebnik *et al.*, 1995), DNA repair enzymes such as poly-ADP-ribose polymerase (PARP) (Lazebnik *et al.*, 1994) and cytoskeletal proteins such as actin (Mashima *et al.*, 1995), fodrin (Cryns *et al.*, 1996) and gelsolin (Kothakota *et al.*, 1997). The exact contribution that the cleavage of the caspase substrates makes to the biochemistry and morphology of apoptosis is unclear. However, fragmentation of DNA during apoptosis is caused in part by an enzyme known as caspase activated DNase (CAD). Under normal cellular circumstances CAD exists in an inactive complex with the inhibitor of CAD (ICAD). During apoptosis ICAD is cleaved from CAD by caspase-3, resulting in the release of

CAD. CAD then enters the nucleus of the cell and fragments the DNA, causing the characteristic "DNA ladder" seen in apoptotic cells (Sakahira *et al.*, 1998).

Relevance to depression:

The proposed role of apoptosis in the neurodegenerative hypothesis of depression is widely discussed in the literature (Manji & Duman, 2001; Fuchs *et al.*, 2004). The noted decrease in neuronal survival signals in depressed patients may ultimately lead to neuronal demise via apoptosis (Smith *et al.*, 1995; Manji *et al.*, 2003). Caspases are the effector proteins in the apoptotic pathway, and therefore, if the reduction in cell survival signals is responsible for the neuronal demise, an increase in caspase expression and activation will be noticeable (Leist & Jaattela, 2001).

2.3.2.4 B-cell lymphoma/leukemia-2 (Bcl-2) gene family

Background:

The prototype of the Bcl-2 gene family, B-cell lymphoma-2 (Bcl-2), was described in 1988 and is seen as a cell death antagonist (Mock *et al.*, 1988). This gene family consists of pro- and anti-apoptotic mediators (Liu & Huang, 2001). Bcl-2 associated X protein (Bax), is a death agonist member of the Bcl-2 protein family. This protein was discovered by co-immunoprecipitation with Bcl-2 and was also the first Bcl-2 homologue to be identified. The Bax-gene shares a 21% identity and 43% similarity with Bcl-2 (Liu & Huang, 2001; Borner, 2003). Cell susceptibility to apoptosis is thought to be largely influenced by the relative ratios of pro-apoptotic and anti-apoptotic Bcl-2 family members (Oltvai *et al.*, 1993; Yang *et al.*, 1995; Sattler *et al.*, 1997). Up to date 15 mammalian Bcl-2 family members have been identified, which can be divided into the following three subfamilies (Adams & Cory, 1998; Tsujimoto, 1998; Borner, 2003):

- Bcl-2 subfamily (anti-apoptotic): Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1.
- Bax subfamily (pro-apoptotic): Bax, Bak and Bok.
- BH3 subfamily (pro-apoptotic): Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL.

The anti-apoptotic proteins of the Bcl-2 family are initially integral membrane proteins found in the mitochondria, endoplasmic reticulum (ER) or nuclear membrane. In contrast with this, a substantial amount of the pro-apoptotic proteins reside in the cytosol or cytoskeleton prior to a death signal (Oltvai *et al.*, 1993; Adams & Cory, 1998; Chao & Korsmeyer, 1998; Borner, 2003)

Mechanism of action:

The Bcl-2 family members are able to regulate the release of cytochrome c, therefore playing a pivotal role in mitochondrial mediated apoptosis (Adams & Cory, 1998; Chao & Korsmeyer, 1998).

Bax and the anti-apoptotic family member Bcl-2 are in a balance which inhibits the action of both these proteins (Oltvai *et al.*, 1993; Yang *et al.*, 1995; Sattler *et al.*, 1997). A change in the ratio of Bax to Bcl-2 can ultimately lead to cellular demise or survival. If the ratio of Bax to Bcl-2 increases, cell death is executed by means of mitochondrial dysfunction and the activation of the caspase cascade (Borner, 2003). The leakage of cytochrome c from the mitochondria is thought to be caused by Bax that forms pores in the mitochondrial membrane (Desagher & Martinou, 2000; Martinou *et al.*, 2000; Zamzami & Kroemer, 2001; Wang, 2001), rather than the rupture of the membrane due to swelling (von Ahsen *et al.*, 2000).

Relevance to depression:

Unpredictable stress (an animal model for depression) significantly reduces the expression of Bcl-2 in the central nucleus of the amygdala, cingulate- and frontal cortices of rats. Hippocampal Bcl-X_L is also reduced in rats after unpredictable stress (Kosten *et al.*, 2007). A number of studies found that the decreased expression of anti-apoptotic Bcl-2 family members in depression is reversible with antidepressant treatment (Manji et al., 2001; Bush & Hyson, 2006; Kosten et al., 2007; Chen et al., 2007). The restoration of the normal expression level of Bcl-2 can promote cellular survival by stabilising the mitochondrial membrane, by inactivating the apoptosome or by preventing the pore formation of Bax. Thus, Bcl-2 family members may contribute to the development of depression as well as antidepressant efficacy.

2.3.2.5 Protein Kinase B (Akt)

Background:

Protein kinase B, commonly known as Akt, is part of the family of serine/threonine kinases and plays a pivotal role in cellular signalling (Dudek *et al.*, 1997; Kim *et al.*, 2001; Schloss & Henn, 2004). Akt is able to promote cellular survival by phosphorylating and inhibiting death-inducing proteins (Kim *et al.*, 2001). This enzyme is involved in signal transduction pathways such as cell proliferation, apoptosis and angiogenesis (Kandel & Hay, 1999). In mammals there are three known isomers of this

substance, Akt1, -2 and -3, exhibiting a high degree of homology, yet differing slightly in the localisation of their regulatory phosphorylation sites (Galetic *et al.*, 1999; Kandel & Hay, 1999).

Mechanism of action:

In general, many membrane receptors make use of second messengers which are able to activate phosphoinositide 3-kinase (PI3K) (Heerssen & Segal, 2002; Coyle & Duman, 2003). Survival and growth factors are some of the substances responsible for the activation of PI3K. PI3K then generates phosphorylated phosphatidylinositides (PI-3,4-P_2 and PI-3,4,5-P_3) which binds to Akt as well as activating phosphoinositide-dependant kinase (PDK), that is responsible for the phosphorylation of membrane-bound Akt (Galetic *et al.*, 1999; Yoganathan *et al.*, 2000). Activated Akt is responsible for the inactivation of glycogen-3β. Because activated glycogen-3β inhibits the transcription of CREB and, in addition, causes apoptosis, activation of the P13K/Akt cascade is important for neuronal survival (Jope & Bijur, 2002). Except for promoting the activity of CREB and BDNF, Akt is also able to inhibit apoptosis by phosphorylating the Bad component (pro-apoptotic) of the Bad/Bcl-X_L complex, and the Bax component (pro-apoptotic) of the Bax/Bcl-2 complex. The phosphorylated Bad and/or Bax binds to 14-3-3 proteins which causes these substances to dissociate from their respective complexes, resulting in cell survival (Galetic *et al.*, 1999; Kandel & Hay, 1999). Akt also activates IKKα that ultimately leads to the activation of NF-κβ and cell survival (Kandel & Hay, 1999; Schloss & Henn, 2004).

Relevance to depression:

As mentioned above, Akt promotes the activity of CREB and BDNF through activation of the PI3K/Akt pathway (Jope & Bijur, 2002). Both CREB and BDNF are of dire importance in maintaining normal neuronal plasticity and survival (Smith *et al.*, 1995; Nibuya *et al.*, 1999). Studies have indicated that both latter neurotrophic factors are reduced in depressed patients (Koch *et al.*, 2003). An increase in Akt activity promotes BDNF activity, which is ultimately responsible for neuronal survival and growth (dendritic arborisation and sprouting of new nerve terminals) (Mamounas *et al.*, 2000; Nestler *et al.*, 2002; Schloss & Henn, 2004). Therefore Akt might be involved in increased neurotrophic factor expression and reversal of hippocampal volume reduction, as is noted during antidepressant therapy.

2.3.2.6 Brain derived neurotrophic factor

Background:

Neurotrophic factors are endogenous soluble proteins that regulate the survival, growth, morphological plasticity and synthesis of new neurons for differentiated functions (Hefti *et al.*, 1993). The neurotrophin family in mammals is composed of four known proteins namely: brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 and neurotrophin-4 (Rosenfeld *et al.*, 1995; Lewin & Barde, 1996). Of the latter proteins, BDNF is the most abundant with the highest levels of mRNA and protein expression found in the hippocampus and prefrontal cortex (Mamounas *et al.*, 2000).

Mechanism of action:

BDNF is a 27 kDa homodimeric protein whose signalling actions are mediated via the tyrosine kinase B receptor (Altar *et al.*, 1997; Heerssen & Segal, 2002). In hippocampal pyramidal neurons, BDNF increases dendritic arborisations, and in the frontal cortex, promotes the sprouting of serotonergic fibres (Mamounas *et al.*, 2000; Nestler *et al.*, 2002). In addition to acting as a trophic factor, BDNF is thought to modulate other signalling molecules, including the monoamine, amino acid and peptide neurotransmitters (Duman *et al.*, 1997; Nestler *et al.*, 2002; Schloss & Henn, 2004).

Relevance to depression:

BDNF plays an important role in the limbic system, due to its involvement in long-term potentiation and learning (Gartner & Staiger, 2002). Due to the involvement of BDNF in the limbic system, a growing number of research results has indicated that BDNF may play a crucial role in mental disorders such as depression (Smith *et al.*, 1995; Nibuya *et al.*, 1995). Studies performed on test animals have indicated that lower levels of BDNF mRNA are expressed during stress and depression (Smith *et al.*, 1995; Nibuya *et al.*, 1999). BDNF levels are also reduced in the serum of depressed patients and this reduction correlates negatively with the severity of the depression (Shimizu *et al.*, 2003). Furthermore, the lower BDNF mRNA levels in depressed patients were found to increase with chronic anti-depressant treatment (Nibuya *et al.*, 1996; Dias *et al.*, 2003; Gonul *et al.*, 2005). A post-mortem study also revealed an increase in hippocampal BDNF immunoreactivity in subjects who had been treated with anti-depressants, thus supporting a role for BDNF in major depressive disorder and its treatment (Chen *et al.*, 2001b). *In vivo* and *in vitro* animal studies have also shown an increase in BDNF levels

in serum and limbic structures in response to chronic anti-depressant treatment (Smith *et al.*, 1995; Chen *et al.*, 2001a).

2.4 Treatment of depression

Depression is commonly treated with a variety of antidepressants. Although antidepressants have been used for more than 40 years, their underlying mechanisms of action are still poorly understood (Sapolsky, 2000). Currently the most common understanding, in line with the monoaminergic hypothesis of depression, is that most antidepressants exert their antidepressant action by improving pathologically impaired monoaminergic neurotransmission (Duman *et al.*, 1999). In particular it is believed that the most commonly used antidepressants, namely the SSRIs and TCAs accomplish this by restoring intrasynaptic concentrations of serotonin (5-HT) and/or *l*-norepinephrine (*l*-NE) (Manji & Duman, 2001). Recent clinical and preclinical studies, however, suggest that major depressive disorder may also be associated with an impairment of neuronal plasticity and resilience in the brain (Manji & Duman, 2001; Sheline *et al.*, 2003). In addition, it is suggested that antidepressants may act by correcting other biochemical imbalances and even stimulate neurogenesis (Duman *et al.*, 1999; Manji *et al.*, 2000; Manji & Duman, 2001). Therefore it is suggested that antidepressants may in part influence hippocampal cell quantity and/or viability in an adaptive manner (Fuchs *et al.*, 2004). The latter view is supported by data from a previous study that has indicated that antidepressants exert neurotrophic/protective effects against various insults (Nibuya *et al.*, 1996; Chen & Chuang, 1999; Manev *et al.*, 2001b; Angelucci *et al.*, 2003; Chen *et al.*, 2007). It was also established that antidepressants can modulate factors involved in cell survival and growth, such as CREB, BDNF and Bcl-2 (Koch *et al.*, 2003; Chen *et al.*, 2007). Some of the classic antidepressants as well as a few of the newer and experimental anti-depression therapeutics are discussed below.

2.4.2 Tricyclic antidepressants

Tricyclic antidepressants (TCAs) are a distinct class of antidepressants, including drugs such as imipramine, amitriptyline and desipramine (Mycek *et al.*, 2000; Katzung, 2004). TCAs are multi-potent drugs that are able to block the muscarinic, H_1-histaminic and α_1-adrenergic receptors; although their main function is to block the reuptake of 5-HT, *l*-NE and DA (Mycek *et al.*, 2000; Katzung, 2004). The latter then results in increased concentrations of these monoamines in the synaptic cleft. The latter effect is thought to

be responsible for the antidepressant effect of the TCAs. However, this theory has been discounted by some observations, including the following (Katzung, 2004):

- The potency of the TCAs in blocking neurotransmitter uptake often does not correlate with clinically observed antidepressant effects.
- Blockade of reuptake of neurotransmitters occurs immediately after administration of the drug, but the antidepressant effect of the TCA requires several weeks of continued treatment.

The above-mentioned observations suggest that decreased uptake of neurotransmitters is an initial event that may not be directly/finally responsible for the antidepressant effect. Prolonged treatment with TCAs may eventually lead to up- and down-regulation of certain CNS receptors and survival/neurotrophic factors (Baldessarini, 1989; Koch *et al.*, 2003; Brink *et al.*, 2004). It has been suggested that monoaminergic receptor densities in the brain may change over a 2 to 4 week period with drug use, which may be important in the onset of antidepressant activity.

All the TCAs have similar therapeutic efficacy, and the choice of drug depends on the tolerance of side-effects and the duration of action.

2.4.2.1 Imipramine (Tofranil®)

Background:

Imipramine was the first of the TCAs and viewed as a prototype TCA. This drug is derived from the neuroleptic drug, chlorpromazine (Katzung, 2004).

Pharmacokinetics:

Imipramine is lipophilic and highly bound to plasma proteins (90.1%) with a peak plasma concentration of 200 ± 137 ng/ml (Goodman & Gilman, 2001). Due to the lipophilicity of this drug, it is well absorbed after oral administration. The lipid solubility of this drug also contributes to its long half-life (4 to 17 hours) (Mycek *et al.*, 2000; Katzung, 2004). It undergoes extensive hepatic oxidation, predominantly to the pharmacologically active *N*-demethylated product desipramine, where after the inactive metabolite is excreted via the kidneys. The therapeutic blood level range of imiprmaine is between 0.8 and 6.3 μM (Goodman & Gilman, 2001).

Mechanism of action:

Imipramine exhibits its effect by blocking the re-uptake of monoamine neurotransmitters, in particular serotonin and *l*-norepinephrine, thus increasing their concentrations in the synaptic cleft. Imipramine also blocks serotonergic, α-adrenergic, histamine and muscarinic receptors (Mycek *et al.*, 2000). The therapeutic benefit of the latter receptor blocking characteristics of imipramine is uncertain.

Adverse reactions:

This drug blocks acetylcholine receptors and therefore imipramine treatment leads to antimuscarinic effects such as blurred vision, xerostomia, urinary retention, constipation, and the aggravation of glaucoma and epilepsy (Mycek *et al.*, 2000). Imipramine can also cause cardiac overstimulation due to increased catecholamine activity, which can be life-threatening in the case of imipramine overdose. The most serious adverse effect of imipramine in the elderly is orthostatic hypotension and reflex tachycardia caused by α-adrenergic receptor blockage. Sedation is one of the most prominent adverse reactions during the first several weeks of treatment (Mycek *et al.*, 2000; Katzung, 2004).

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

A study performed on Neuro-2A cells indicated that imipramine is able to significantly increase the levels of phosphorylated Akt, without changing total Akt levels (Basta-Kaim *et al.*, 2005). During the same study it was also found that imipramine did not change the phospho-CREB levels. A study performed on jurkat cells revealed that imipramine is able to increase CREB-phoshorylation after long-term treatment of 3 weeks, without inducing apoptosis. In the same study, no changes was observed in the overall CREB expression (Koch *et al.*, 2003). Therefore, it can be assumed that imipramine induces CREB-phosporylation via signal transduction pathways that are different from those inducing apoptosis (Koch *et al.*, 2003). Another study indicated that imipramine increases the production of ROS *in vitro*, which then leads to the over-expression of the anti-apoptotic protein Bcl-2, thus preventing apoptosis (Xia *et al.*, 1999). The latter action of imipramine does not affect the generation of ROS. It is also documented that chronic imipramine treatment significantly reduces plasma corticosterone concentrations and increases CREB and BDNF mRNA and protein levels in the hippocampus (Song *et al.*, 2006). Imipramine is able to induce significant apoptotic cell

death at a 100 μM concentration. A concentration of 150 μM imipramine was found to be sufficient to reduce the number of cultured jurkat cells to half (Koch *et al.*, 2003).

2.4.3 Selective serotonin-reuptake inhibitors

This chemically unique group of antidepressants is relatively new and function by specifically inhibiting the reuptake of serotonin. Drugs belonging to this group include fluoxetine, sertraline, citalopram and paroxetine (Mycek *et al.*, 2000). The SSRIs are generally better tolerated compared to the TCAs, are associated with fewer anticholinergic effects and are less cardiotoxic (Katzung, 2004).

2.4.3.1 Fluoxetine (Prozac®)

Background:

Fluoxetine is the prototype of the SSRI drug group and has a bicyclic structure. Fluoxetine is also the only member of this group which forms an active metabolite with a half-life of several days (Katzung, 2004).

Pharmacokinetics:

Fluoxetine is well absorbed after oral administration (≥ 80%), is highly bound to plasma protein (±94%) and has a peak plasma concentration of 200 – 531 ng/ml *in vivo* (Goodman & Gilman, 2001). It has a large volume of distribution and undergoes biotransformation to metabolites such as the pharmacologically active N-demethylation product norfluoxetine. Both fluoxetine and norfluoxetine, is characterised by long half-lives (t½ 1-3 days for fluoxetine and 7-15 days for norfluoxetine) and therefore this drug can be administered once daily (Goodman & Gilman, 2001). The clinical onset of the action of this drug is, as with other antidepressants, delayed, but it is as effective as TCAs in the treatment of depression. Fluoxetine has a nonlinear pharmacokinetic profile, so that it must be used with caution in patients with hepatic dysfunction (Baldessarini, 2001). Fluoxetine is a hepatic enzyme inhibitor and therefore it is able to increase the concentration of co-administered medications by inhibiting their metabolism (Mycek *et al.*, 2000).

Mechanism of action:

Fluoxetine exerts its effect by specifically inhibiting the reuptake of 5-HT from the synaptic cleft in the central nervous system (Lee *et al.*, 2001; Cipriani *et al.*, 2005) thereby increasing the 5-HT concentration in the synaptic cleft (Baldessarini, 2001).

Adverse reactions and drug interactions:

Fluoxetine is known to interact with lithium, tryptophan and monoamine oxidase inhibitors (Baldessarini, 2001). The latter interactions are potentially serious and may cause serotonergic syndrome, a rare and severe syndrome, characterised by symptoms and signs that correspond with an increase in 5-HT in the CNS (Katzung, 2004). The most frequent symptoms of serotonergic syndrome are myoclonia, diarrhoea, confusion, psychomotor agitation, hyperreflexia, shivers, motor in-coordination, fever, tremors, nausea, vomiting and blood pressure alterations. This syndrome is usually caused by high levels of serotonergic drugs and its symptoms usually disappear within 24 hours after the administration of the causative drug has been ceased. Furthermore, the interaction between fluoxetine and monoamine oxidase inhibitors can lead to hypothermia, confusion and in extreme cases death. Because of the latter interaction a 5 week wash-out period is recommended if fluoxetine is to be replaced with a monoamine oxidase inhibitor (Baldessarini, 2001; Katzung, 2004). Fluoxetine is associated with fewer antimuscarinic, cardiovascular and CNS adverse events, but a greater number of gastrointestinal adverse events than those reported for TCAs (Mycek *et al.*, 2000).

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

Recent research has found that a part of the neuroprotective action of fluoxetine may be attributed to the ability of this drug to increase CREB and BDNF (measuring mRNA expression) in the hippocampus (Chang *et al.*, 2006). As discussed above, CREB is an intra-cellular transcription factor, responsible for the induction of cell survival signals such as BDNF, as well as promoting synaptic plasticity (Walton & Dragunow, 2000). CREB has been shown to be up-regulated in both human and rodent studies after chronic administration of fluoxetine (Chang *et al.*, 2006). Up-regulation of BDNF mRNA and its receptor trkB, parallels CREB activation after chronic administration of fluoxetine (Nibuya *et al.*, 1996; Thome *et al.*, 2000). Furthermore, chronic administration of fluoxetine influences the proliferation, as well as the maturation, differentiation and

survival of neurons after neurogenesis. Fluoxetine is also able to inhibit the levels of the pro-inflammatory cytokines IL-6, TNFα and IL-1β (Chiou *et al.*, 2006).

Recent studies have also demonstrated that chronic fluoxetine treatment is able to increase neurogenesis in the hippocampus (Lee *et al.*, 2001; Santarelli *et al.*, 2003). Furthermore, Manev and colleagues (2001) reported that fluoxetine can increase cell proliferation in the hippocampus of adult rats *in vivo* and in primary neural cultures *in vitro* (Manev *et al., 2001a).* Fluoxetine has also been reported to have varying effects on increasing cell proliferation, preventing apoptosis in the dentate gyrus, stimulating DNA synthesis, inhibiting ultra violet (UV) induced DNA fragmentation, and enhancing responses to chemo thermotherapy (Wright *et al.*, 1994; Lee *et al.*, 2001; Peer *et al.*, 2004). Chronic fluoxetine treatment is also able to up-regulate the neurotrophic factor S100β, which plays a crucial role in the modulation of adult neurogenesis (Manev *et al.*, 2001b). It is also stated in the literature that fluoxetine is able to interact with the voltage-dependent anion channels of the mitochondria, inhibiting cytochrome c release and protecting against apoptotic cell death (Nahon *et al.*, 2005). Additionally, fluoxetine also inhibits dendrite atrophy of hippocampal neurons by decreasing nitric oxide synthase expression in a rat depression model (Luo & Tan, 2001). Song and colleagues noted that chronic fluoxetine treatment significantly reduces plasma corticosterone levels in mice (Song *et al.*, 2006). A study conducted by Koch and colleagues indicated that fluoxetine is able to induce significant apoptosis in jurkat cells within 24 hours in a dose-dependant way. Significant fluoxetine-induced apoptosis was noted from a 20 μM concentration. A concentration of 30 μM fluoxetine was sufficient to terminate half of the sample cells (Koch *et al.*, 2003).

Thus, the effectiveness of fluoxetine as antidepressant may be partially attributed to the ability of this drug to increase cell survival signals and to inhibit substances associated with cellular damage if used at the pharmacological concentration (97 nM).

2.4.4 Monoamine oxidase inhibitors

Monoamine oxidase (MAO) is a mitochondrial enzyme found in neuronal and other tissues, such as the gut and liver. In the neuron, MAO oxidatively deaminates and inactivates any excess neurotransmitter molecules that may leak out of synaptic vesicles when the neuron is at rest (Baldessarini, 2001). MAO inhibitors (such as phenelzine, tranylcypromine and isocarboxazid) are structurally related amphetamines

and are orally active (Goodman & Gilman, 2001). They inhibit both MAO-A (enzyme which metabolizes norepinephrine, serotonin, and tyramine) and MAO-B (enzyme which metabolizes dopamine). The MAO inhibitors may irreversibly or reversibly inactivate the above-mentioned enzymes, permitting neurotransmitter molecules to escape degradation (Mycek *et al.*, 2000; Katzung, 2004). This causes the accumulation of neurotransmitters in the synaptic space. The latter increase in neurotransmitters is responsible for the activation of adrenergic and serotonergic receptors, and may be responsible for the antidepressant action of this drug group (Baldessarini, 2001). Meclobemide, on the other hand, is a selective and reversible inhibitor of MAO-A inhibitor and causes less severe adverse reactions than the non-selective MAO inhibitors (such as a minimal response to dietary tyramine – "cheese reaction"). All the MAO inhibitors decrease the activity of hepatic enzymes and cause many drug interactions and therefore their use is very limited (Katzung, 2004).

2.4.5 Lithium salts

2.4.5.1 Lithium Chloride (Lithobid®)

Background:

The use of lithium as a mood stabiliser was first described in 1949 by John Cade (Cade, 2000), and in 1970 it was approved by the American Food and Drug administration as an intervention for mania (Jefferson & Greist, 1977). Lithium is also used in prophylactic treatment as well as in short- and long-term treatment of manic episodes in patients suffering from bipolar disorder, and is effective in treating 60 to 80% of patients exhibiting mania and hypomania (Barlow & Durand, 2002). This drug is also used to treat TCA-resistant or recurrent major depression.

Pharmacokinetics:

Lithium is an alkali metal and a monovalent cation, which is minimally bound to plasma proteins. Lithium does not undergo biotransformation, thus elimination is primarily via renal excretion (Ward *et al.*, 1994; Baldessarini & Tondo, 2000; Kilts, 2000; Goodman & Gilman, 2001). Lithium is available in a variety of preparations, including lithium carbonate, lithium citrate, lithium chloride and as immediate- and slow release formulations (Baldessarini & Tarazi, 2001). Lithium chloride, the most commonly used of the lithium salts, is orally administered and readily absorbed. After administration,

this drug distributes rapidly to the liver and kidneys, and more slowly to the muscles, brain and bone structure. Lithium reaches its peak plasma concentration after 1-2 hours with rapid-release formulations and 4-5 hours with slow-release preparations (Finley *et al.*, 1995), and is excreted renally after approximately 24 hours (Baldessarini & Tarazi, 2001). Lithium chloride has a maximal plasma concentration of 1 – 2 mM. After 5 days of treatment, lithium reaches steady-state concentrations (Keck, Jr. & McElroy, 2002). This drug enters the cells via sodium channels and it is extruded by the active sodium pump at about one-tenth the rate of sodium. The latter occurrence causes lithium to accumulate within the cells. In fact, it causes the concentration of lithium within the thyroid to be 2 to 5 times higher than its serum concentration. The monitoring of lithium concentrations is essential because of lithium's narrow therapeutic index (Baldessarini & Tarazi, 2001).

Mechanism of action:

Although many cellular processes are altered by lithium treatment, the mode of action is not entirely known. Lithium has a stabilising effect on neurotransmission and receptor systems (Cade, 2000). In fact, in animal studies, lithium has been shown to increase tryptophan uptake and 5-HT synthesis, while it also enhances 5-HT release, increases 5-HT_1 receptor-mediated behaviour and attenuates 5-HT_2 receptor-mediated responses (Kilts, 2000). Moreover, lithium enhances noradrenergic neurotransmission and alters phosphatidylinositol turnover, which mediates signal transduction at a number of central receptors, such as 5-HT_2, α_1-noradrenergic, muscarinic and glutamate receptors (Baraban *et al.*, 1989). Lithium's action on 5-HT function, though it is not yet well documented in humans, may explain its antidepressant activity and potential interaction with TCAs (Ward *et al.*, 1994). Lithium reduces the brain levels of free inositol by inhibiting *myo*-inositol monophosphatase (IMPase) (Parthasarathy *et al.*, 1997; Shaldubina *et al.*, 2006). This enzyme is responsible for the hydrolysis of *myo*-inositol 1-phosphate to free inositol in the presence of magnesium (Mg^{2+}), in order to maintain a precursor supply for the formation of inositol second messenger signalling molecules. Therefore, its therapeutic function in mania might be a result of the depletion of inositol dependant intra-cellular messengers which slows down the phosphatidylinositol (PI) cycle (alters the cellular concentration of the second messenger IP_3) in overactive synaptic systems (Baraban *et al.*, 1989; Berridge & Irvine, 1989; Snyder, 1992; Kofman & Belmaker, 1993). Therefore, the effectiveness of lithium as antidepressant is thought to be connected to the subsequent increase in brain *myo*-inositol 1-phosphate (Brandish

et al., 2005; Eden *et al.*, 2006), a lipid metabolite with the ability to increase the phospholipid transfer activity of phosphatidylinositol transfer protein-alpha (Komatsu *et al.*, 2004). This transfer protein is a bifunctional phosphatidylinositol and phosphatidylcholine transfer protein (Wirtz, 1997; Cockcroft, 1998), and plays critical roles in polyphosphoinositide signaling (Cunningham *et al.*, 1996), constitutive and regulated vesicular trafficking among intracellular organelles (Tuscher *et al.*, 1997; Jones *et al.*, 1998) and in secretory trafficking pathways (Ohashi *et al.*, 1995; Paul *et al.*, 1998).

Adverse reactions:

Lithium may interfere with renal sodium excretion, can alter thyroid function and may cause heart conduction disturbances. Lithium's side effect profile includes ataxia, tremors, confusion and convulsions (Freeman & Freeman, 2006). Rare side effects of this drug include nausea, diarrhoea, abdominal pain and weakness (Katzung, 2004).

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

Lithium has been reported to exert neuroprotective activity against glutamate toxicity in neuronal cell cultures and *in vivo* models by inhibiting NMDA receptor-mediated Ca^{2+} influx (Nonaka *et al.*, 1998). Thus, it is suggested that lithium may partially impart its neuroprotective effect by reducing glutamate excitotoxicity. Indeed, earlier studies have described an effect of lithium salts on NO signalling, a critical down-stream messenger activated by the NMDA receptor and strongly implicated in neurotoxicity and plasticity (Harvey *et al.*, 1994). Manji and colleagues found that lithium is able to augment synaptic plasticity and they postulated that this might be attributed to lithium's ability to block neuronal death, enhance neurogenesis, stimulate the proliferation of dendrites and facilitate synaptic transmission (Manji *et al.*, 1999). These cellular actions of lithium have been shown by other studies to be associated with the up-regulation of a diverse number of neurotrophic and neuroprotective factors. Lithium affects the expression of transcription factors such as Akt and CREB, which are responsible for the regulation of many fundamental neuroprotective proteins, such as Bcl-2 (Chen *et al.*, 1999; Chen & Chuang, 1999; Chen *et al.*, 2000; Bush & Hyson, 2006). This drug is not just able to increase the expression of Bcl-2, but also to decrease this protein's pro-apoptotic counterpart Bax (Chen *et al.*, 1999; Chen & Chuang, 1999). It has been proven that lithium increases the expression of glia-derived neurotrophic factor (GDNF) (Manji *et al.*,

2001; Fukumoto *et al.*, 2001; Angelucci *et al.*, 2003). The above-mentioned molecules are known to play an essential role in maintaining normal synaptic transmission and plasticity (Silva *et al.*, 1998; McAllister *et al.*, 1999; Fields & Stevens-Graham, 2002). Brain imaging studies have also indicated that chronic lithium administration is able to increase the volume of grey matter in bipolar patients (Moore *et al.*, 2000b; Sassi *et al.*, 2002). This increase might be associated with the proliferation of dendrites, suggesting that lithium may enhance synaptic connections (Moore *et al.*, 2000a). All these neuroplastic actions of lithium may lead to the enhancement of neuronal plasticity and reverse impaired synaptic communications in neuronal circuits involved in the pathophysiology of mood disorders - and eventually contribute to therapeutic efficacy (Manji *et al.*, 2001).

2.4.6 Atypical drugs used in the treatment of depression

During recent years much research has been done in the field of depression in an attempt to fully understand and determine the underlying factors involved in this disorder. With the additional information regarding this disease, alternative and newer drugs are being considered. Some atypical mood stabilising drugs are discussed below.

2.4.6.1 *myo*-Inositol

Background:

myo-Inositol (*m*Ins) is the most abundant, naturally occurring stereoisomer of the inositols (hexahydroxycyclohexanes or six-carbon sugar-alcohols) and a derivative of glucose (Harvey *et al.*, 2002; Groenen *et al.*, 2006). *m*Ins is a essential constituent of living cells, including brain cells, and is obtained through dietary intake (Holub, 1986) or via *de novo* synthesis from D-glucose-6-phosphate (Hauser & Finelli, 1963). Approximately 1 g/day of inositol is provided by normal dietary intake (Petroff *et al.*, 1989; Colodny & Hoffman, 1998).

Concentrations of inositol is higher in the human brain than in plasma or in the cerebrospinal fluid (CSF) and have been calculated to range between 2 and 15 mM, but may be higher in certain neuronal cells (Fisher *et al.*, 2002). *m*Ins is also an essential nutrient for cultured human cell lines (Holub, 1986). Therefore, *m*Ins is a compulsory

part of cultured cell growth medium with a concentration of 0.1 mM (18 mg/L) in Ham's F12 medium.

The *in vivo* role of *m*Ins:

*m*Ins serves as an organic osmolyte and its levels increase when a cell is subjected to a hypertonic environment. *m*Ins also plays a pivotal role in intracellular signalling pathways, intracellular calcium regulation and membrane integrity (Harvey *et al.*, 2002; Groenen *et al.*, 2006). The signal transduction molecule phosphatidylinositol (PI) is formed trough phosphorylation of *m*Ins (Moore *et al.*, 1999). Phosphorylation of the hydroxyl groups of *m*Ins at different positions (position 3, 4, 5 or a combination thereof) can generate a number of different phosphatidylinositol phosphates (PIPs) each with an important physiological function (Hu *et al.*, 2001). The PI cycle is of utmost importance during G protein-coupled receptor signalling of several neurotransmitters (Baraban *et al.*, 1989). Following G protein-coupled receptor activation by extracellular chemical messengers, the enzyme phospholipase C is activated (Berridge, 1993; Kim *et al.*, 2005), which is responsible for the conversion of the membrane component phosphatidylinositol bisphosphate (PIP_2) (Harvey, 1997; Groenen *et al.*, 2006), to the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Berridge, 1993; Hu *et al.*, 2001). IP_3 causes the mobilisation of stored Ca^{2+} within the cell. Under normal cellular conditions Ca^{2+} acts as a second messenger, activating Akt, which regulates cell survival and proliferation by phosphorylating a number of downstream targets, such as Bad (anti-apoptotic protein) (Hu *et al.*, 2001).

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

*m*Ins concentrations have been reported in the literature to be reduced in depressed patients (Frey *et al.*, 1998). The latter statement is confirmed by a study conducted by Shimon and colleagues who compared the frontal cortex *m*Ins concentrations in the post-mortem brains of suicide victims and bipolar patients with normal control subjects. They found that the mIns concentrations were reduced by 23% in the suicide victims and 32% in the bipolar patients compared to the control group (Shimon *et al.*, 1997).

Although the penetration of exogenous inositol through the blood-brain barrier is limited (only about 3% of plasma *m*Ins concentration) (Spector & Lorenzo, 1975; Einat *et al.*, 1999), recent research has indicated that chronic oral administration of high doses *m*Ins significantly elevate *m*Ins levels in specific rat brain regions (27% elevation in the

hippocampus) (Kofman *et al.*, 1998). Exogenous administration of *m*Ins has been proven to be clinically effective in the treatment of depression in humans (Levine, 1997). A study by Levine *et al.* indicated that chronic *m*Ins intake (12 g/day for 4 weeks) significantly reduces the scoring of depressed patients on the Hamilton Depression Rating Scale (Levine *et al.*, 1995). Another study by Levine and colleagues also indicated that dietary supplements of *m*Ins increase the cerebrospinal fluid *m*Ins levels with up to 60% (Levine *et al.*, 1993).

The activity of inositol in human patients and studies in animals appears to be similar to that of SSRIs. Indeed, inositol has been found to exert distinct effects on dopamine and serotonin receptors following chronic administration (Harvey *et al.*, 2001), inducing a significant increase in striatal D_2 receptor density with a slight, albeit insignificant, increase in $5HT_2$ receptor density. This suggests that D_2 receptor upregulation may play an important role in the behavioural effects of inositol. However, the mechanism of inositol appears to be related to post-receptor intracellular mechanisms rather than to receptor and pre-synaptic mechanisms as is the case with SSRIs (Einat *et al.*, 1999). A study performed on human neuroblastoma cells indicated that *m*Ins inhibits mAChR function and that the PI metabolic pathway seems to play a pivotal role in the latter action (Brink *et al.*, 2004). This action of mIns correlates with the cholinergic super sensitivity hypothesis of depression, stipulating that the inhibition of mAChR produces antidepressant effects (Daws & Overstreet, 1999). An overall increase of *myo*-inositol 1-phosphate in the brain after chronic *myo*-inositol administration might also contribute to the antidepressant effect of this substance, which correlates well with the suspected anti-depressive mechanism of lithium (Parthasarathy *et al.*, 1997; Brandish *et al.*, 2005; Eden *et al.*, 2006).

2.4.6.2 Tianeptine (Stablon®)

Background:

Tianeptine is a novel antidepressant which has been available in France and other countries since 1988, and has a modified tricyclic structure (Gaulier *et al.*, 2000; McEwen & Olie, 2005). This drug has clinically proven to be an effective antidepressant which is well tolerated in the elderly and does not have the adverse effects of SSRIs (Saiz-Ruiz *et al.*, 1998). This drug is also used to treat depression in post-alcohol-withdrawal patients (Uzbay *et al.*, 2006).

Pharmacokinetics:

Tianeptine is highly plasma protein bound (95%) at therapeutic concentrations of 0.3 – 1 µM (Zini *et al.*, 1990). Multiple dosing of tianeptine after oral administration is well tolerated. Tianeptine and its MC5 metabolite (C5 acid analogue of tianeptine) reach maximum plasma levels after 1.8 ± 0.99 and 2.96 ± 1.44 hours, respectively (Goodman & Gilman, 2001). Tianeptine follows linear pharmacokinetics, has a half-life of 2.5 ± 1.1 hours, and is eliminated from plasma mainly via the extra-renal route. Its antidepressant and anxiolytic properties appear to be similar to amitriptyline, imipramine and fluoxetine and even superior to maprotiline. Tianeptine has been shown to be well tolerated in long- and short-term therapy (up to one year) (Wagstaff *et al.*, 2001). Clinical studies have indicated that depressed patients receiving tianeptine experience symptomatic improvement after 7 days of treatment (Novotny & Faltus, 2003).

Mechanism of action:

In contrast with the monoamine hypothesis of depression and treatment efficiency, this drug stimulates the uptake of 5-HT in brain synaptosomes and platelets and reduces serotonergic-induced behaviour (Whitton *et al.*, 1991; Wagstaff *et al.*, 2001). An increase in 5-hydroxyindoloacetic acid (5-HIAA) in cerebral tissue and plasma has also been noted in patients using this drug. Tianeptine also reduces the hypothalamic-pituitary-adrenal response to stress and it has proven its efficacy in major depression, bipolar disorder (with or without melancholia) and in dystymic disorder (Conrad *et al.*, 1999).

Adverse reactions:

Dry mouth, constipation, dizziness, syncope, drowsiness and postural hypotension are less frequent side effects with tianeptine than with tricyclic antidepressants. Frequent side effects of tianeptine include insomnia and nightmares (Loo *et al.*, 1999). Given this drugs' relative lack of sedation, anticholinergic and cardiovascular adverse effects, it is particularly suitable in the treatment of depression (Saiz-Ruiz *et al.*, 1998; Wagstaff *et al.*, 2001).

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

Depressive states lead to a decrease in dendritic length and branching of hippocampal neurons (Magarinos *et al.*, 1999). Tianeptine is able to restore the dendritic length and branching of the hippocampal neurons. Tianeptine has also been shown to modulate

the neurodegenerative effects of stress in the hippocampus (Magarinos *et al.*, 1999; Alfonso *et al.*, 2006). This drug prevents stress- and corticosterone-induced atrophy of hippocampal CA3 neurons (McEwen & Olie, 2005). Tianeptine has also been found to prevent stress-induced reorganisation of glutamatergic synaptic vesicles and to reverse spatial memory dysfunction caused by stress or glucocorticoids. In addition, tianeptine also reverses the deleterious effects of partial medial septal lesions to the CA3 area of the hippocampus on memory (Morris *et al.*, 2001). A study done on cells seeded from foetal rats indicated that tianeptine does not reduce neuronal damage caused by high concentrations of glutamate. Thus, the neuroprotective effects of this drug are not related to simple antagonism of the NMDA receptor. During animal studies it was found that tianeptine treatment totally reversed stress induced down-regulation of CREB and BDNF mRNA in the hippocampus (Alfonso *et al.*, 2006; Song *et al.*, 2006). Tianeptine also normalises the expression of nerve growth factor (NGF), improves neuroplasticity and restores hippocampal volume. This drug increases neurogenesis in the dentate gyrus as well as regulate apoptosis in the hippocampus and temporal cortex. In contrast with the latter findings, another animal study indicated that chronic tianeptine treatment did not alter the mRNA expression of BDNF in the rat hippocampus (Kuroda & McEwen, 1998). Yet, another study also showed that tianeptine exerted protective effects in neurons after co-incubation with the pro-inflammatory cytokine, IL-1β (Castanon *et al.*, 2001). The above-mentioned studies indicate that there is still controversy about the specific neuroprotective mechanisms of tianeptine, although all these studies postulate that tianeptines' antidepressant efficacy is better explained by its ability to improve neuroplasticity.

2.4.6.3 Gabapentin (Neurontin®)

Background:

The route to discovering new drugs often leads to unexpected findings and, simultaneously, the advancement of pharmacological science, as illustrated by compounds such as gabapentin (Dooley *et al.*, 2007). Gabapentin (or 2-[1-(aminomethyl)cyclohexyl]acetic acid) was originally synthesised in 1977 by Hartenstein and Satzinger (Satzinger, 1994), with an empirical formula of $C_9H_{17}NO_2$ and a molecular weight of 171.237 g/mol. This drug was synthesised as an anticonvulsant that mimicked the structure of the inhibitory neurotransmitter gamma amino-byturic acid (GABA) for the treatment of epilepsy (Carta *et al.*, 2003). The original concept of

Hartenstein and Satzinger was to increase the lipophilicity of GABA by addition of a cyclohexyl substituent, thereby increasing its propensity to penetrate the CNS while retaining a similar pharmacological profile (Dooley *et al.*, 2007). More recently this drug has been used in the treatment of bipolar disorder. Some investigators claim that gabapentin is just as effective in bipolar treatment with the advantage of fewer side effects than more conventional bipolar drugs such as lithium (Carta *et al.*, 2003)

Pharmacokinetics:

Gabapentin is not significantly bound to plasma protein (less than 3%) and has a bioavailability of 59%. The major route of elimination of gabapentin is via renal excretion of the unchanged compound (64-68% urinary excretion) (Goodman & Gilman, 2001). Due to the fact that this drug is not metabolised, it has no effect on the hepatic drug metabolising enzymes, which reduces drug interactions of this substance (Goodman & Gilman, 2001; Carta *et al.*, 2003). Because of the latter characteristic of gabapentin, it may be co-administered with lithium in the treatment of bipolar disorder (Carta *et al.*, 2003). Gabapentin is well absorbed after oral administration, although the absorption seems to be reduced with dosages of 1800 mg/day or higher. A recommended initial dose for depressive disorder is 300 mg/day, with a dose increase of 300 mg/day every 1-3 days. Maintenance dosages usually range from 900-2400 mg/day (Carta *et al.*, 2003). Gabapentin has a half-life of 6.5 ± 1.0 hours and therefore multiple daily doses are required (Goodman & Gilman, 2001). If gabapentin is administered three times daily at a dose of 800 mg until steady state is reached, the drug's peak concentration will be reached 2-3 hours after administration. The peak concentration of gabapentin has been measured as 4 µg/ml, although this drug is still affective at concentrations lower than 2 µg/ml (Gee *et al.*, 1996; Goodman & Gilman, 2001). If this medication is taken after a meal, the absorption of gabapentin can increase with up to 14% (Katzung, 2004).

Mechanism of action:

Several mechanisms of action have been proposed over the past few years to explain the efficacy of gabapentin in epilepsy, neuropathic pain and depression (Taylor *et al.*, 1998; Bryans & Wustrow, 1999). The most parsimonious mechanism to date is considered to be the ability of gabapentin to bind to the auxiliary $\alpha_2\delta$ sub-unit of voltage-sensitive Ca^{2+} channels. The latter action of gabapentin causes physiological effects

such as modulation of Ca^{2+} influx and neurotransmitter release (such as increasing GABA levels in the brain) (Gee *et al.*, 1996; Dooley *et al.*, 2007).

Adverse reactions:

The adverse reactions of gabapentin include somnolence, fatigue, dizziness and ataxia. Tolerance to the above-mentioned adverse effects of gabapentin develops rapidly, adding to its tolerance and success in therapy.

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

The previously-mentioned ability of gabapentin to reduce cellular calcium influx is thought to be responsible for the neuroprotective characteristics of gabapentin, although little research has been done into the protective mechanism of this drug (Gee *et al.*, 1996; Dooley *et al.*, 2007).

2.4.6.4 Mirtazapine (Remeron®)

Background:

Mirtazapine is a tetracyclic piperazinoazepine, and is the first of a new class of antidepressants, the noradrenergic and serotonin-specific antagonists (NaSSA) (Holm & Markham, 1999; Ptacek *et al.*, 2003; Wenzel *et al.*, 2006). In comparative studies, patients receiving mirtazapine showed significantly greater improvement as measured by scores on the Hamilton Rating Scale for depression (HAM-D) and Montgomery-Asberg Depression Rating Scale scores compared with placebo. During these studies mirtazapine proved to be just as effective as the tricyclic antidepressants clomipramine, amitriptyline and doxepin and the SSRI fluoxetine in the treatment of depression. Mirtazapine also has been shown to have a beneficial effect on anxiety and sleep disturbances. This drug demonstrated superior tolerability to tricyclics because of the relative absence of anticholinergic effects (Holm & Markham, 1999; Katzung, 2004).

Pharmacokinetics:

Mirtrazapine is well-adsorbed from the gastrointestinal tract and reaches its peak plasma concentration level 2 hours post dosing, with a linear pharmacokinetic profile. Fed or fasting conditions have no effect on the rate and extension of mirtazapine absorption (Timmer & Voortman, 1996). This drug has an elimination half-life of 20-40 hours, thus allowing once daily administration (Mycek *et al.*, 2000). Steady state is

reached within 3 – 5 days, and approximately 100% of the administered dose is excreted in the faeces and urine within 4 days (Timmer & Voortman, 1996). Mirtazapine is renally excreted after extensive metabolism by the cytochrome-P450 enzyme; therefore its clearance is reduced in patients with hepatic and renal impairment. Mirtazapine has no inducing or inhibiting effect on cytochrome P450, and is therefore unlikely to cause interactions with other hepatically metabolised drugs. Mirtazapine is significantly bound to plasma albumin *in vivo* (Mycek *et al.*, 2000; Goodman & Gilman, 2001; Katzung, 2004), and has an absolute bioavailability of approximately 50% (Timmer & Voortman, 1996).

Mechanism of action:

The efficacy of this antidepressant appears to be linked to the enhancement of central *l*-NE and 5-HT_1 receptor-mediated serotonergic neurotransmission. In fact, mirtazapine blocks pre-synaptic α_2-adrenergic auto receptors as well as post-synaptic 5-HT_2 and 5-HT_3 receptors (Mycek *et al.*, 2000; Baldessarini, 2001; Katzung, 2004).

Adverse reactions:

The most commonly reported adverse effects of mirtazapine include somnolence, increased appetite, weight gain and dizziness (Mycek *et al.*, 2000).

Previous research and hypotheses regarding anti-depressive and neuroprotective properties:

The neurodegenerative hypothesis of depression states that the reduction in hippocampal volume might be caused by increased glucocorticoids (cortisol) and a reduction in anti-apoptotic mediators (McEwen, 1999; Sapolsky, 2000; McEwen, 2000; Appelhof *et al.*, 2006). Therefore, the protective properties and clinical efficacy of mirtazapine might be partially attributed to this drug's ability to decrease corticosterone-induced gene transcription as well as increasing BDNF mRNA levels with chronic treatment (Rogoz *et al.*, 2005).

2.5 Synopsis

According to the World Health Organisation, the incidence of depression is vastly increasing with each passing year. It is also speculated that if this increase persists, depression would be second on the scale of most debilitating disorders in the world by 2020. Although the treatment regimes of depression had progressed greatly over the

past few years, most of them are still plagued by a delayed onset of action, unfavourable side effect profiles and treatment resistance. Recent research has demonstrated that major and prolonged depression correlate with a progressive volume loss in the implicated brain regions (such as the hippocampus) and that a number of prototype and novel antidepressants are able to counter or reverse this volume loss. This loss of brain volume is thought to be connected to a number of changes in the brain during depression, including increased glutamate neurotransmission, increased pro-inflammatory cytokines and malfunctioning of the HPA-axis. The most current depression hypothesis, the neurodegenerative hypothesis, states that the cytotoxic changes in the brain cause a decrease in neuronal survival and growth signals resulting in a decreased hippocampal volume and depression. The mainstream of research regarding the latter hypothesis has been aimed at the levels of BDNF in depressed patients, before and after antidepressant therapy. From this research sprouted findings that BDNF is significantly decreased in depressed patients and that this decrease is reversible with antidepressant therapy. After this discovery, other survival signals such as CREB and Bcl-2 have also been identified as being involved in depression and antidepressant efficacy. From these observations emerged the idea that the neurodegenerative effects in depression might be a result of apoptosis and decreased neuronal plasticity and viability. Therefore, the current study was aimed at determining the role of mitochondrial activity and the expression of pro- and anti-apoptotic genes in the mechanism of action of a diverse range of known and experimental antidepressants during glutamate induced neuronal stress. The results from the current study may also contribute to a better understanding of the intra-cellular processes of depression and the mechanisms of action of the drugs used in the study, which may in turn lead to the discovery of alternative drug targets and enhanced treatment regimes.

Chapter 3

Article: Molecular Psychiatry

This chapter is compiled according to the "Instructions to Authors" for "Molecular Psychiatry" available for perusal in *Appendix C*. This article will discuss the effects of pharmacological antidepressant concentrations on cell viability, and apoptotic gene expressions in human SH-SY5Y cells. An overview of all the study data is given in Chapter 4, while a complete and detailed discussion of all the study results is covered in *Appendix B*.

TITLE PAGE

Title of article

Prototypical and putative antidepressants display neuroprotective properties in cultured human neuroblastoma cells via distinct mechanisms.

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Running title

Neuroprotective mechanisms of antidepressants

Keywords

Antidepressants, neurodegeneration, apoptosis, depression, neuroprotective, mitochondrial activity

Abstract

The neurodegenerative hypothesis of depression postulates that major depression is associated with impaired neuroplasticity in particularly the hippocampus and prefrontal cortex, and that antidepressants are neuroprotective. Therefore the objective of this study was to determine the effects of a diverse range of antidepressant drugs on the survival of cultured human neuroblastoma cells, before and after glutamate-induced excitotoxicity, as well as determining the effects of these drugs on pro- and anti-apoptotic pathways. The free fraction of plasma protein bound drugs was determined in Ham's F12 medium + 10% foetal bovine serum by means of HPLC analysis. Cultured neuroblastoma cells were pre-treated for 24 hours with and without 10 mM glutamate plus a pharmacological concentration of fluoxetine, mirtazapine, tianeptine, imipramine, *myo*-inositol, gabapentin or lithium. Thereafter mitochondrial activity was determined with the MTT cell viability assay. The effects of the pre-treatments on genes encoding for pro-apoptotic factors Bax, caspase-3 and caspase-8, and anti-apoptotic factors BDNF, NF-κβ, Akt, CREB and Bcl-2, were determined utilising quantitative rt^2-PCR. Results from HPLC analyses enabled the quantification of drug-plasma albumin binding in culture medium and to calculate the free drug concentrations. The pre-treatment regime of lithium, *myo*-inositol, imipramine and gabapentin, but not of fluoxetine, mirtazapine or tianeptine, protected against glutamate induced excitotoxicity, as measured with the MTT assay. Quantitative rt^2-PCR indicated that fluoxetine, imipramine, tianeptine and *myo*-inositol increases anti-apoptotic gene expression (fluoxetine: CREB, Akt & Bcl-2; imipramine: NF-κβ; tianeptine: CREB, BDNF, Akt & Bcl-2; *myo*-inositol: CREB & BDNF) in human neuroblastoma cells after glutamate-induced excitotoxicity, while pro-apoptotic gene expression is decreased with gabapentin, imipramine, lithium, mirtazapine, *myo*-inositol and tianeptine (gabapentin, mirtazapine & *myo*-inositol: Caspase-3 & Caspase-8; imipramine: Bax & Caspase-3; lithium: Bax, Caspase-3 & Caspase-8). We conclude that pharmacological concentrations of diverse classes of antidepressant display different effects on cellular resilience pathways. Of these, some, but not others, involve protection against excitotoxicity.

Introduction

Depression is the second most ubiquitous disorder in the Western world, after cardiovascular disorders, with a prevalence of 9-18%, and is also among the most common causes of morbidity, disability and suicide around the world [1,2]. While current drug treatments are plagued by unfavourable side-effect profiles, delayed onsets of action and a high incidence of treatment resistance, there remains a significant and urgent need for advanced treatments, as well as a better understanding of the complex neurobiological basis of depression.

Leading hypotheses of the neurobiological basis of depression postulate aberrant concentrations of monoamines (i.e. serotonin, norepinephrine and dopamine) in the synaptic cleft, and conclude that antidepressants exert their primary bio-chemical effects by readjusting the intrasynaptic concentrations of these neurotransmitters [3,4]. In stark contrast with the latter hypothesis, more recent neuro-imaging studies have revealed selective structural changes and a reduction in the volume of certain limbic and non-limbic brain regions in several stress-related neuropsychiatric illnesses, such as major depressive disorder [5,6,7]. The hippocampus, one of the major brain areas implicated in depression, has shown up to 20% reduction in volume in neuro-imaging studies of severely depressed patients [8,9]. These findings, along with the fact that certain pharmacologically unrelated drugs are not only effective in treating depression, but are also effective in reversing the aforementioned neuroanatomical changes associated with the illness, has lead to the formulation of the neurodegenerative hypothesis of depression [3,4,8].

The mainstream of research regarding the neurodegenerative hypothesis has been focussed on the reduction of neurotrophic factors such as brain derived neurotrophic factor (BDNF) and cyclic adenosine monophosphate response element binding protein (CREB) in the brain of depressed patients [10,11,12]. Studies have indicated that prolonged and acute stress is able to significantly reduce the expression of BDNF in the hippocampal pyramidal cells and dentate gyrus of rodents [10]. A reduction in the neurotrophic transcription factor CREB, which is responsible for the transcription of BDNF expression *in vivo* and *in vitro*, has also been detected during depression [13,14] [11,12]. In fact, the chronic administration of almost all classes of antidepressant is able to increase CREB and BDNF expression in *in vitro* and in humans and animals [15,16].

A substantial number of antidepressants also prevent stress-induced decreases in the expression of CREB and BDNF [17]. Antidepressant-like effects is produced in study animals (rodents) after *in vivo* administration of BDNF [18], and the injection of a CREB-encoding virus into the hippocampal dentate gyrus (thereby stimulating the expression of CREB) [17]. Although decreased expression of CREB and BDNF is the main focus of this hypothesis, stress-related malfunction of the HPA-axis, increased pro-inflammatory cytokines and increased glutamate neurotransmission are also postulated to be involved.

Except for the fact that HPA-axis malfunction increases cortisol levels, which has been shown to account for the decreased BDNF and CREB levels in depression [10,11,12], increased cortisol can also damage neurons by inducing oxidative stress [19]. High cortisol levels increase brain activity by directly influencing neurotransmission [10,20,21] leading to increased cellular metabolism of glucose and the formation of free radicals as by-products [22,23,24]. The amount of free radicals may then exceed the anti-oxidant defence capacity of the brain, damaging the neurons by means of oxidative stress [25,26,27]. Increased cortisol may also lead to increased glutamate neurotransmission [28], where excessive glutamate, via activation of *N*-methyl-*D*-aspartate (NMDA) receptor channels, is associated with excessive Ca^{2+} influx and neuronal dysfunction, cell damage or even cell death [8]. Also, elevated levels of extracellular glutamate activate neuronal nitric oxide synthase and the production of reactive nitrogen species such as nitric oxide and peroxynitrite [29], while glutamate also inhibits cysteine uptake, both processes resulting in the induction of oxidative stress and neuronal damage [22]. The balance between the intracellular anti-apoptotic protein B-cell CLL/lymphoma 2 (Bcl-2) and the pro-apoptotic protein Bcl2-associated X protein (Bax) can also be disturbed by high glutamate levels. This disturbance causes the Bcl-2 levels to decrease and Bax to increase, which ultimately leads to neuronal death via apoptosis [30,31,32].

Chronic stress may also cause an inappropriate rise in pro-inflammatory cytokines [33,34]. Pro-inflammatory cytokines enhance the activity of the indoleamine-2,3-dioxygenase (IDO) enzyme, leading to an excessive formation of quinolinic acid, a neurotoxic metabolite with an agonistic effect at the NMDA receptor [35,36]. Pro-inflammatory cytokines can also bind to cellular receptors and activate proteins that mediate cellular demise [37,38].

Several prototype and experimental anti-depressants have been proven to prevent and/or reverse hippocampal atrophy [7]. These include tianeptine (effective antidepressant that affects structural plasticity in the hippocampus [39,40,41]), fluoxetine (down-regulates neurodegenerative enzymes, rendering it anti-apoptotic [42]), lithium (neuroprotective [43,44,45] and anti-apoptotic [46,47,48]), imipramine (increases hippocampal neurogenesis and neuroprotective [30,42,49]) and mirtazapine (decreases corticosterone-induced gene transcription and neuroprotective [47]). Although multitudes of data strongly support the proposed protective properties of antidepressants, their precise mechanisms of action in this regard are still poorly understood [50]. Also, a better understanding of the neuroprotective properties of antidepressants, and the role of neurodegeneration in depression, may eventually stimulate the discovery of new antidepressant targets, strategies and treatment regimes [47].

The main objective of the current study was to determine the effect of selected prototypes of different classes of clinical and experimental anti-depressants on neuroplasticity in a human neuroblastoma cell line (SH-SY5Y) in the presence and absence of glutamate-induced excitotoxicity. Antidepressants studied include fluoxetine (a serotonin reuptake inhibitor; SRI), imipramine (a tricyclic antidepressant), lithium (mood stabiliser), mirtazapine (noradrenergic and serotonin-specific antagonist), and the atypical compounds, gabapentin, *myo*-inositol and tianeptine. Biological markers of the neuroplasticity selected include mitochondrial activity and the expression of genes involved in neuronal survival and growth, viz. CREB, BDNF, Akt, NF-κβ, Bcl-2, Bax, caspase-3 and caspase-8.

Materials and methods

Cells and medium used for cell cultures and cell culture maintenance: The current study was performed on human neuroblastoma (SH-SY5Y) cells obtained from Adcock Ingram® Scientific Group, South Africa. The study cells were maintained in Ham's F12 medium enriched with 10% foetal bovine serum (FBS) at 37°C and 5% CO_2. Penicillin (100 units/ml), streptomycin (100 µg/ml) and fungizone (0,25 µg/ml) were added to the above-mentioned culture medium for culture maintenance prior to the pre-treatment. The above-mentioned culture medium, FBS and antibiotics were obtained from Gibco (Invitrogen).

Study drugs

Fluoxetine (97 nM): Fluoxetine, a generous gift from Eli Lilly, South Africa, has a therapeutic peak plasma concentration of 200 – 531 ng/ml in humans and is 94% bound to plasma protein [51]. A total plasma concentration of 500 ng/ml therefore represents 30 mg/ml (97 nM) free drug. We calculated the free fraction in culture medium (Ham's F12 + 10% FBS) as 32.2%, as from measured total and free drug with HPLC analyses before and after micro-filtration [52]. Therefore, we used a total concentration of 301.2 nM in culture medium to yield a 97 nM free drug concentration, as administered to SH-SY5Y cells for 24 hours.

Gabapentin (0.23 µM): Gabapentin, purchased from Sigma-Aldrich, South Africa, is not significantly bound to plasma protein (less than 3%) and therefore the reported therapeutic peak plasma concentration of 0.23 µM in humans was administered in culture medium to SH-SY5Y cells [51].

Imipramine (71 nM): Imipramine, purchased from Sigma-Aldrich, has a therapeutic peak plasma concentration of 200 ± 137 ng/ml in humans and is 90.1% bound to plasma protein [51]. A total plasma concentration of 200 ng/ml therefore represents about 20 mg/ml (71 nM) free drug. We calculated the free fraction in culture medium (Ham's F12 + 10% FBS) as 56.4%, as from measured total and free drug with HPLC analyses before and after micro-filtration [52]. Therefore, we used a total concentration of 125.9 nM in culture medium to yield a 71 nM free drug concentration, as administered to SH-SY5Y cells for 24 hours.

Lithium (1 mM): Lithium chloride, obtained from Sigma-Aldrich, has a therapeutic peak plasma concentration of 1 – 2 mM [51] and is not significantly bound to plasma protein. Because of its narrow therapeutic window and high toxicity, 1 mM was administered in culture medium to SH-SY5Y cells for 24 hours [51].

Mirtazapine (169 nM): Mirtazapine, obtained from Sigma-Aldrich, is 85% bound to plasma protein and has a therapeutic peak plasma concentration of 41.8 ± 7.7 ng/ml in humans [51]. We calculated the free fraction in culture medium (Ham's F12 + 10% FBS) as 46%, as from measured total and free drug with HPLC analyses before and after micro-filtration [52]. Therefore, we used a total concentration of 367.4 nM in culture medium to yield a 169 nM free drug concentration, as administered to SH-SY5Y cells for 24 hours.

myo-Inositol (2 mM): *myo*-Inositol, a derivative of glucose, was obtained from Sigma-Aldrich. Its normal physiological concentration range in the brain has been determined to vary between 2 and 15 mM [53]. It is not significantly bound to plasma protein [54,55].

Tianeptine (1 µM): Tianeptine, obtained from Servier, is significantly bound to plasma albumin [56]. A study conducted by Plaisant and colleagues (2003) used 1 µM, 10 µM and 100 µM tianeptine concentrations for the pre-treatment of cortical cell cultures [39]. Therefore, a 1 µM tianeptine concentration was used for the pre-treatment of the study cells. We calculated the free fraction in culture medium (Ham's F12 + 10% FBS) as 61.9%, as from measured total and free drug with HPLC analyses before and after micro-filtration [52]. Therefore, we used a total concentration of 1.62 µM in culture medium to yield a 1 µM free drug concentration, as administered to SH-SY5Y cells for 24 hours.

Pre-assay layout (cell seeding): Human neuroblastoma SH-SY5Y cells were maintained in Ham's F12 medium, 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone in culture flasks (Corning) at 37°C in 5% CO_2 until 90% confluent. Cells were detached from culture flasks by means of trypsination and seeded into 24-well plates at a density of ± 5 x 10^5 cells/well. A haemocytometer (0.1 mm depth, 0.0025 cm^2) and Nikon TMS inverted microscope (model number: 31771) were employed for cell counting. The well plates (Corning) were then incubated

for 48 hours at 37°C and 5% CO_2, to allow the cells to attach to the well bottoms and further multiply to about two million cells per well.

Drug pre-treatments: After the 48 hour incubation period (See '*Pre-assay layout*') the cells were treated with a pre-determined 10 mM concentration of glutamate (L-glutamic acid sodium salt hydrate from Sigma-Aldrich) to induce excitotoxicity. Along with the glutamate, the seeded cells were also treated with a pharmacological concentration of an experimental or clinical antidepressant (as discussed above) for 24 hours. Thereafter the culture medium with drug was aspirated, the cells rinsed twice and the appropriate assay performed (i.e. MTT test or RNA extraction for real-time PCR).

High performance liquid chromatography (HPLC) determination of [free drug]: For the purpose of this study the free fraction of plasma protein bound drugs (fluoxetine, imipramine, mirtazapine and tianeptine) had to be determined in Ham's F12 medium + 10% foetal bovine serum This was achieved by preparing a known concentration series for each drug (see ***Table 1***). These concentration ranges were prepared in such a manner that they would include the study concentrations.

(Table 1 about here)

Each concentration was prepared in duplicate: one in culture medium with 10% foetal bovine serum (FBS), and one without FBS. The samples containing FBS were centrifuged in microcentrifuge tubes from Sigma-Aldrich with a pore size of 30 kDa, to remove the albumin (66.3 kDa to 69 kDa), together with protein-bound drug [57]. The drug concentrations of all samples were analysed by HPLC, using a mobile phase of 30% (v/v) acetonetrile/0.005 M heptane sulphonic acid sodium salt in water (pH 3.5) and a flow rate of 1.0 ml/min, with a 150 x 4.6 mm Luna C18-2 column (100 Å pores), and a UV detection wavelength of 228 nm. The drug concentrations were calculated form the areas under the curves (AUC), relative to a standard. The regression lines of the total versus free concentration curves were used to determine the free fraction of the implicated drugs.

Determination of optimum glutamate co-treatment concentration: A 24-hour incubation of cultured neuronal cells has been used before to investigate the effects of drugs on biomolecular markers of cell function [58]. Since excessive glutamatergic activity has been associated with anxiety-related disorders, such as depression, *in vivo*

glutamate-induced excitotoxicity was selected as neuronal stressor for the current study, to evaluate neuroprotective effects of antidepressants. Prior to the commencement of the study experiments, an appropriate glutamate concentration had to be established for the *in vitro* induction of sufficient excitotoxicity in cultured human SH-SY5Y cells within the 24-hour drug treatment period. The standard MTT assay (measuring mitochondrial activity) was used as read-out of cell viability (i.e. relative quantification of excitotoxicity). A target decrease in cell viability was chosen as 50%, thereby to enable the observation of either neuroprotective (improved function) or neurotoxic (decreased function) effects by the experimental drug treatments. It has been reported that 24-hour incubation with 1 - 10 mM glutamate induces significant cytotoxicity in cultured cells [19]. Thus, we investigated the degree of excitotoxicity, as measured by the MTT assay, of 2 mM, 5 mM, 10 mM and 15 mM glutamate for 24 hours. The MTT assay was repeated twelfe times (n=12) for each of the above mentioned test concentrations.

MTT cell viability assay: The standard MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) [59] was employed to measure cell viability (mitochondrial activity). Briefly, the SH-SY5Y cells were seeded and treated with drugs as described above. Additional wells were also seeded to allow for positive controls (100% cell death via lyses with 0.33% formic acid (Saarchem, Unilab) for 30 minutes) and negative controls (no glutamate, no cell lyses). After incubation the formic acid/culture medium (containing a study drug) was aspirated from the cells. Following the 24-hour drug-treatments, the other cells were washed twice with 1 ml phosphate-buffered saline (PBS) per well. Thereafter 200 µl of 0.5% MTT reagent in PBS was added to each well and incubated at 37°C for 2 hours in the dark. Thereafter the MTT-reagent was aspirated and 250µl *iso*-propanol (2-propanol from Sigma) added to each well. The well-plates were then incubated at room temperature for 5 minutes to dissolve the purple formazan crystals, whereafter 100 µl of the formazan-containing iso-propanol per well was transferred to a corresponding well in a 96-well plate (Corning) and the spectrophotometric absorbance read at 560 nm (Ladsystems Multiscan RC).

The mitochondrial activity was determined and expressed as a percentage of the negative control (100% cell viability) by means of the following equation (where Abs = absorbance as read by the spectrophotometer):

$$\% \text{ Viable Cells} = 100 \times \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{positive control})}}{\text{Abs}_{(\text{negative control})} - \text{Abs}_{(\text{positive control})}}$$

Determination of primer efficiency and sample comparability: Equal volumes of each study sample lysate were mixed to form one sample containing lysate of all the different study conditions. This "mixed" pre-treatment sample was diluted to form a concentration range of four (4) samples. These samples were then analysed in the same manner as the study samples for each study primer. After rt^2-PCR analysis, graphs were constructed for each study primer by plotting the obtained C_T value against the Log function of the dilution factor. The slopes of the latter graphs were used to determine the efficiency of each primer by means of the following equation:

$$E = 10^{-1/Slope}$$

Where *E* is the primer efficiency and *Slope* is the slope of the C_T value against the Log of the dilution factor graph.

The primer efficiency for each primer was respectively used to determine its relative expression ratio in the study samples (see '*Real-time reverse transcriptase polymerase chain reaction*' for equation). Furthermore, the ubiquitin C gene (UBC) was used as a reference (house-keeping) gene in order to ensure that all data were comparable, since the expression thereof has been shown not to be influenced by the respective drug treatments (data not shown).

Real-time reverse transcriptase polymerase chain reaction: Real-time reverse transcriptase polymerase chain reaction (rt^2-PCR) was employed to determine gene expression (cDNA from mRNA) before and after drug treatments. The expression of selected genes encoding for the expression of pro-apoptotic (Bax, Caspase-3 & 8) and anti-apoptotic factors (BDNF, NF-κβ, Akt, CREB & Bcl-2) were investigated. We employed the validated Quigen Fastlane cDNA kit® for mRNA extraction and cDNA synthesis. BioRad SYBR Green® and QuantiTec Primer assay® (specific for each study gene, including the house-keeping gene UBC) were added for cDNA amplification during the rt^2-PCR process. The rt^2-PCR samples were analysed in 96-well format, utilising the iCycler® from BioRad®, and using 25 µl reaction volumes and the run-time conditions supplied by Quigen® and BioRad®.

All the rt^2-PCR results data were standardised according to the primer efficiency of the respective primer and the UBC expression, using the following equation:

$$Ratio = \frac{(E_{sample})^{Ct(control-sample)}}{(E_{ref})^{Ct(control-sample)}}$$

Where *Ratio* is the relative expression ratio of the study gene to the implicated control, E_{sample} is the efficiency of the implicated gene in the study sample and E_{ref} is the efficiency of the reference gene in the study sample. The C_T values were obtained from the rt^2-PCR analyses.

Statistical analysis: Basic processing of data was performed with Microsoft Excel 2003®, while all statistical analyses were performed with GraphPad Prism® (version 5.01 for Microsoft Windows, GraphPad Software, San Diego, CA, U.S.A.). All study data were analysed using a one-way analysis of variance (ANOVA), followed by Dunnett's Multiple Comparison post test (multiple comparisons of experimental groups to control) or the Tukey-Kramer post test (multiple comparisons between experimental groups). MTT data were obtained from triplicate observations from at least three separate, comparable experiments, while rt^2-PCR data were obtained from duplicate observations from at least four separate, comparable experiments. Data are expressed as the mean ± SEM with $p < 0.05$ regarded as statistically significant.

Results

The MTT results for the determination of the optimum glutamate co-treatment concentration are depicted in ***Figure 1***. It can be seen in ***Figure 1*** that the mitochondrial activity (cell viability) is not significantly altered by 24-hour incubation with 2 mM glutamate (7 ± 2.7% increase, not significant, n = 12), but significantly reduced relative to control by a 24-hour incubation with 5 mM glutamate (17 ± 4.5% decrease, $p < 0.05$, n = 12), 10 mM glutamate (45 ± 4.3% decrease, $p < 0.001$, n = 12) or 15 mM glutamate (89 ± 2.1% decrease, $p < 0.001$, n = 12).

(Figure 1 about here)

The HPLC analysis of the free concentration of protein-bound study drugs (fluoxetine, imipramine, mirtazapine and tianeptine) in Ham's F12 medium + serum, indicated that these drugs are bound moderately to plasma protein in the culture medium (***Figure 2***). ***Figure 2A-D***, illustrates the proportional increase in the free and bound drug concentrations as the total concentration of the drug increases, also suggesting that a fraction of the drug is bound to the protein of the foetal bovine serum. From ***Figure 2E-H*** it can be seen that a significant fraction of roughly between 25% and 75% of the study drugs is bound to the culture medium plasma protein at the concentration ranges of total drug employed. It should be noted that, at the lowest concentration measured for each drug, the concentration of the free fraction was lower than the measurable range, so that the free fraction for these low concentrations were calculated by extrapolation from determined fractions at the three higher concentrations of each drug respectively.

(Figure 2 about here)

From ***Figure 3A*** it can be seen that, in the absence of excitotoxicity (i.e. no glutamate), a 24-hour incubation of neuroblastoma cells with a pharmacological concentration of tianeptine (121.0 ± 27.4% cell viability, $p < 0.05$), but not the other antidepressants, increases mitochondrial function above control. However, from ***Figure 3B*** it becomes evident that in the presence of excitotoxicity (i.e. 10 mM glutamate), a 24-hour incubation of the neuroblastoma cells with a pharmacological concentration of the prototype antidepressants imipramine (54.06 ± 9.14% cell viability, $p < 0.05$)) and lithium (107.23 ± 5.88% cell viability, $p < 0.001$)) and the experimental antidepressant

myo-inositol (57.84 ± 14.18% cell viability, $p < 0.001$), protect against excitotoxicity. A similar trend was seen with gabapentin, but statistical significance was not reached. Fluoxetine, mirtazapine and tianeptine did not protect against excitotoxicity in this model.

(Figure 3 about here)

It can be seen from ***Figures 4A-G*** that, in the presence of glutamate, select gene expressions after all drugs were significantly, but differentially altered relative to control. ***Figure 4A*** indicates that statistically significant differences in gene expression after a pharmacological concentration of fluoxetine, relative to no drug (control) , are induced for **Akt** 214.75 ± 58.10% v/s 100 ± 10.10% control ($p < 0.01$); **CREB** 401.15 ± 109.02% v/s 100 ± 9.77% control ($p < 0.001$); **NFκβ** 332.81 ± 83.68% v/s 100 ± 6.58% control ($p < 0.001$) and **Bcl-2** 231.23 ± 56.64% v/s 100 ± 6.05% control ($p < 0.05$). ***Figure 4B***, indicates that significant differences in gene expression after a pharmacological concentration of imipramine, relative to no drug (control) , are induced for **NF-κβ** 263.02 ± 75.91% v/s 100 ± 6.58% control ($p < 0.05$); **Bax** 35.64 ± 11.74% v/s 100 ± 6.60% control ($p < 0.01$) and **Caspase-3** 20.36 ± 9.31% v/s 100 ± 6.69% control ($p < 0.001$). ***Figure 4C*** indicates that significant differences in gene expression after a pharmacological concentration of lithium, relative to no drug (control) are induced for **Bax** (60.70 ± 6.84% v/s 100 ± 6.60% control, $p < 0.01$), **Caspase-3** (60.75 ± 7.36% v/s 100 ± 6.69% control, $p < 0.001$) and **Caspase-8** (79.89 ± 4.99% v/s 100 ± 6.27% control, $p < 0.001$). ***Figure 4D*** indicates that significant differences in gene expression after a pharmacological concentration of tianeptine, relative to no drug (control) are induced for **BDNF** (263.88 ± 108.91% v/s 100 ± 6.74% control, $p < 0.05$), **Akt** (252.60 ± 46.51% v/s 100 ± 10.10% control, $p < 0.001$), **CREB** (275.22 ± 61.74% v/s 100 ± 9.77% control, $p < 0.05$), **Bcl-2** (329.36 ± 119.95% v/s 100 ± 6.05% control, $p < 0.001$), **Caspase-3** (15.89 ± 4.58% v/s 100 ± 6.69% control, $p < 0.001$) and **Caspase-8** (51.13 ± 20.97% v/s 100 ± 6.27% control, $p < 0.05$). ***Figure 4E*** indicates that significant differences in gene expression after a pharmacological concentration of *myo*-Inositol, relative to no drug (control) are induced for **BDNF** 507.42 ± 134.95% v/s 100 ± 6.74% control ($p < 0.001$); **CREB** 450.00 ± 88.93% v/s 100 ± 9.77% control ($p < 0.001$); **Caspase-3** 22.48 ± 8.17% v/s 100 ± 6.69% control ($p < 0.001$); and **Caspase-8** 6.55 ± 3.99% v/s 100 ± 6.27% control ($p < 0.001$). ***Figure 4F*** indicates that significant differences in gene expression after a pharmacological concentration of gabapentin,

relative to no drug (control) are induced for **Caspase-3** (45.38 ± 8.30% v/s 100 ± 6.69% control, $p < 0.001$) and **Caspase-8** (14.48 ± 7.68% v/s 100 ± 6.27% control, $p < 0.001$). ***Figure 4G*** indicates that significant differences in gene expression after a pharmacological concentration of mirtazapine, relative to no drug (control) are induced for **Caspase-3** (55.85 ± 8.47% v/s 100 ± 6.69% control, $p < 0.01$) and **Caspase-8** (33.66 ± 11.56% v/s 100 ± 6.27% control, $p < 0.01$).

(Figure 4 about here)

Discussion

Optimum glutamate co-treatment concentration: A decrease in glutamate-induced cell viability was set at 50% (see methods section), to be able to observe any drug-induced protection against excitotoxicity, or a drug-induced amplification thereof. It follows from the data in ***Figure 1*** that this target was achieved after a 24-hour incubation of human neuroblastoma SH-SY5Y cells with 10 mM glutamate. Therefore 10 mM glutamate was selected for the induction of excitotoxicity in the current study.

HPLC determination of free drug concentrations: For fluoxetine the % free drug varied between 35.47% at 80 nM and 43.56% at 12.68 µM for imipramine between 60.83% at 70 nM and 71.04% at 11.47 nM, for tianeptine between 64.40% at 1 µM and 60.45% at 12 µM, and for mirtazapine between 49.61% at 150 nM and 58.53% at 11.55 µM (compare ***Figure 2A-H***). Interestingly, all the study drugs (fluoxetine, imipramine, mirtazapine and tianeptine) are approximately 90% bound to plasma albumin *in vivo* [51], and the free percentage of these drugs in Ham's F12 + 10% serum is in the region of 50%. While the lower concentrations of the drugs had free concentrations below the detectable range, we calculated the expected free drug concentrations by means of extrapolation from linear regression. The % free drug at the pharmacological concentrations of the drugs were calculated respectively as 32.2% for fluoxetine (i.e. 301.24 nM total yields 97 nM free; r^2=0.92), as 56.4% for imipramine (i.e. 125.9 nM total yields 71 nM free; r^2=0.92), as 61.9% for tianeptine (i.e. 1.62 µM total yields 1 µM free; r^2=0.79), as 46% for mirtazapine (i.e. 367.4 nM total yields 169 nM free; r^2=0.91). These total concentrations were therefore employed when incubating the culture cells for 24 hours with the respective drugs.

Mitochondrial Activity: As is evident from ***Figure 3A***, there was no significant change in mitochondrial activity following a 24-hour pre-treatment with the pharmacological concentration of fluoxetine in the presence or absence of glutamate. Therefore it can be concluded that fluoxetine does not exert any effect on mitochondrial activity in the absence of a stressor such as glutamate. However, in the presence of 10 mM glutamate (excitotoxicity) a pharmacological concentration of fluoxetine (97 nM) showed a tendency towards potentiating the reduction of mitochondrial activity by glutamate (38.12 ± 9.78% reduction, not significant) versus control (***Figure 3B***).

Compared to the glutamate-free control it is evident that the pharmacological concentration of imipramine tended to increase mitochondrial activity (***Figure 3A***) although the increase was not significant (18.70 ± 26.70% increase, not significant). In the presence of 10 mM glutamate the pharmacological concentration of imipramine was able to yield a significant increase in mitochondrial activity (8.62 ± 9.14%, $p < 0.05$) compared to the glutamate control (***Figure 3B***). From these results can be speculated that the protective ability of imipramine might not be stressor dependant but, that the presence of a stressor enhances the protectiveness of this drug.

As is evident from ***Figure 3A***, there was no significant change in mitochondrial activity following a 24-hour pre-treatment with the pharmacological concentration of lithium in the absence of glutamate. Therefore, it can be concluded that lithium does not exert any protective effect on mitochondrial activity in the absence of a stressor such as glutamate. However, in the presence of 10 mM glutamate (excitotoxicity) the pharmacological lithium concentration (1 mM) showed a significant increase in mitochondrial activity (61.79 ± 5.88% increase, $p < 0.001$) comparing to the glutamate control (45.44 ± 8.85%). Based on the latter result it can be postulated that the pharmacological lithium concentration protects against the damaging and deleterious effects of glutamate on neuronal cells.

The pharmacological tianeptine concentration significantly increased the cell viability (21.0 ± 27.4%, $p < 0.05$) compared to a glutamate-free control (100 ± 18.2% cell viability) in the absence of glutamate (***Figure 3A***). No significant effect on mitochondrial activity was noted with 1 µM (0.08 ± 5.28% increase, not significant) compared to a 10 mM glutamate control (45.44 ± 8.85% cell viability) (***Figure 3B***). Therefore, it can be postulated that tianeptine may exhibit mitochondria related protective properties in the absence of a stressor but, during stressor involved situations, i.e. depression, this drug may exert its neuroprotective characteristics via an alternative intra-cellular mechanism.

The normal physiological *myo*-inositol concentration in the human brain is determined to range between 2 mM and 15 mM, and the lower limit of this concentration range was employed as the *myo*-inositol study concentration (2 mM). No significant change was observed with the MTT results for the incubation of SH-SY5Y cells with the physiological *myo*-inositol concentration (2.3 ± 31.51% increase, not significant) (***Figure 3A***). As seen in ***Figure 3B,*** the co-incubation of *myo*-inositol and glutamate indicated a significant increase in cell viability with *myo*-inositol pre-treatment (12.4 ± 14.18%

increase, $p < 0.001$) compared to a 10 mM glutamate control (45.44 ± 8.85% cell viability). Based on the effect of *myo*-inositol on mitochondrial activity, it can be said that the protective ability of *myo*-inositol is stressor dependant for the study concentration of this substance.

Gabapentin has no effect on the viability of the human SH-SY5Y cells when administered without glutamate (***Figure 3A***) after a 24-hour incubation period. The pharmacological gabapentin concentration tended to increase cell viability (7.49 ± 6.95% increase, not significant) when co-incubated with glutamate (***Figure 3B***). The protective effects of gabapentin on the mitochondria can thus be said to be stressor dependant.

Mirtazapine, administered with of without 10 mM glutamate, had no effect on the viability of human SH-SY5Y cells, as can be seen from ***Figure 3A and B***. Therefore, based on the literature which confirms the clinical efficacy and neuroprotectivity of mirtazapine, it can be postulated from the current MTT results that the protective ability of this drug employs a neuroprotective pathway which is not primarily or necessarily influenced by the cell's mitochondria.

Gene Expression: The results on gene expression suggested an overall tendency for neuroprotection by all antidepressants (***Figure 4***). The neuroprotective mechanisms involved, however, seem to differ among the antidepressants. While fluoxetine only up-regulated the expression of genes involved in anti-apoptotic pathways, lithium, mirtazapine and gabapentin only down-regulated the expression of genes involved in pro-apoptotic pathways, and imipramine, *myo*-inositol and tianeptine did both. Importantly, the specific genes that were up- and down-regulated by these respective antidepressants differed. The data therefore suggests that, while all the prototypes of clinical and experimental antidepressants from different pharmacological classes induce changes in gene expressions that are associated with neuroprotective effects, they do so via differential mechanisms.

Fluoxetine significantly (***Figure 4-fluoxetine***) increases the expression of the genes encoding for the anti-apoptotic proteins **Akt**, **CREB**, **NFκβ** and **Bcl-2**, with a tendency also for **BDNF**. These data suggest that fluoxetine may exert neuroprotective effects. Previous studies also indicated that fluoxetine increases the expression of the anti-apoptotic transcription factor **CREB** [14,42,] as well as of **BDNF** [42]. It is interesting to

note that, in the current study, fluoxetine did not significantly change the expression of the pro-apoptotic genes, compared to the glutamate control. Based on the latter observation it can be postulated that the neuroprotective ability of fluoxetine is rooted in the promotion of cell survival signals, rather than inhibiting signals responsible for cellular demise.

Figure 4-imipramine, which compares the effect of the imipramine pre-treatment regime (71 nM imipramine + 10 mM glutamate) to a glutamate (10 mM) control, indicates that the pharmacological imipramine concentration is able to significantly increase the expression of the anti-apoptotic transcription factor **NF-κβ** (163.02 ± 75.91% increase). The imipramine data for **Akt** expression correlates well with previous studies, which found that no significant change in overall **Akt** expression (19.74 ± 30.20% decrease, not significant) is brought about by imipramine pre-treatment [60]. **CREB**, **BDNF** and **Bcl-2** gene up-regulation was also noted in previous imipramine studies however, these results were not confirmed by this study, though a tendency for **Bcl-2** up-regulation (48.87 ± 54.28% increase, not significant) was observed [14,30,49]. Imipramine was also able to significantly reduce the expression of **Bax** (64.36 ± 11.74% decrease) and **Caspase-3** (79.64 ± 9.31% decrease), which are both pro-apoptotic mediators. From the above-mentioned results can be speculated that imipramine exerts its neuroprotective characteristics mainly by inhibiting pro-apoptotic mediators, although an increase in selected anti-apoptotic substances might also play a pivotal role.

Lithium (***Figure 4-lithium***) seems to exert its protective abilities by decreasing the gene expressions of pro-apoptotic proteins during the co-administration with glutamate. Past research found that lithium significantly increases the expression of **CREB** and **Bcl-2**, and decreases the expression of **Bax** [45,46,48]. Although the above-mentioned increases in anti-apoptotic gene expression were not observed in the current study, the decrease seen in **Bax** (60.70 ± 6.84% decrease, significant) expression correlates with previous research [45]. A significant decrease is also noted in **Caspase-3** (60.75 ± 7.36% decrease) and **Caspase-8** (79.89 ± 4.99% decrease) expression compared to their corresponding expressions in the 10 mM glutamate control. Because lithium is an inhibitor of the IMPase enzyme [61], the anti-apoptotic characteristics of this drug might be caused by an increase in *myo*-inositol 1-phosphate as well as reduced metabolic oxidative stress in the brain [62]. Based on the current data it can be said that the

dominant route for lithium induced neuroprotection is via the inhibition of cell death inducing factors.

Figure 4-tianeptine compares a 10 mM glutamate control to the specified tianeptine pre-treatment regime, which indicated significant increases in the anti-apoptotic genes **BDNF**, **Akt**, **CREB** and **Bcl-2**. The expression of **NF- κβ** was not significantly increased, although, a very strong tendency for **NF- κβ** gene up-regulation (235.31 ± 112.15% vs. 100 ± 6.58% control) can be noted. The pharmacological tianeptine concentration was also able to significantly decrease the expression of the pro-apoptotic genes **Caspase-3** and **Caspase-8**, in the presence of glutamate (***Figure 4-tianeptine***). From these rt^2-PCR results can be concluded that tianeptine exerts remarkable protective properties in the presence of glutamate which correlates well with findings in the literature [56,63].

Compared to a glutamate-control the experimental antidepressant *myo*-inositol demonstrated an excellent neuroprotective potential (***Figure 4-myo-inositol***). This substance significantly increased the anti-apoptotic gene expression of **BDNF** and **CREB**; and decreased the expression of pro-apoptotic **Caspase-3** and **Caspase-8**. The up-regulation of both **CREB** and **BDNF** correlates well with the fact that **CREB** is a transcription factor responsible for the regulation of **BDNF** expression. *myo*-Inositol administration ultimately leads to an increase in brain inositol metabolites and the second messenger molecule IP_3, which increases the activation of anti-apoptotic Akt and Bad, and might be responsible for the antidepressant properties of this substance [64]. Another possibility is that the increase in *myo*-inositol 1-phosphate is responsible for the antidepressant capacity of this substance, and this assumed mechanism also correlates well with the suspected antidepressant effectivity of lithium (reduces inositol dependant intra-cellular signalling for the treatment of mania whilst accumulating *myo*-inositol 1-phasphate in the brain for the treatment of depression) [61,62].

Significant pro-apoptotic gene down-regulation can be observed when the gabapentin pre-treatment data are compared to the 10 mM glutamate control (***Figure 4-gabapentin***). The expression of the pro-apoptotic factors **Caspase-3** and **Caspase-8** decreases significantly in comparison with their respective glutamate-controls. Thus, the protective characteristics of gabapentin can be attributed to the ability of this drug to significantly decrease pro-apoptotic gene expression at the pharmacological concentration, in the presence of glutamate.

From ***Figure 4-mirtazapine*** it is evident that the neuroprotective characteristics of mirtazapine is at least partially attributed to this drug's ability to significantly decrease the expression of the pro-apoptotic genes **Caspase-3** and **Caspase-8** compared to the their respective glutamate controls.

It can be concluded that the data from the current study support the hypothesis that the therapeutic efficacy of antidepressants may at least partially be attributable to their neuroprotective effects. The current study is, as far as we know, the first to investigate neuroprotective properties of a series of prototypes of clinical and experimental antidepressants from different pharmacological classes in a single neuronal model, allowing direct comparison. In this regard the study furthermore suggests that all antidepressants may potentially possess neuroprotective effects, but that they do so via different mechanisms. The current findings warrant further investigations to delineate in more detail the mechanisms underlying the neuroprotective effects of antidepressants, to confirm these results in *in vivo* animal studies and eventually, where possible, from human studies.

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Tables

Table 1

Concentration ranges used for determination of free drug fractions					
Plasma albumin bound drug	**Study concentration**	**Total concentrations, as spiked into Ham's F12 ± 10% FBS (nM)**			
		1	**2**	**3**	**4**
Fluoxetine	97 nM	80	4280	8480	12680
Imipramine	71 nM	70	3870	7670	11470
Mirtazapine	169 nM	150	3950	7750	11550
Tianeptine	1 µM	1000	4000	8000	12000

Figures

Figure 1

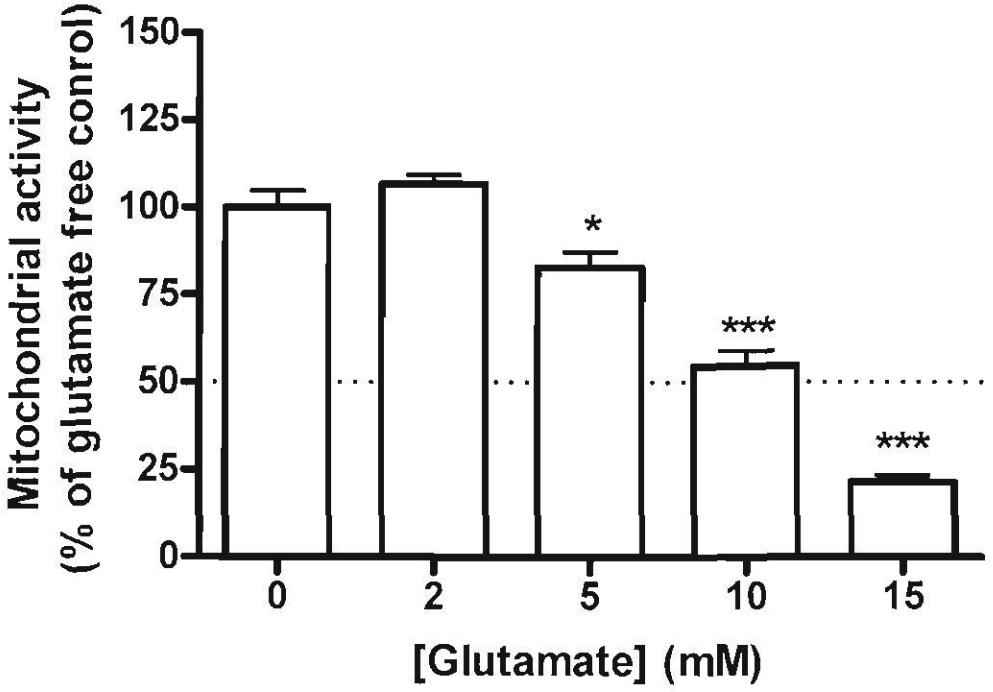

Figure 2

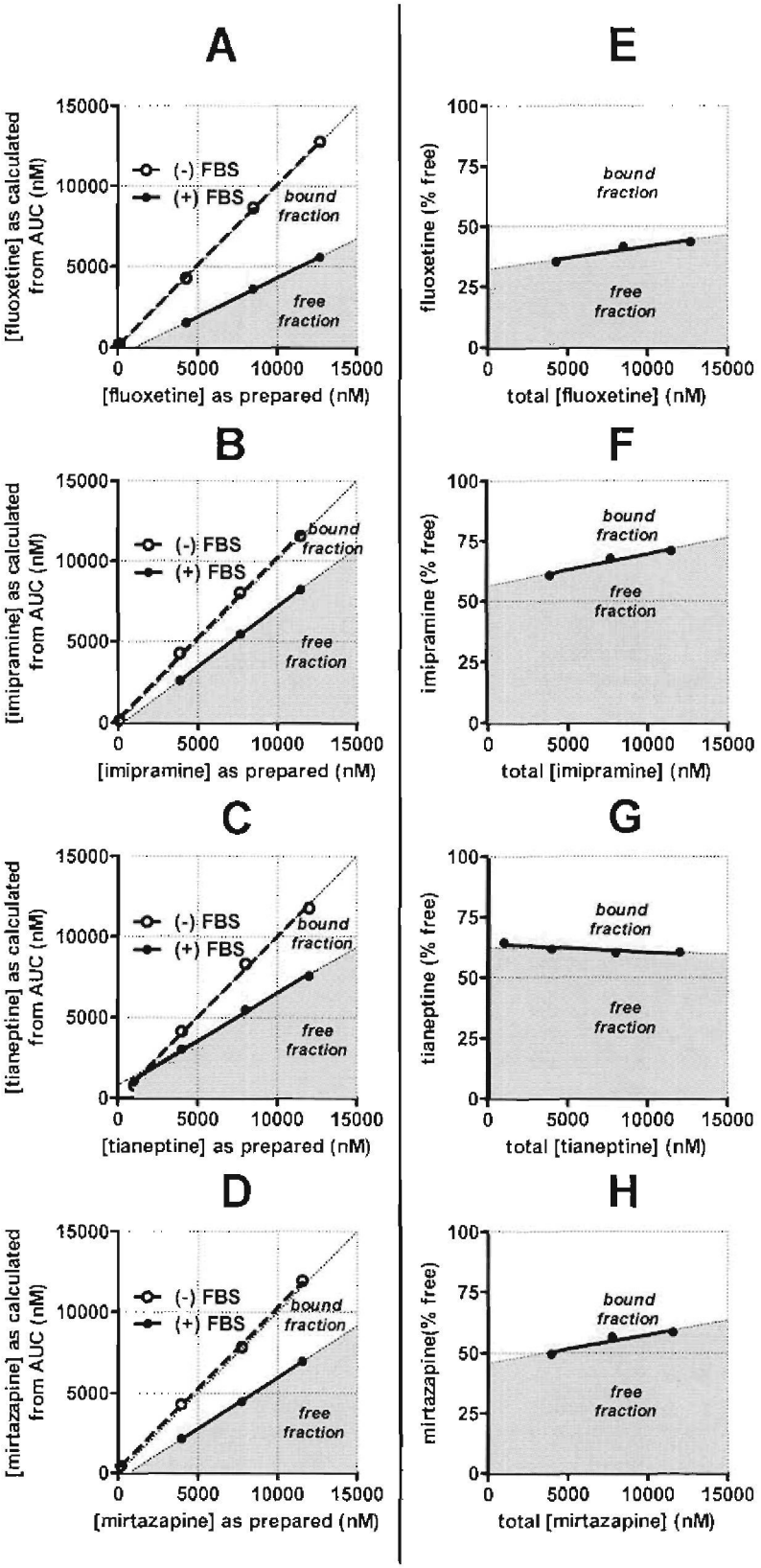
A
[fluoxetine] as calculated from AUC (nM)
(-) FBS
(+) FBS
bound fraction
free fraction
[fluoxetine] as prepared (nM)
E
fluoxetine (% free)
bound fraction
free fraction
total [fluoxetine] (nM)
B
[imipramine] as calculated from AUC (nM)
[imipramine] as prepared (nM)
F
imipramine (% free)
total [imipramine] (nM)
C
[tianeptine] as calculated from AUC (nM)
[tianeptine] as prepared (nM)
G
tianeptine (% free)
total [tianeptine] (nM)
D
[mirtazapine] as calculated from AUC (nM)
[mirtazapine] as prepared (nM)
H
mirtazapine(% free)
total [mirtazapine] (nM)

Figure 3

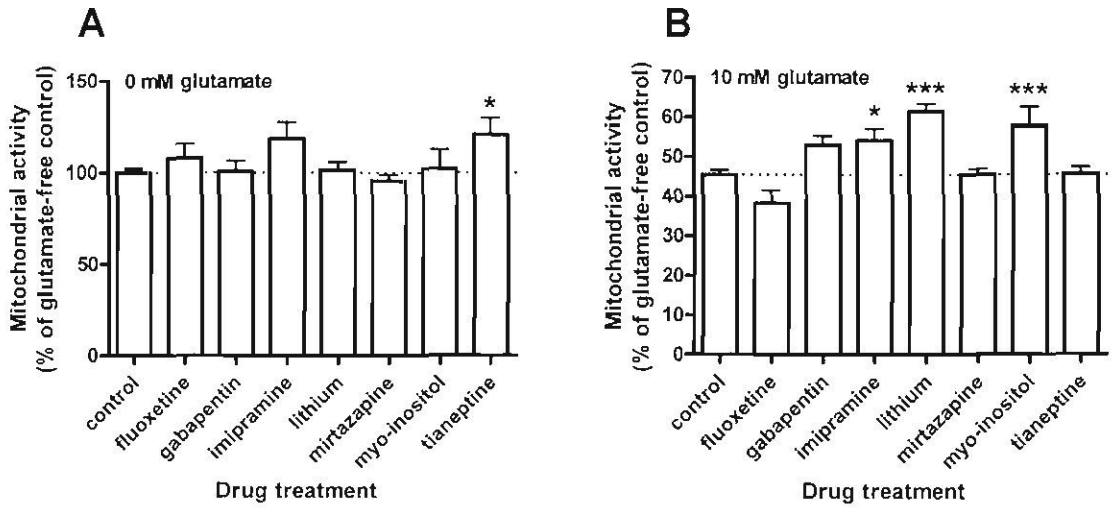
A
0 mM glutamate
Mitochondrial activity
(% of glutamate-free control)
150
100
50
0
*
control
fluoxetine
gabapentin
imipramine
lithium
mirtazapine
myo-inositol
tianeptine
Drug treatment
B
10 mM glutamate
Mitochondrial activity
(% of glutamate-free control)
70
60
50
40
30
20
10
0
*
***
***
control
fluoxetine
gabapentin
imipramine
lithium
mirtazapine
myo-inositol
tianeptine
Drug treatment

Figure 4

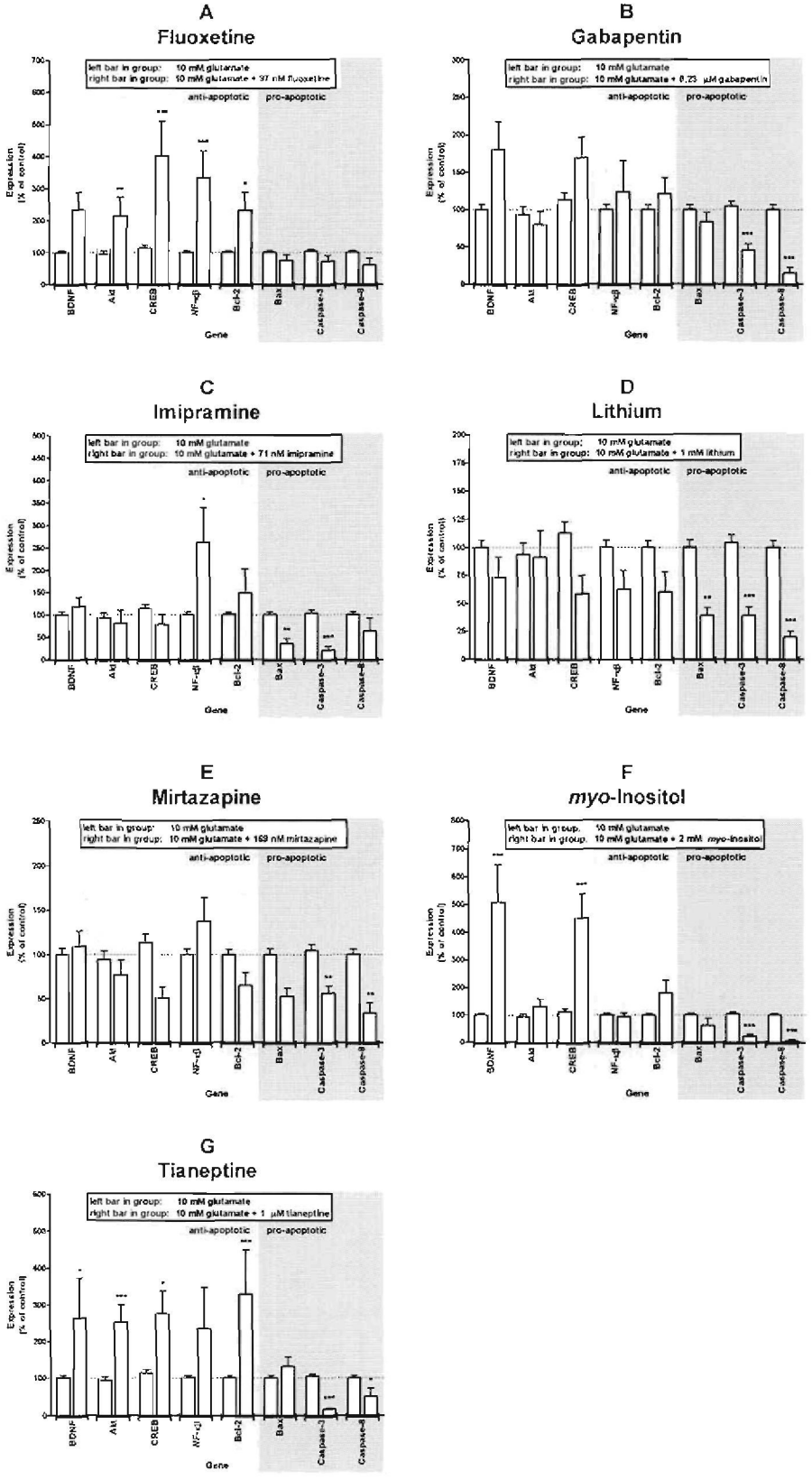
A
Fluoxetine
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 97 nM fluoxetine
anti-apoptotic
pro-apoptotic
Expression (% of control)
BDNF
Akt
CREB
NF-κβ
Bcl-2
Bax
Caspase-3
Caspase-8
Gene
B
Gabapentin
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 8.23 μM gabapentin
C
Imipramine
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 71 nM imipramine
D
Lithium
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 1 mM lithium
E
Mirtazapine
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 169 nM mirtazapine
F
myo-Inositol
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 2 mM myo-inositol
G
Tianeptine
left bar in group: 10 mM glutamate
right bar in group: 10 mM glutamate + 1 μM tianeptine

Figure and Table legends

Figure 1: Determination of the optimum glutamate co-treatment concentration for human neuroblastoma (SH-SY5Y) cells via the standard MTT cell viability assay using a concentration range of 2 mM, 5 mM, 10 mM and 15 mM glutamate. The data are expressed as percentage increase/decrease compared to a glutamate-free control. Data are averages of two independent experiments, each with triplicate observations and expressed as the mean ± SEM, with statistical significance given as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Figure 2: (A-D) The difference in total and free concentrations of the protein bound study drugs in Ham's F12 medium as measured by HPLC analysis. (E-H) Demonstration of the relationship between the concentration of the protein bound study drugs and the percentage of free and bound study drug in Ham's F12, enriched with 10% FBS.

Figure 3: Effect of pharmacological concentrations prototype and experimental antidepressants on mitochondrial activity of human neuroblastoma (SH-SY5Y) cells, either (A) without glutamate or (B) in the presence of 10 mM glutamate. Data are averages of three independent experiments, each with triplicate observations and expressed as the mean ± SEM, with statistical significance indicated as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Figure 4: Effect of 24-hour incubation of human neuroblastoma (SH-SY5Y cells) with prototype and experimental antidepressants on pro- and anti-apoptotic gene expression, after glutamate induced excitotoxicity. Data are averages of four independent experiments, each with duplicate observations and expressed as the mean ± SEM, with statistical significance given as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Table 1: Concentration ranges used for determination of free drug fractions in Ham's F12 culture medium + 10% foetal bovine serum. These concentration ranges were chosen to cover the concentration ranges needed for drug treatment.

Chapter 4 Summary and Conclusions

4.1 Summary and discussion of results

The current study was aimed at determining the intracellular changes as well as the pathways by which prototype and experimental antidepressants exert their neuroprotective characteristics. In order to obtain these results human SH-SY5Y cells were pre-treated for 24 hours with a predetermined pharmacological, low or high concentration of fluoxetine, imipramine, lithium, gabapentin, mirtazapine, tianeptine or *myo*-inositol, respectively. Firstly an optimum glutamate concentration was determined for the induction of cytotoxic effects in the human SH-SY5Y cells via MTT cell viability assay. Hereafter the free percentage of the plasma albumin bound study drugs (fluoxetine, imipramine, mirtazapine and tianeptine) was determined by HPLC analyses of respective concentration ranges, with of without the addition of 10% FBS. The effect of the different study drug concentrations on cell viability was determined in the presence or absence of 10 mM glutamate by means of the standard MTT cell viability assay. After this, the effect of the pharmacological/low drug concentrations, in combination with 10 mM glutamate, was determined on gene expressions of selected pro- and anti-apoptotic proteins (anti-apoptotic: CREB, BDNF, Bcl-2, NF-κβ and Akt; pro-apoptotic: Bax, caspase-3 and caspase-8) via quantitative rt^2-PCR.

The MTT assay results indicated that the optimum concentration of glutamate needed to induce a significant decrease of about 50% in the viability of the human SH-SY5Y cells after a 24-hour incubation period is 10 mM (45 ± 4.3%). Glutamate concentrations of 2 mM, 5 mM, and 15 mM were also tested but the cell viability results were unfavourable in the context of this study (2 mM: 7 ± 2.7% increase; 5 mM: 17 ± 4.5%; 15 mM: 89 ± 2.1%)

The HPLC results of the free and bound percentage drug versus the study drug concentration of fluoxetine, imipramine, mirtazapine and tianeptine in Ham's F12 + 10% FBS are listed in ***Table 4-1***.

Table 4-1: *The HPLC determined free and bound fractions (% of total) of the plasma albumin-bound study drugs.*
The "Concentration used" column represents the concentration of the drug added to the Ham's F12 medium + 10% FBS to obtain the needed free concentration ("Concentration needed" column).

Free and bound drug (%) in Ham's F12 enriched with 10% FBS				
Drug	**Concentration needed (free)**	**Free drug (%)**	**Bound drug (%)**	**Concentration used (total)**
Fluoxetine	97 nM	32.16	67.84	301.62 nM
	10 µM	41.69	58.31	23.99 µM
Imipramine	71 nM	56.35	43.65	126.00 nM
	10 µM	69.70	30.30	14.35 µM
Mirtazapine	169 nM	45.99	54.01	367.47 nM
	10 µM	57.52	42.48	17.39 µM
Tianeptine	1 µM	61.95	38.05	1.61 µM
	10 µM	60.47	39.53	16.54 µM

After the human SH-SY5Y cells were pre-treated for 24 hours with a pharmacological/low or high antidepressant concentration ± 10 mM glutamate, the cells were analysed via MTT cell viability assay. Cells were also incubated with a pharmacological/low antidepressant concentration and 10 mM glutamate for 24 hours, and analysed by means of quantitative rt^2-PCR. The latter results were statistically analysed by comparing the study sample values to those of a 0 M and 10 mM glutamate control. The MTT results as well as the rt^2-PCR results are summarised in ***Table 4-2***.

Table 4-2: *The effect of a 24-hour incubation period with the respective drug pre-treatment regimes, on the viability and apoptotic gene expression of human SH-SY5Y cells as measured via the standard MTT cell viability assay and quantitative rt^2-PCR.*
A horizontal arrow denotes no effect, a downward arrow a reduction and an upward arrow an increase in the mitochondrial activity. The amount of arrows correlates with the degree of significance of the change in cell viability or gene expression.

Drug (24 hours)	MTT				rt^2-PCR													
					anti-apoptotic										*pro-apoptotic*			
					BDNF		Akt		CREB		NF-κβ		Bcl-2		Bax		Casp3		Casp8	
	10 mM glutamate?																			
	[low]		[high]		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
	-	+	-	+																
fluoxetine	↔	↔	↔	↓↓↓	↔	↔	↔	↑↑	↔	↑↑↑	↔	↑↑↑	↔	↑	↔	↔	↔	↔	↔	↔
gabapentin	↔	↔	↔	↑	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓↓↓	↔	↓↓↓
Imipramine	↔	↑	↓↓	↔	↑↑	↔	↔	↔	↔	↔	↔	↑	↔	↔	↔	↓↓	↔	↓↓↓	↔	↔
lithium	↔	↑↑↑	↓	↑↑↑	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓↓	↔	↓↓↓	↔	↓↓↓
mirtazapine	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↓↓	↔	↓↓
myo-inositol	↔	↑↑↑	↔	↔	↔	↑↑↑	↔	↔	↔	↑↑↑	↔	↔	↑	↔	↔	↔	↔	↓↓↓	↔	↓↓↓
tianeptine	↑	↔	↑	↔	↔	↑	↔	↑↑↑	↔	↑	↔	↔	↔	↑↑↑	↔	↔	↔	↓↓↓	↔	↓

In the current study the pharmacological/low concentration of the prototype antidepressants imipramine and lithium, as well as *myo*-inositol, protected against the glutamate-induced reduction of mitochondrial activity, as depicted in ***Table 4-2***. A common reason for the protective characteristics of lithium and myo-inositol might be that chronic administration of these substances cause an increase in *myo*-inositol 1-phosphate in the brain. However, the results suggest that all three these drugs may possess neuroprotective properties during neuronal stress. Nevertheless, these drugs had no significant effect on neuronal viability in the absence of glutamate, suggesting that their protective abilities are stress-dependant. Tianeptine, on the other hand, increased the viability of the cells in the absence of glutamate, but had no significant effect on mitochondrial activity in the presence of glutamate. Therefore, considering the evidence in the literature that this antidepressant does indeed have neuroprotective effects (Kuroda & McEwen, 1998; Wagstaff *et al.*, 2001; McEwen & Olie, 2005), we conclude that tianeptine exerts its neuroprotective characteristics via an alternative intra-cellular mechanism that is not directly evident in our neuronal cell culture model.

In the presence of glutamate, the high concentration (± 10 X pharmacological concentration) of fluoxetine significantly reduced mitochondrial activity, which suggests that high fluoxetine concentrations augment the damaging effects of glutamate and is cytotoxic. The high lithium concentration significantly decreased mitochondrial activity however, the co-administration of a high lithium concentration along with glutamate caused a significant increase in the viability of the cells. This indicates that high lithium concentrations are only neuroprotective in the presence of a stressor, such as glutamate. High gabapentin concentrations also proved to be helpful in protecting against glutamate induced excitotoxicity.

The results in ***Table 4-2*** also indicate that high imipramine concentrations influence the viability of cells in a negative manner.

The rt^2-PCR results indicated an overall tendency for neuroprotection by all antidepressants (***Table 4-2***). Fluoxetine significantly increases the expression of CREB, which correlates with results from previous studies (Nibuya *et al.*, 1996; Koch *et al.*, 2003). Previous research found that fluoxetine significantly increases the expression of BDNF, although the results from this study only indicated a tendency for BDNF gene up-regulation (Song *et al.*, 2006). The genes encoding for the anti-apoptotic

transcription factors Akt and NF-κβ also significantly increased with fluoxetine pre-treatment.

Gabapentin, lithium and mirtazapine seem to exert their protective abilities by decreasing the gene expressions of pro-apoptotic proteins. Past research found that lithium significantly increased the expression of CREB and Bcl-2, and decreased the expression of Bax (Nonaka & Chuang, 1998; Fukumoto *et al.*, 2001; Bush & Hyson, 2006). Although increases in anti-apoptotic gene expression were not observed, the decrease seen in Bax expression correlates with previous research (Chen *et al.*, 1999; Chen & Chuang, 1999; Bush & Hyson, 2006). Lithium also inhibits the enzyme *myo*-inositol monophosphatase (IMPase) (Parthasarathy *et al.*, 1997), a key enzyme in the formation of free inositol (Brandish *et al.*, 2005). Because lithium inhibits *myo*-inositol 1-phosphate to be further transformed to free inositol and ultimately intra-cellular signalling molecules (reduces inositol dependant neuronal signalling in the treatment of mania) it causes *myo*-inositol 1-phosphate to accumulate in the brain, which might contribute to its effectiveness in the treatment of depression (Eden *et al.*, 2006). The imipramine data for Akt expression correlates well with previous studies, which found that no significant change in overall Akt expression is brought about by imipramine pre-treatment (Basta-Kaim *et al.*, 2005). CREB, BDNF and Bcl-2 gene up-regulation was also noted in previous imipramine studies however, these results were not confirmed by this study though a tendency for Bcl-2 up-regulation was observed (Koch *et al.*, 2003; Van Hoomissen *et al.*, 2003). The experimental antidepressant *myo*-inositol demonstrated an excellent potential as neuroprotective agent, by increasing anti- and decreasing pro-apoptotic gene expression. These observations might be caused by an increase in *myo*-inositol metabolites and a corresponding increase in the intra-cellular second messenger molecule IP_3, which activates anti-apoptotic Akt and Bad (Hu *et al.*, 2001), and might enhance neuronal synaptic communication and overall plasticity (Berridge & Irvine, 1989; Berridge, 1993; Groenen *et al.*, 2006). The antidepressant effectivity of *myo*-inositol might also be attributed to the corresponding increase in *myo*-inositol 1-phosphate, which correlates well with the suspected antidepressant efficacy of lithium (Parthasarathy *et al.*, 1997; Brandish *et al.*, 2005; Shaldubina *et al.*, 2006). Tianeptine has previously been found to be a superior neuroprotective agent, as is evident from the results regarding this drug's ability to significantly increase genes encoding for neuronal survival as well as decreasing the genes encoding for neuronal

demise (Kuroda & McEwen, 1998; Wagstaff *et al.*, 2001; Plaisant *et al.*, 2003; Alfonso *et al.*, 2006).

4.2 Conclusions

In the current study, it was demonstrated that the free fraction of the implicated study drugs can be determined via HPLC analysis. Being able to treat cultured cells with drugs in medium containing bovine serum albumin, prevent acute stress on cultured cells due to serum deprivation. In addition, the use of pharmacological concentrations (peak plasma concentrations) of drugs renders the results obtained in the *in vitro* study more applicable and comparable to an *in vivo* situation.

This study also established a glutamate co-treatment regime (10 mM glutamate for 24 hours), whereby the viability of human SH-SY5Y cells can be significantly reduced, which enables researchers to determine the putative neuroprotective or neurodegenerative properties of drugs in the implicated cell line. Importantly, glutamate has also been postulated to play a role in stress-related central disease, such as depression and post-traumatic stress disorder (PTSD), therefore the glutamate co-treatment of cells enables the testing of drugs in a stress-related environment.

The main objective of the current study was to determine the role of mitochondrial activity and apoptosis in the neuroprotective actions of the selected novel and prototype antidepressants. The current data indicate that all the study drugs (prototype and experimental antidepressants) induce changes in intra-cellular biochemistry, which is likely to ultimately enhance neuronal resilience. In the current study evidence is provided that all the study antidepressants (experimental and clinical prototype) are neuroprotective, either by increasing mitochondrial activity, decreasing pro-apoptotic gene expression, increasing anti-apoptotic gene expression or a combination thereof. Importantly, most prominent results suggesting neuroprotective activity of the antidepressants drugs, measuring both mitochondrial activity and gene expression, were found in the presence of excitotoxicity (glutamate), suggesting that the antidepressants may selectively display neuroprotective activity in states of neuronal stress.

Moreover, the current study corroborates previous data in the literature, as well as provides useful and novel information regarding the intra-cellular pathways by which

antidepressants exert their neuroprotective effects. Furthermore, the results support the neurodegenerative hypothesis of depression, which states that the effectiveness of an antidepressant is at least partially connected to its neuroprotective properties.

4.3 Recommendations and prospective studies

Although the majority of results indicate significant differences when compared to the applicable control, a reasonable number of trends are also observed. These trends could prove to be significant if the number of assay repetitions and/or the number of similar samples (n) in each assay is increased.

Due to practical limitations of this study, the employed cell line could only be pre-treated for 24 hours. Adapting the pre-treatment method in such a manner that it would allow for longer pre-treatment periods could increase the accuracy and applicability of the results to an *in vivo* situation. A possible solution for this would be to pre-treat the cells in culture flasks rather than seeding the cells into 24-well plates before pre-treatment. The latter would allow regular splitting of cells, while excluding the problem of growth inhibition or cell death due to overgrowth, especially if the pre-treatment is started on a relatively small (diluted) cell culture colony. If the pre-treatment is handled correctly, the cells in the culture flask could be treated for up to a week before the cell capacity limit of the flask will start to affect the study results. If pre-treatment is approached in this manner the obtained results may provide a clearer picture of the effects of chronic treatment, and hence clinical *in vivo* effects. This may be particularly relevant since antidepressants have a delayed onset of action of about two weeks. Alternatively brain tissue of animals treated chronically with antidepressants can also be analysed. An additional advantage of this approach is that differential effects in different areas of the brain can be investigated, in particular regions that are associated with depression and antidepressant action.

The current study may also be repeated on other neuronal cell lines in order to determine the difference in cellular response between the cell lines, as well as determining the optimum cell line for this type of research.

The possible involvement of stress (glucocorticoids) and pro-inflammatory cytokines in the development of depression is widely discussed in the literature. Therefore, it would

be useful to repeat the current study using an implicated glucocorticoid or pro-inflammatory cytokine as cellular stressor, in stead of glutamate.

Due to constraints during this study, no Western Blots were conducted to verify the quantitative rt^2-PCR results. Therefore, the effect of the altered gene expressions on the expression of the corresponding proteins can be determined by means of Western Blotting in prospective studies.

Appendix A
Materials and Methods

A.1 Materials

A.1.1 Cells and medium used for cell culture maintenance

For this study a human neuroblastoma cell line (SH-SY5Y cells) from ATCC® in the United States of America (ATCC Number: CRL-2266™) was used.

The following media and additives were purchased from Gibco, Invitrogen for cell culture maintenance:

- Ham's F12 culture medium
- 10% Foetal bovine serum (FBS)
- Trypsin/versin (1:250 + 0.38% EDTA)
- Penicillin, streptomycin and fungizone

A.1.2 Chemicals applied for study preparation and assays

Reagents and substances obtained from Sigma:

- *myo*-Inositol, lithium chloride (LiCl), imipramine hydrochloride, mirtazapine and gabapentin.
- MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
- *Iso*-propanol (2-propanol)
- L-glutamic acid sodium salt hydrate (glutamate)

From Saarchem, Unilab:

- Formic acid
- Absolute ethanol

Fastlane cDNA kits were purchased from Qiagen, DNase and RNase free water from Celtic Molecular Diagnostics (Bioline), peroxide from Medicolab, CybrGreen from Bio

Rad and rt^2-PCR primer assays from Quantitec. Fluoxetine hydrochloride was a kind gift from Eli Lilly and tianeptine was obtained from Servier.

A.1.3 Consumables

From Corning Incorporated:

- Culture flasks (150 cm^2)
- 24- and 96-well plates
- Sterile conical tubes (15 ml and 50 ml)

From Bio Rad:

- 96-well rt^2-PCR plates
- Optical tape

Pipette tips (10 µl, 100 µl, 1 ml, 5 ml) were bought from Eppendorf, DNase and RNase free eppendorf tubes and pipette tips were from WhiteSci Quality Scientific Plastics and Ultrafree-MC Microcentrifuge tubes (30 kDa) from Sigma-Aldrich.

A.1.4 Instrument and software

The following software and instruments were implicated in the experimental execution.

- Haemocytometer (0.1 mm depth, 0.0025 cm^2) and Nikon TMS inverted microscope (model number: 31771) were employed for cell counting.
- Analytical measurements were made with quantitative Eppendorf micropipettes (10 – 100 µl and 100 – 1 000 µl).
- A 96-well plate reader (Ladsystems Multiscan RC) with a 560 nm filter was used to determine the absorbance of the MTT formazan product.
- A 3K15 bench top centrifuge from Sigma-Aldrich (Johannesburg, South Africa) was used for sample centrifugation.
- The BioRad iCycler® was used for quantitative rt^2-PCR quantification.

A.2 Methods

A.2.1 Cultured cell preparation

Human SH-SY5Y cells (neuroblastoma cells) were maintained in Ham's F12 culture medium, 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone in culture flasks at 37°C in 5% CO_2 and humified atmosphere, and grown to 90% confluency. Cells were detached from culture flasks by means of trypsination (& EDTA) and seeded into 24-well plates at a density of ± 5 x 10^5 cells/well. The well plates were then incubated for 48 hours at 37°C and 5% CO_2, to allow the cells to attach to the well bottoms, while cell growth and multiplication during the 48 hours also resulted in a cell quantity of approximately two million cells per well.

A.2.2 Drug treatments

After the 48 hour preparation of cultured cells, the cells were treated (incubated) for 24 hours with specified concentrations of the study drugs in the presence or absence of 10 mM glutamate (sufficient to reduce mitochondrial activity with 50%, as determined by MTT assay – see § A.2.5). The concentrations selected for each drug was the reported peak plasma concentration (denoted the pharmacological or "low" concentration) and a higher concentration (usually 10x the "low" concentration, denoted the "high" concentration). Drug concentrations represented the free (not bound to FBS protein – as determined by HPLC) concentration.

The cell line employed in this study was of a cancerous origin, and has a duplication time of about 30 hours. The 24-hour incubation period of the study cell lines under artificial *in vitro* conditions may be sufficient to elicit cellular responses seen in chronic *in vivo* drug treatments. Several studies also indicated that 24 hours is sufficient for anti-depressant therapeutics to alter gene expression and increase overall cellular resilience (Brink *et al.*, 2004).

A.2.3 Concentrations used in pre-treatment

A.2.3.1 Fluoxetine (97 nM):

This anti-depressant was obtained from Eli Lilly and has a peak plasma concentration of 200 – 531 ng/ml *in vivo* and is 94% plasma protein bound in humans (Goodman &

Gilman, 2001). Thus, for a concentration of 500 ng/ml, the 6% free fraction fluoxetine can be calculated as 30 ng/ml (97 nM). It was furthermore determined with HPLC analyses (see § A.2.4) that the free fraction of fluoxetine ranges between 32.1% - 46.5% for a concentration range of 80 nM – 15 µM. Therefore, to obtain a free concentration of 97 nM in culture medium enriched with 10% FBS, 301.24 nM fluoxetine was added to the cells (67.8% bound to plasma proteins rendering 32.2% free or unbound drug). A concentration of 10 µM was used as a high concentration. This concentration was adapted to 24 µM after HPLC analysis (58.3% bound to plasma proteins rendering 41.7% free or unbound drug) to yield 10 µM free drug in Ham's F12 medium + 10% FBS.

A.2.3.2 Gabapentin (0.23 µM):

Gabapentin is not significantly plasma protein bound (less than 3%), therefore the normal pharmacological concentration of 0.23 µM and a high concentration of 10 µM were administered without any concentration adaptation. Gabapentin was purchased from Sigma.

A.2.3.3 Imipramine (71 nM):

Imipramine was purchased from Sigma and has a peak plasma concentration of 200 ± 137 ng/ml and is approximately 90.1% plasma protein bound. The study concentration for this drug was calculated as 9.9% of 200ng/ml which yielded a concentration of about 20ng/ml (71 nM). A concentration of 10 µM was used as the high study concentration for imipramine. Because of the highly plasma protein nature of this drug, the free fraction of this drug was determined in Ham's F12 media via HPLC analysis. The HPLC analyses indicated that the free fraction of imipramine ranged between 56.4% - 76.4% for a concentration range of 70 nM – 15 µM. The administered drug concentration was then adapted correspondingly to yield a free drug concentration of 71 nM (free fraction 56.4%; total drug administered 125.9 nM) and 10 µM (free fraction 69.7%; total drug administered 14.3 µM) respectively, in the implicated culture medium.

A.2.3.4 Lithium (1 mM):

Lithium chloride has a maximal plasma concentration of 1 – 2 mM. Because of the narrow therapeutic window and high toxicity of lithium, 1 mM and 10 mM were administered as the pharmacological and high pre-treatment doses respectively.

Lithium's concentration is unaffected by the presence of plasma proteins, therefore no concentration adaptation was needed. Lithium chloride obtained from Sigma was utilised in the current study.

A.2.3.5 Mirtazapine (169 nM):

This drug was obtained from Sigma and is 85% plasma protein bound and has a peak concentration of 41.8 ± 7.7 ng/ml. A concentration of 169 nM was used as the low, and 10 µM as the high study concentration. The administered concentrations were adapted by means of HPLC analysis due to the plasma protein bound nature of this drug. The HPLC results indicated that the free fraction of mirtazapine is between 45.8% - 63.4% for a concentration range of 150 nM – 15 µM. Therefore the administered concentrations were adapted to 367.4 nM (46% free) and 17.4 µM (57.5% free) to yield free concentrations of 169 nM and 10 µM respectively.

A.2.3.6 Myo-inositol (2 mM):

The normal brain concentration of *myo*-inositol has been determined to range between 2 and 15 mM. Therefore, a concentration of 2 mM was administered as the low and 15 mM as the high concentration in the pre-treatment regime. *myo*-Inositol is a derivative of glucose and protein binding has no effect on the effective concentration of this compound. Sigma was used as supplier for this substance.

A.2.3.7 Tianeptine (1 µM):

Tianeptine, obtained from Servier, is 94% bound to plasma albumin (Zini *et al.*, 1990). A study conducted by Plaissant and colleagues (2003) used 1 µM, 10 µM and 100 µM tianeptine concentrations for the pre-treatment of cortical cell cultures. Therefore 1 µM and 10 µM tianeptine concentrations were used for the pre-treatment of the study cells. Because of the influence of plasma proteins on the concentration of this drug, a concentration range of 1 µM – 15 µM was HPLC analysed to determine the respective free fractions of tianeptine. The results indicated that the free fraction of tianeptine is 61.9% - 59.6% for the implicated concentration range. Hereafter the administered drug concentration was adapted to 1.62 µM (61.9% free) and 16.5 µM (60.5% free) in order to yield a free fraction of 1 µM and 10 µM, respectively in Ham's F 12 enriched with 10% FBS. The adapted concentrations were determined via HPLC analysis.

A.2.4 HPLC determination of free drug concentration

The free fraction of plasma protein-bound drugs (fluoxetine, imipramine, mirtazapine and tianeptine) were determined by spiking known concentrations of these drugs into Ham's F12 medium ± 10% FBS. The samples containing FBS were centrifuged in microcentrifuge tubes from Sigma-Aldrich with a pore size of 30 kDa to remove the protein-bound drug. Bovine albumin has a molecular weight of 66.3 kDa to 69 kDa and therefore a pore size of 30 kDa is sufficient to remove all the plasma albumin and bound drug in the implicated samples (Tanaka *et al.*, 2001). The drug concentrations of all samples were analysed by HPLC, using a 30% acetonetrile/0.005 M heptane sulphonic acid sodium salt in water (v/v) mobile phase (pH 3.5), flow rate 1.0 ml/min, 150 x 4.6 mm Luna C18-2 column (100 Å pores), at a UV detection wavelength of 228 nm. The obtained areas under the curves were used to determine the free fraction of the plasma albumin bound drugs.

A.2.5 MTT cell viability assay

The MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) was developed by T Mosmann in 1983. This test is a standard, sensitive colorimetric test for measuring cellular proliferation. The MTT assay is based on the cleavage of the yellow tetrazolium salt, MTT, to a purple formazan crystal by the mitochondrial enzyme, succinic dehydrogenase, of metabolically active cells (Mosmann, 1983). The latter reduction of tetrazolium salt can only take place when mitochondrial reductase enzymes are active, and therefore the concentration of formazan is directly related to the number of viable cells. The formazan concentration is thereafter determined by lysing the cells to release and dissolve the formazan in a suitable solvent, such as *iso*-propanol (2-propanol), and reading the optical density with a spectrophotometer. The colorimetric signal is proportional to the number of viable cells.

A.2.5.1 MTT-assay Aim

This assay was performed in order to determine the protective capacity of selected prototype and experimental anti-depression treatments on cell viability (mitochondria activity), after glutamate induced excitotoxicity. The optimum glutamate concentration needed for co-treatment was also determined using the MTT-assay.

A.2.5.2 MTT-assay Protocol

The SH-SY5Y cells were seeded and treated as described in § A.2.1 - A.2.3. Additional wells were also seeded to allow for positive controls (0% cell viability) and negative controls (100% cell viability). The cells designated as positive controls were incubated with freshly prepared 0.33% formic acid in ddH_2O for 30 minutes, while the cells designated as negative controls received no drug and were incubated in normal growth medium.

After the 24-hour drug treatments (in the presence or absence of the pre-determined 10 mM glutamate concentration), and following the 30 minute incubation with formic acid when indicated (positive control only), the formic acid or culture medium was aspirated from the cells. The cells in each well were then washed twice with 1 ml phosphate buffered saline (PBS). The MTT reagent was diluted to a 0.5% solution using PBS. Each well then received 200 µl of the 0.5% MTT solution and the well plates were then incubated at 37°C in 5% CO_2 for 2 hours. All assay steps with MTT were executed in low light and incubated in the dark, due to the light sensitivity of the MTT-reagent. After the 2 hour incubation period the MTT-reagent was aspirated from the wells and 250 µl *iso*-propanol (2-propanol) added to each well. The well-plates were then incubated at room temperature for 5 minutes to dissolve the purple formazan crystals. Thereafter 100µl of the formazan-containing iso-propanol were transferred into a 96-well plate and the spectrophotometric absorbance (Abs) read at 560nm. The mitochondrial activity was determined and expressed as percentage of the negative control (100% cell viability) by means of the following equation:

$$\%\ \text{Viable Cells} = 100 \times \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(\text{positive control})}}{\text{Abs}_{(\text{negative control})} - \text{Abs}_{(\text{positive control})}}$$

Where $\text{Abs}_{(\text{sample})}$ is the absorbance of the study sample, $\text{Abs}_{(\text{positive control})}$ is the absorbance of the formic acid treated cells (0% cell viability) and $\text{Abs}_{(\text{negative control})}$ is the absorbance of the untreated cells (100% cell viability), as spectropotometrically determined at 560nm.

A.2.6 Real-time reverse transcriptase polymerase Chain Reaction

Real-time reverse transcriptase polymerase chain reaction (rt^2-PCR) is a laboratory technique used for the simultaneous amplification and quantification of a specific gene (or other section) of a given DNA or RNA (converted to cDNA) molecule. This assay is used to determine whether or not a specific cDNA sequence is present in a sample

(qualitative), and if it is present, the absolute or relative number of copies (quantitative) in the sample. The Quigen Fastlane cDNA kit® (a standardised kit) was used for mRNA extraction from drug-treated SH-SY5Y cells, and the subsequent cDNA synthesis. BioRad SYBR Green® and QuantiTec Primer assay® (specific for each study gene) were added for cDNA amplification during the rt^2-PCR process. The rt^2-PCR samples were analysed using 25 µl reaction volumes and the run-time conditions (§ A.2.6.4) supplied by Quigen®. A RT-blank sample (received no RT Master mix; see § A.2.6.4) and two PCR-blank samples (received no cDNA; see § A.2.6.4) were included into each run to ensure that there was respectively no RT Master mix or cDNA contamination on the PCR-plate.

A.2.6.1 Quantitative rt^2-PCR assay aim

This assay was performed in order to determine the effect of the study pre-treatments on the expression of genes responsible for the regulation of cellular survival (*anti-apoptotic:* BDNF, CREB, Akt, Bcl-2 and NF-κβ; *pro-apoptotic:* Bax, caspase-3 and caspase-8). Thus, it could be determined what the effects of the drugs are on these markers of cellular resilience and therefore suggest a possible mechanism of the putative neuroprotective actions of the study antidepressants.

A.2.6.2 Determination of primer efficiency

A sample was comprised of equal volumes of each study condition lysate. This "mixed" pre-treatment sample was diluted to form a concentration range of four (4) samples. These samples were then analysed in the same manner as the study samples for each study primer (see § A.2.6.4 – A.2.6.5). After rt^2-PCR analysis, graphs were constructed for each study primer by plotting the obtained C_T value against the Log function of the dilution factor. The slopes of the latter graphs were used to determine the efficiency of each primer by means of the following equation:

$$E = 10^{-1/Slope}$$

Where *E* is the primer efficiency and *Slope* is the slope of the C_T value against the Log of the dilution factor graph.

A.2.6.3 Pre-assay Preparation

Following the drug treatments of cells as described in § A.2.2, the preparation for the rt^2-PCR was initiated in a laminar flow cabinet, pre-cleaned with a 70% ethanol and 30% peroxide solution. The cabinet was also radiated with an ultra violet light for at least 30 minutes before each assay run to ensure that the cabinet is DNase and RNase free.

A.2.6.4 mRNA extraction and cDNA synthesis

The culture medium was aspirated from the drug-treated cells. Thereafter the cells in each well were washed using 500 µl FCN buffer (supplied in Quigen Fastlane Kit®). The FCN buffer was then aspirated and 200 µl FCP buffer was added to each well (supplied in Quigen Fastlane Kit®), and incubated at room temperature for 10 minutes to lyse the cells. The lysates were then transferred into DNase- and Rnase-free eppendorf tubes. The gDNA wipe-out buffer, supplied in the Fastlane kit, was diluted by adding 8 µl RNAse free water for each 2 µl gDNA wipe-out buffer (25% dilution). The diluted gDNA wipe-out buffer was then vortexed for no longer than 5 second and briefly centrifuged. Enough of the latter 25% gDNA wipe-out buffer was made to add 10 µl to each lysate sample, as well as 10 µl to an additional real-time blank (RT-blank) sample. Ten micro litres (10 µl) of the buffer dilution was then added to 4 µl of each sample lysate. A randomly chosen lysate sample was used in the RT-blank sample. The samples were then briefly vortexed and centrifuged (no longer than 5 seconds) and placed in a heating block set at 42°C for 5 minutes. Template RNA was formed during the latter step. After the 5 minute incubation the samples were removed from the heating block and placed on crushed ice until the end of the reaction.

A real-time (RT) Master mix stock solution, which amplifies the expression of the implicated gene, was then prepared in a clean eppendorf tube as follows:

- RT-Buffer (kit): 4 µl per sample (preferably add this first).
- Universal primer (kit): 1 µl per sample.
- RT (kit): 1 µl per sample.

The latter stock solution was then flicked 5 times and centrifuged. All the lysate samples then received 6 µl of the RT-Master mix stock except the RT-blank sample. The samples were then flicked (no more than 5 times), briefly centrifuged and placed in a heating block set at 42°C for 30 minutes (cDNA was formed during this step).

A.2.6.5 rt^2-PCR amplification and quantification

After the 30 minute incubation period, the samples were incubated at 95°C for another 3 minutes. This step was included to denaturise the polymerase enzyme, which is responsible for cDNA formation. A primer stock solution was made for each study primer using:

- 2.5 µl primer per sample.
- 12.5 µl Cybergreen Master mix per sample.
- 9 µl RNAse free water per sample.

Two extra eppendorf tubes were marked as "PCR-blank" (no cDNA will be added to these samples), and enough of the above-mentioned primer stock solution was prepared to include these two samples. Hereafter, 24 µl of the primer stock solution was pipetted into each well of a PCR plate. One micro litre (1 µl) of the sample (cDNA) was pipetted into each well containing primer stock. The 2 wells containing the random primer stock for the PCR-blank samples did not receive any cDNA, to equalise the volume of these two wells with the volume of the other sample wells, 1 µl RNAse free water was added to them. The PCR plate was then sealed using optical tape and centrifuged at 3000 rpm (1238 gs) and 4°C for 1 minute.

The PCR run time protocol was adapted to be compatible with the Sybr Green® supplied by BioRad®. The implemented iCycler® conditions are stipulated below.

Table A-1: *The conditions used for rt^2-PCR sample analysis of 25 µl reaction volumes.*

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial activation step	5 min	95°C	Maximal / fast mode	HotStarTaq Plus DNA Polymerase is activated by this step
Two-step cycling				
Denaturation	10 s	95°C	Maximal / fast mode	
Combined annealing/ extension	30 s	60°C	Maximal / fast mode	Perform fluorescence data collection
Number of cycles	35 - 50			The number of cycles needed depends on the original amount of template DNA

A.2.6.6 Determination of gene expression

All the rt^2-PCR data were standardised according to the primer efficiency value of the respective primer. The primer efficiency was determined by means of the following equation:

$$\text{Ratio} = \frac{(E_{\text{sample}})^{\text{Ct(control-sample)}}}{(E_{\text{ref}})^{\text{Ct(control-sample)}}}$$

Where *Ratio* is the relative expression ratio of the study gene to the implicated control, E_{sample} is the efficiency of the implicated gene in the study sample and E_{ref} is the efficiency of the reference gene (Ubiquitin C gene) in the study sample. The C_T values were obtained from rt^2-PCR analysis.

Ubiquitin C (UBC) was used as a reference (house-keeping) gene to ensure that the data obtained from the different rt^2-PCR runs are comparable. None of the treatments of the cells affected the expression of UBC (see results in § B.2.9). The data also suggest that experimental errors (such as variations in pipetting volumes or the number of cells per well) were insignificant and that the measured relative expression of the test genes are valid.

The expression levels of UBC were determined with the same equation as was used for the determination of the study sample gene expressions above.

Appendix B
Complete results and discussion

This appendix contains a more extensive discussion of the article presented data as well as additional results from experimental data that were not incorporated in the article (Chapter 3), such as pilot studies, studies to confirm validity and results that were not considered worth reporting in a peer reviewed article.

As stipulated in *Chapter 1*, the current study was performed to investigate the potential neuroprotective properties of selected clinical and experimental antidepressants in an *in vitro* model, i.e. a cultured human neuroblastoma cell line. The rationale for this is based on the neurodegenerative hypothesis of depression, postulating that the efficacy of antidepressants may be partially attributed to their neuroprotective characteristics. Since decreased neuroplasticity during depression is thought to be attributed to an increase in glutamate neurotransmission, the study investigated the neuroprotective potential of selected antidepressants in the presence of glutamate-induced excitotoxicity.

B.1 Pilot study

Glutamate is believed to play a significant role in neuronal stress, as seen in anxiety-related disorders. Therefore, the neuroblastoma cells were treated with glutamate to induce excitotoxicity, together with prototypes of different classes of antidepressants. Prior to the commencement of the study experiments, an appropriate glutamate concentration had to be established for the *in vitro* induction of excitotoxicity in cultured human SH-SY5Y cells.

Secondly, while SH-SY5Y cells are grown in culture medium with 10% foetal calf serum, and since some of the drugs bind significantly to plasma proteins, it was necessary to determine the free fraction of these drugs in the culture medium.

B.1.1 Development of a model for glutamate-induced excitotoxicity

The extent of glutamate-excitotoxicity was determined at different concentrations of glutamate, measuring cell viability with the standard MTT assay (mitochondrial activity). Furthermore, a study conducted by Brink *et al.* in 2004 indicated that a 24-hour treatment of cultured human neuroblastoma (SH-SY5Y) cells with antidepressants is sufficient to induce pharmacologically relevant intra-cellular changes. Therefore, the target was to determine the glutamate concentration that would decrease cell viability with about 50% in 24 hours. Thereby it would be possible to observe neuroprotective or neurotoxic effects of the study drugs during co-incubation.

It is reported in literature that an incubation of jurkat cells with 1 - 10 mM glutamate for 24 hours induces significant cytotoxicity. Thus, a concentration range of 2 mM, 5 mM, 10 mM and 15 mM was employed in the current pilot study. The MTT assay was repeated four times in triplicate for each of the latter concentrations (n=12). **Figure B-1** depicts the percentage changes in mitochondrial activity after a 24-hour incubation with the above-mentioned concentration range (compared to a glutamate-free control).

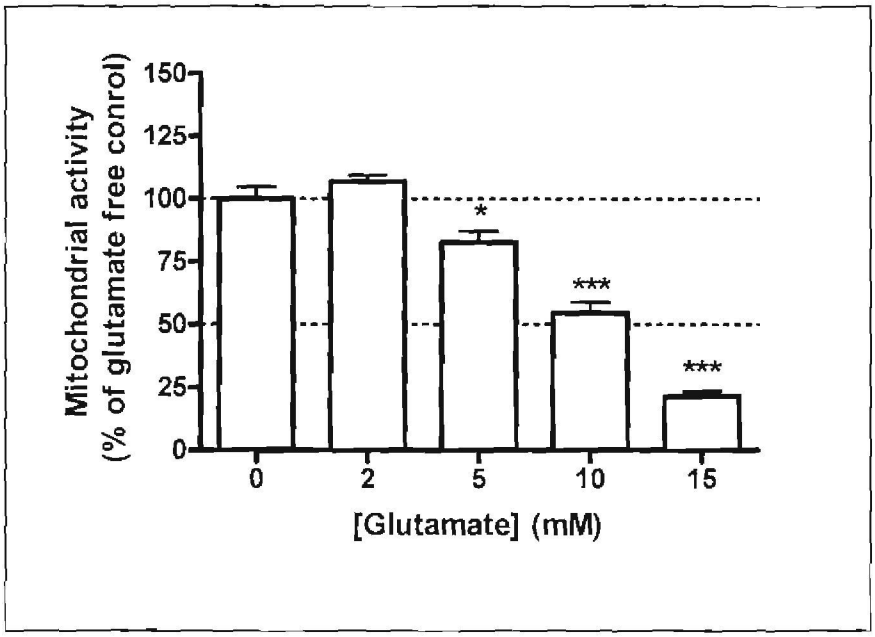

Figure B-1: *Mitochondrial activity of human neuroblastoma (SH-SY5Y) cells after a 24-hour treatment with 0, 2, 5, 10 and 15 mM glutamate, as determined with the standard MTT cell viability test.*
Data are calculated as percentage of control (without glutamate) and represent averages of two independent experiments, each with triplicate observations and expressed as the mean ± SEM. Statistical analyses were performed using the one-way ANOVA followed by the Dunnett's multiple comparison post test, with statistical significance given as $p < 0.05$ (*); $p < 0.001$ (***).

It can be seen in ***Figure B-1*** that a 24-hour incubation of the neuroblastoma cells with 2 mM glutamate does not result in any significant change in mitochondrial activity relative to control (7 ± 2.7% increase, not significant), while mitochondrial activity is significantly decreased relative to control with 5 mM glutamate (17 ± 4.5% decrease, $p < 0.05$), 10 mM glutamate (45 ± 4.3% decrease, $p < 0.001$) and 15 mM glutamate (89 ± 2.1% decrease, $p < 0.001$). A target decrease of about 50% in mitochondrial activity is thus obtainable after a 24-hour incubation period with 10 mM glutamate in a human SH-SY5Y cell line and this concentration was therefore selected for further co-incubation with antidepressants.

B.1.2 Determination of the free fraction of plasma protein-bound drugs

For the purpose of this study, it was necessary to determine the fraction of serum protein binding of the experimental drugs in the culture medium (Ham's F12 + 10% foetal bovine serum), in order to determine the effective free drug concentration. A concentration series was prepared for each drug (see ***Table B-1***). These concentration ranges were determined in such a manner that they include the study concentrations. Each concentration was prepared in duplicate: one in culture medium with 10% foetal bovine serum (FBS), and one without FBS. The samples of the concentration ranges containing FBS were centrifuged in microcentrifuge tubes (30 kDa pore size) to filter out the protein-bound fraction and to collect the free fraction (molecular weight of bovine albumin ranges between 66.3 kDa and 69 kDa). Samples of both concentration ranges were analysed by HPLC and the areas under the curves (AUC) used to determine the corresponding concentration curves. The regression lines of the total versus free concentration curves were used to determine the free fraction of the implicated drugs.

Table B-1: *The concentrations spiked into Ham's F12 ± 10% FBS for each plasma albumin bound study drug.*
These concentration ranges were determined to cover the concentration range needed for pre-treatment.

Concentration ranges used for determination of free drug fractions						
Plasma albumin bound drug	**Study concentration**		**Concentrations spiked into Ham's F12 ± 10% FBS**			
	Low	**High**	**1**	**2**	**3**	**4**
Fluoxetine	97 nM	10 µM	80 nM	4280 nM	8480 nM	12680 nM

Plasma albumin bound drug	Study concentration		Concentrations spiked into Ham's F12 ± 10% FBS			
	Low	High	1	2	3	4
Imipramine	71 nM	10 µM	70 nM	3870 nM	7670 nM	11470 nM
Mirtazapine	169 nM	10 µM	150 nM	3950 nM	7750 nM	11550 nM
Tianeptine	1 µM	10 µM	1000 nM	4000 nM	8000 nM	12000 nM

According to literature, fluoxetine, imipramine, mirtazapine and tianeptine are significantly bound to plasma proteins, with the percentage plasma protein binding in human blood reported as 94% for fluoxetine, 90.1% for imipramine, 85% for mirtazapine 85% and 95% for tianeptine.

The differences in the HPLC readings of the free and total drug concentration ranges of each of the implicated study drugs are depicted in ***Figure B-2A***, whereas the difference in percentage free drug over the analysed concentration range is illustrated by ***Figure B-2B.***

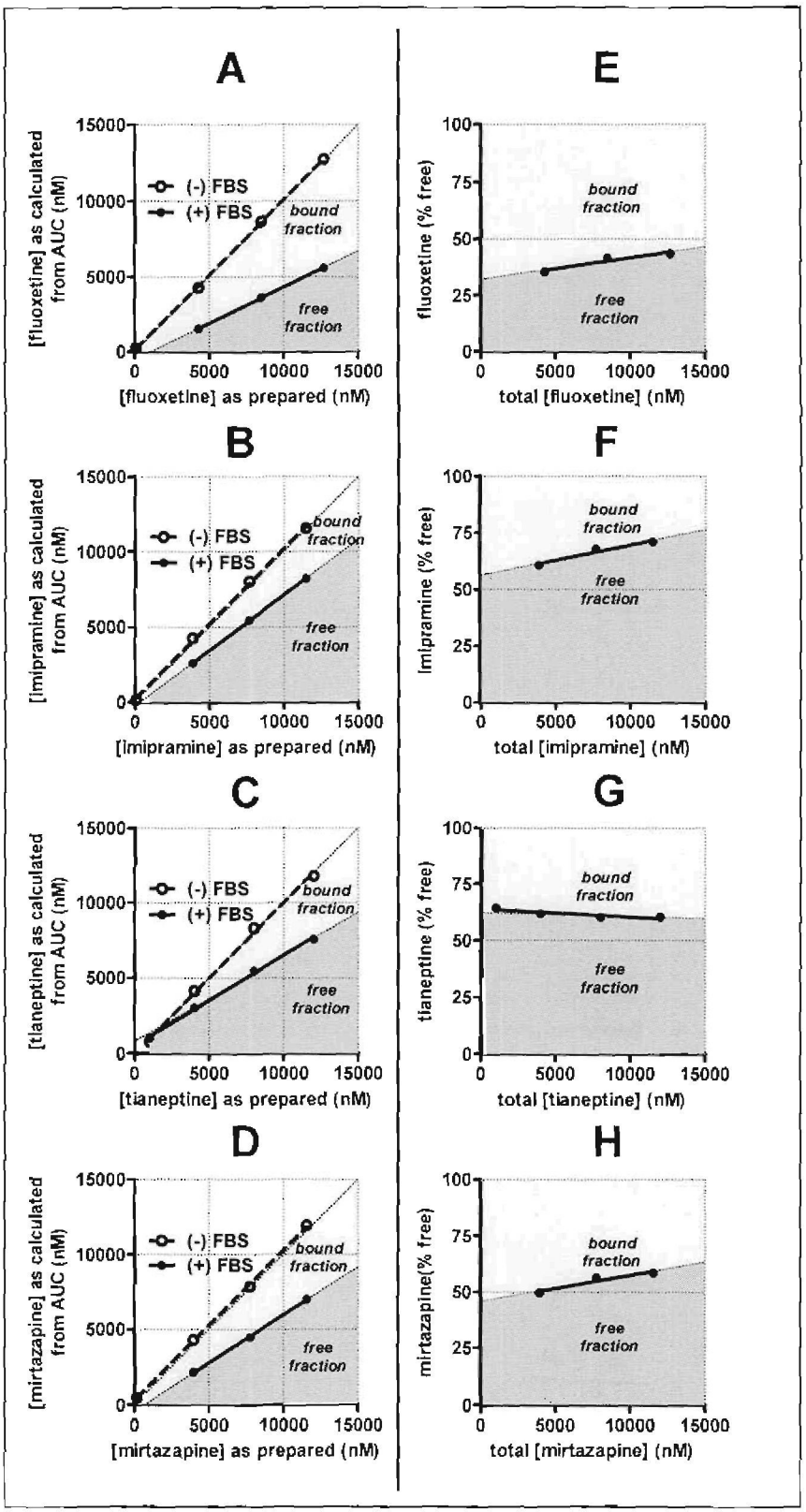

Figure B-2: *Plasma-protein binding of the test drugs fluoxetine, imipramine, mirtazapine and tianeptine in culture medium with 10% FBS, as determined by HPLC analysis.*
***(A-D)** The total and free concentrations of the drugs, as calculated from the area under the curve (AUC) of HPLC measurements, against the total concentration as prepared.*
***(E-H)** The calculated percentage free drug against the total concentration as prepared.*

Each data point represents the mean of (very accurately repeatable) duplicate observations.

As can be seen in ***Figure B-2(A-D)***, there is a proportional increase in the AUC of the free and total drug as the concentration of the drug increases. The AUC of the free drug is always smaller than the AUC of the total drug, suggesting that a fraction of the drug is bound to protein of the foetal bovine serum. The linear regression value for all of the latter graphs was determined to be equal to 0.99.

The free fraction of each drug, expressed as percentage, at the various total drug concentrations is depicted in ***Figure B-2(E-H)***. For fluoxetine, the % free drug varied between 32.1% and 46.5% (r^2=0.92), for imipramine between 56.4% and 76.4% (r^2=0.96), for tianeptine between 61.9% and 59.6% (r^2=0.76), and for mirtazapine between 45.8% and 63.4% (r^2=0.91). It should be noted that, at the lowest concentration measured for each drug, the concentration of the free fraction was lower than the measurable range, so that the free fraction could only be measured for the three higher concentrations of each drug. The free fraction at concentrations lower than the measurable range was consequently calculated by extrapolation.

Interestingly, all implicated study drugs (fluoxetine, imipramine, mirtazapine and tianeptine) are in the order of 90% bound to plasma albumin *in vivo*, and the free percentage of these drugs is in the region of 50% in FBS enriched Ham's F12 medium. As can be seen in ***Figure B-2B*** the slope of the concentration to free percentage graph is positive for all the implicated drugs except tianeptine. Possible reasons for the decrease in free drug with an increase in tianeptine concentration could be many. A possible reason for this might be that the protein binding pharmacokinetics of tianeptine might decrease with increased concentrations. Because of the fact that the slope only declines slightly, the decline could also be due to method variation. If the latter reason is found to be the causative factor, it can be concluded that tianeptine has uniform protein binding characteristics for the implicated concentration range, and the precise free drug percentage could be determined with an increased number of analytical runs, or a more sensitive analytical method.

B.2 Study objective experiments

All the experiments contributing to achievement of the study objectives were performed during Phase II. These experiments were aimed at investigating the effects of a

pharmacological and higher concentration of prototype and experimental antidepressants on neuroplasticity before and after glutamate-induced excitotoxicity. The effect of the pre-treatment regimes on the SH-SY5Y cell viability was determined by performing the standard MTT cell viability assay (i.e. a measure of mitochondrial activity). Thereafter, quantitative real-time reverse transcriptase PCR (rt^2-PCR) was performed to determine the effect of the drugs on expression of genes encoding for proteins implicated in pro-apoptotic and anti-apoptotic pathways. All rt^2-PCR data were standardised according to the corresponding primer efficiencies.

B.2.1 Fluoxetine

Fluoxetine is a prototype of the group of antidepressants known as the selective serotonin re-uptake inhibitors (SSRIs). In the current study SH-SY5Y cells were incubated for 24 hours with or without 10 mM glutamate, plus 0 M, 97 nM (pharmacological concentration) or 10 μM (high concentration) of fluoxetine, whereafter mitochondrial activity was measured with the standard MTT cell viability assay. ***Figure B-3*** depicts the effect of fluoxetine on the viability of human SH-SY5Y cells after these drug treatments.

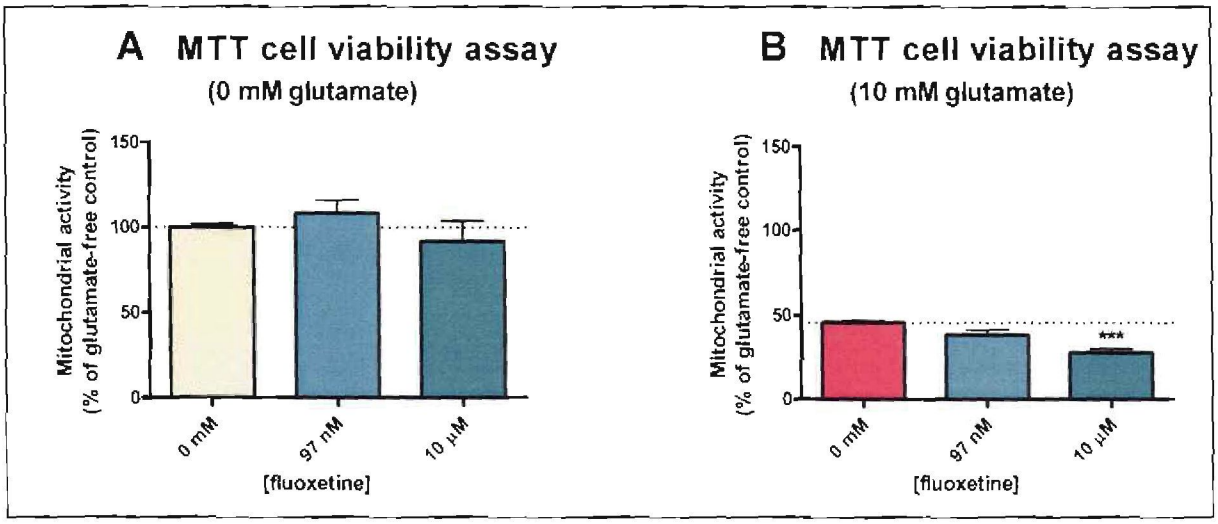

Figure B-3: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no fluoxetine (control), a pharmacological and a high concentration fluoxetine, **(A)** in the absence or **(B)** presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test, with statistical significance indicated as $p < 0.001$ (***).

As is evident from ***Figure B-3A***, there was no significant change in mitochondrial activity following a 24-hour treatment with a pharmacological concentration (97 nM) of fluoxetine in the presence or absence of glutamate. It can therefore be concluded that a pharmacological concentration of fluoxetine does not exert any effect on mitochondrial activity in the absence of a stressor such as glutamate.

However, in the presence of 10 mM glutamate (excitotoxicity) a pharmacological concentration of fluoxetine (97 nM) showed a tendency towards potentiating the reduction of mitochondrial activity by glutamate (7.32 ± 9.78% reduction, not significant), while 10 μM caused a significant potentiation of the effect of glutamate (17.60 ± 6.43% reduction, $p < 0.001$). Based on the latter result, it can be postulated that high fluoxetine concentrations (± 100 times the pharmacological concentration) potentiate the cytotoxic effects of glutamate on neuronal cells and that this high concentration does not protect against the neuronal stress of excitotoxicity.

The effects of a pharmacological concentration of fluoxetine (97 nM) on the indicated study genes, as determined by quantitative rt^2-PCR in SH-SY5Y cells, are illustrated in ***Figure B-4***, either in the absence (***Figure B-4A***) or presence (***Figure B-4B***) of glutamate.

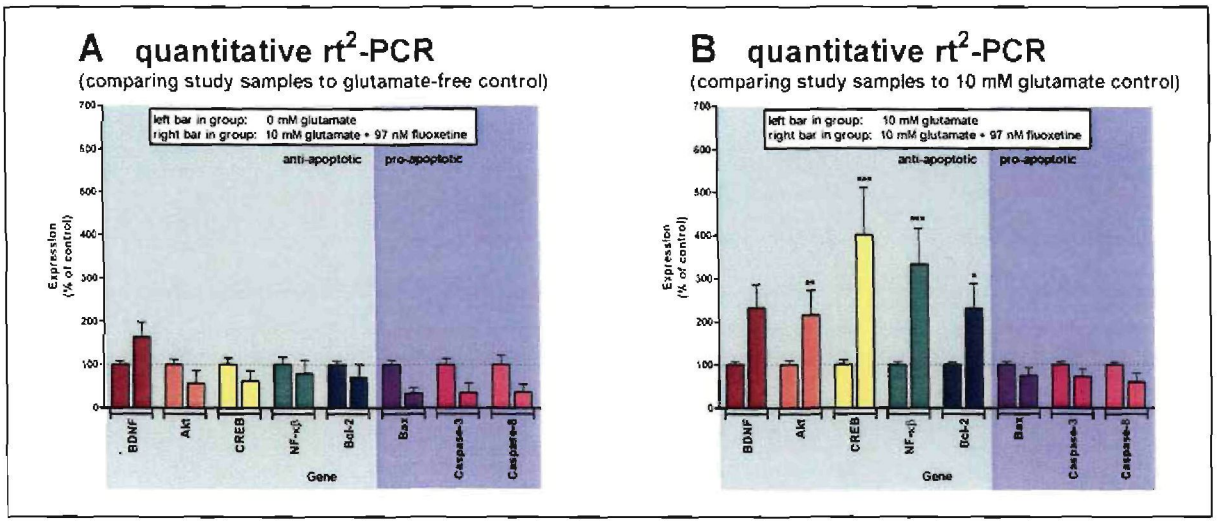

Figure B-4: *The effect of a 24-hour incubation of SH-SY5Y cells with a pharmacologic concentration (97nM) of the prototype antidepressant fluoxetine, on the pro- and anti-apoptotic gene expression. Data were obtained in* ***(A)*** *the absence or* ***(B)*** *presence of 10 mM glutamate (excitotoxicity).*
All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical significance indicated as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison post test.

From ***Figure B-4A*** it is evident that, in the absence of glutamate (i.e. no excitotoxicity) 97 nM fluoxetine does not alter gene expression significantly, as predicted from the MTT data (see ***Figure B-3***). Even though the results were not statistically significant, the tendency for lower expression of the pro-apoptotic factors Bax, caspase-3 and caspase-8 and the increased expression of the anti-apoptotic transcription factor BDNF suggests potential neuroprotective effects of fluoxetine, as reported in the literature (Nibuya *et al.*, 1996; Manev *et al.*, 2001b; Koch *et al.*, 2003; Dias *et al.*, 2003).

Importantly, however, it can be seen in ***Figure B-4B*** that, in the presence of glutamate, gene expressions were significantly altered relative to control. The expression levels after fluoxetine administration were as follows: **BDNF** 231.5 ± 54.1% v/s 100 ± 6.7% control (not significant); **Akt** 214.8 ± 58.1% v/s 100 ± 10.1% control ($p < 0.01$); **CREB** 401.2 ± 109.0% v/s 100 ± 9.8% control ($p < 0.001$); **NFκβ** 332.8 ± 83.7% v/s 100 ± 6.6% control ($p < 0.001$); **Bcl-2** 231.2 ± 56.6% v/s 100 ± 6.0% control ($p < 0.05$); **Bax** 74.1 ± 18.8% v/s 100 ± 6.6% control (not significant); **Caspase 3** 72.4 ± 17.8% v/s 100 ± 6.7% control (not significant); **Caspase 8** 60.4 ± 20.8% v/s 100 ± 6.3% control (not significant). Therefore, fluoxetine increases the expression of the genes encoding for the anti-apoptotic proteins **Akt**, **CREB**, **NFκβ** and **Bcl-2**, with a tendency also for **BDNF**. These data suggest that a pharmacological concentration of fluoxetine (97 nM) may exert neuroprotective effects. Previous studies also indicated that fluoxetine increases the expression of the anti-apoptotic transcription factor **CREB** as well as of **BDNF** (Nibuya *et al.*, 1996; Conti *et al.*, 2002; Koch *et al.*, 2003). It is interesting to note that, in the current study, fluoxetine did not significantly change the expression of the pro-apoptotic genes, compared to the glutamate control. Based on the latter observation it can be postulated that the neuroprotective ability of fluoxetine is rooted in the promotion of cell survival signals, rather than inhibiting signals responsible for cellular demise.

B.2.2 Imipramine

Imipramine falls in a group of antidepressants known as tricyclic antidepressants (TCAs). The ability of this drug to counter the deleterious effects of glutamate on human SH-SY5Y cells was measured via the standard MTT assay followed by quantitative rt^2-PCR. The results obtained via the standard MTT cell viability assay for the co-incubation of 0 M or 10 mM glutamate along with 0 M, 71 nM (pharmacological

concentration) or 10 µM (high concentration) imipramine for 24 hours, are depicted in ***Figure B-5***.

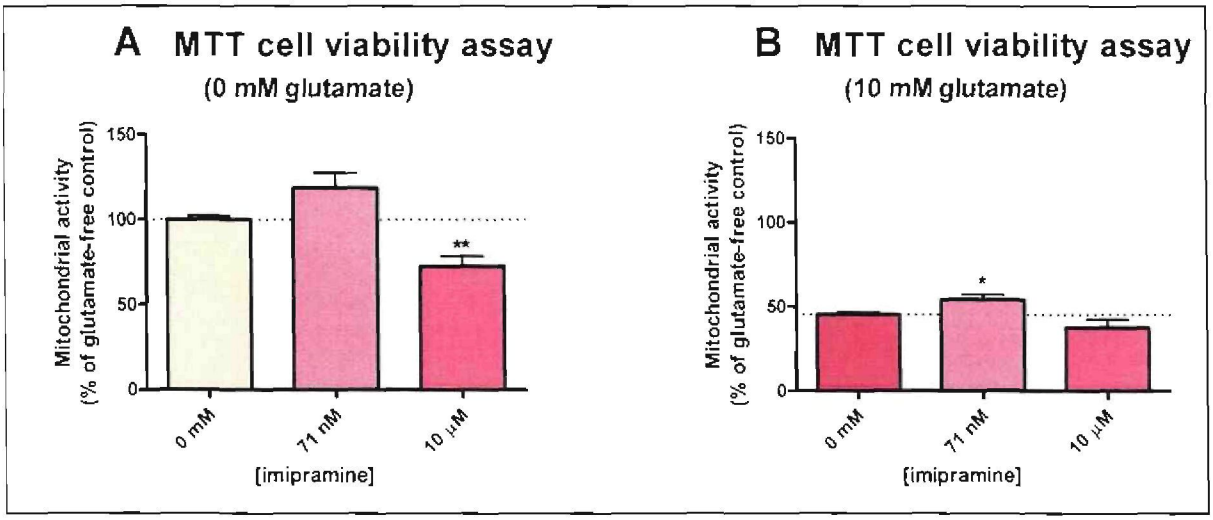

Figure B-5: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no imipramine (control), a pharmacological and a high concentration imipramine, **(A)** in the absence or **(B)** presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test, with statistical significance indicated as $p < 0.05$ (*); $p < 0.01$ (**).

Compared to the glutamate-free control there is a tendency for a pharmacological concentration (71 nM) of imipramine to increase mitochondrial activity (***Figure B-5A***) although the increase was not significant (18.70 ± 26.70% increase, not significant). In the presence of 10 mM glutamate, however, the pharmacological concentration of imipramine was able to yield a significant increase in mitochondrial activity (8.62 ± 9.14% increase, $p < 0.05$) compared to the glutamate control (***Figure B-5B***). From these results it can be speculated that imipramine possesses protective properties that are enhanced in the presence of a stressor, such as glutamate. In the absence of glutamate, the high concentration of imipramine caused a significant decrease in cell viability (27.28 ± 17.45% decrease, $p < 0.01$) compared to the glutamate-free control (***Figure B-5A***). In the presence of glutamate, a tendency for such a decrease is also noted with the high concentration of imipramine (10 µM) (***Figure B-5B***), although not statistically significant (7.88 ± 14.95% decrease, not significant). From these results it can be concluded that a high concentration of imipramine displays cytotoxic effects in the human SH-SY5Y cells in the absence of a stressor (glutamate).

The effect of the 24-hour treatment with a pharmacological concentration of imipramine (71 nM) (in the absence and presence of 10 mM glutamate) on the expression of various pro- and anti-apoptotic genes is depicted in ***Figure B-6***.

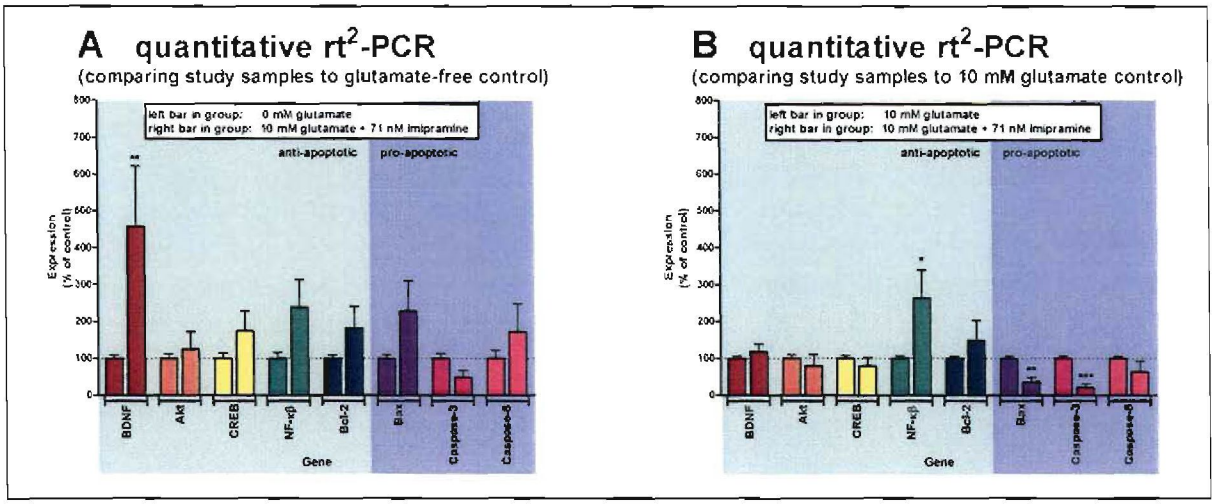

Figure B-6: *The effect of a 24-hour incubation of SH-SY5Y cells with a pharmacologic concentration (71nM) of the prototype antidepressant imipramine, on the pro- and anti-apoptotic gene expression.*
Data were obtained in ***(A)*** *the absence or* ***(B)*** *presence of 10 mM glutamate (excitotoxicity). All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical significance indicated as p < 0.05 (*); p < 0.01 (**); p < 0.001 (***). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison assay.*

In the presence of glutamate, as shown in ***Figure B-6A***, the pharmacological concentration of imipramine was able to significantly increase the expression of the anti-apoptotic transcription factor **BDNF** (358.11 ± 164.54% increase, $p < 0.01$). Comparing the imipramine + glutamate pre-treated samples to the untreated control, the anti-apoptotic factors **CREB** (74.05 ± 54.02% increase, not significant), **NF-κβ** (138.29 ± 74.29% increase, not significant) and **Bcl-2** (80.61 ± 61.21% increase, not significant) and the pro-apoptotic factor **Bax** (128.18 ± 81.45% increase) indicated a slight tendency for increased gene expression, although none of these increases was significant (***Figure B-6A***).

It can be seen in ***Figure B-6B*** that a pharmacological concentration of imipramine in the presence of glutamate significantly increases the expression of the anti-apoptotic transcription factor **NF-κβ** (163.02 ± 75.91% increase, $p < 0.05$) and reduced expression of the pro-apoptotic factors **Bax** (64.36 ± 11.74% reduction, $p < 0.01$) and **Caspase-3** (79.64 ± 9.31% decrease, $p < 0.001$) compared to their respective 10 mM glutamate controls. The data on **Akt** expression (19.74 ± 30.20% decrease, not significant) correlate well with previous studies, which also found no significant

imipramine-induced change in overall Akt expression (Basta-Kaim *et al.*, 2005). The expression of the genes for **CREB**, **BDNF** and **Bcl-2** was not changed by imipramine, while other studies reported increased gene expressions (Koch *et al.*, 2003; Van Hoomissen *et al.*, 2003). The current study only indicated a tendency for **Bcl-2** gene up-regulation (48.87 ± 54.28% increase, not significant). The lower expression of the pro-apoptotic factors **Bax** (64.36 ± 11.74% decrease, $p < 0.01$) and **Caspase-3** (79.64 ± 9.31% decrease, $p < 0.001$) and the increased expression of the anti-apoptotic transcription factors **BDNF** and **NF-κβ** suggest that imipramine might be neuroprotective, as reported in the literature (Nibuya *et al.*, 1996; Manev *et al.*, 2001a; Basta-Kaim *et al.*, 2005).

B.2.3 Lithium Chloride

Lithium chloride (LiCl, in short lithium) is one of the lithium salts used for the treatment of mania and hypomania in bipolar disorder. As with the antidepressants above, the effect of a 24-hour treatment with lithium on the survival of human SH-SY5Y cells was determined via the standard MTT assay, before and after glutamate induced excitotoxicity, as depicted in ***Figure B-7***.

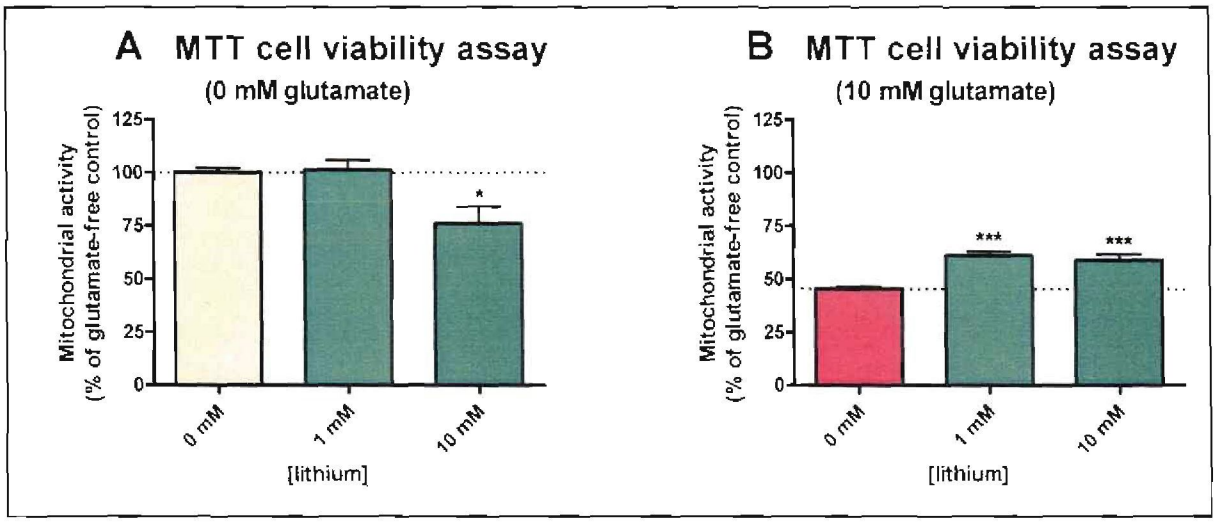

Figure B-7: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no lithium (control), a pharmacological and a high concentration lithium, **(A)** in the absence or **(B)** presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test, with statistical significance indicated as $p < 0.05$ (*); $p < 0.001$ (***).

As is evident from ***Figure B-7A***, there was no significant change in mitochondrial activity following a 24-hour pre-treatment with the pharmacological concentration (1 nM)

of lithium in the absence of glutamate. In the absence of glutamate the high concentration lithium caused a significant decrease in cell viability (23.88 ± 24.00% decrease, $p < 0.05$). Therefore, it can be concluded that lithium does not exert any protective effect on mitochondrial activity in the absence of a stressor such as glutamate.

However, in the presence of 10 mM glutamate (excitotoxicity) the pharmacological and high concentrations of lithium (1 mM and 10 mM) displayed a significant increase in mitochondrial activity (1 mM: 61.79 ± 5.88%, $p < 0.001$; 10 mM: 58.97 ± 8.40%, $p < 0.001$) compared to the glutamate control (45.44 ± 8.85%) (***Figure B-7B***). Based on the latter result it can be postulated that the pharmacological and high concentrations of lithium protect against glutamate-induced excitotoxicity in neuronal cells.

Thus, it can be postulated that the neuroprotective effectiveness of lithium may be stressor-dependant and that high concentrations of lithium can be cytotoxic under certain normal (unstressed) conditions, such as in the absence of the cellular stressor glutamate.

The effect of the pharmacological lithium treatment regimes (1 mM lithium ± 10 mM glutamate) on the expression of the selected study genes is depicted in ***Figure B-8*** below.

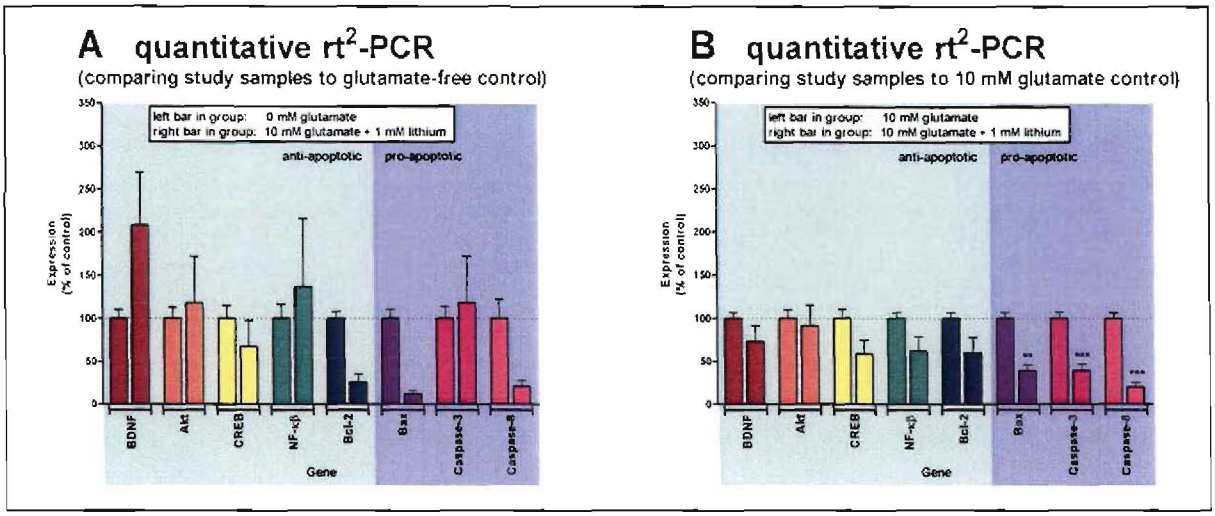

Figure B-8: *The effect of a 24-hour incubation of SH-SY5Y cells with a pharmacologic concentration (1 mM) of the antidepressant lithium, on the pro- and anti-apoptotic gene expression. Data were obtained in (A) the absence or (B) presence of 10 mM glutamate (excitotoxicity). All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical*

significance indicated as $p < 0.01$ (**); $p < 0.001$ (***). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison assay.

Compared to the glutamate-free control, the pharmacological concentration of lithium in the absence of glutamate had no significant effect on gene expression (***Figure B-8A***). A trend for up- and down-regulations was seen, noticeably for **BDNF** (108.12 ± 61.43% increase, not significant), **NF-κβ** (36.34 ± 79.43% increase, not significant), **Bcl-2** (74.51 ± 9.67% decrease, not significant), **Bax** (88.07 ± 3.86% decrease, not significant) and **Caspase-8** (79.24 ± 7.20% decrease, not significant). A prominent trend for **BDNF** gene up-regulation and pro-apoptotic gene down-regulation is observed, which might be significant with an increased number of analysis repetitions and which could contribute to the neuroprotective characteristics of lithium.

However, in the presence of glutamate, the significant down-regulation of **Bax** (60.70 ± 6.84% decrease, $p < 0.01$) expression correlates with research data previously reported (Nonaka & Chuang, 1998). A significant decrease is also noted in **Caspase-3** (60.75 ± 7.36% decrease, $p < 0.001$) and **Caspase-8** (79.89 ± 4.99% decrease, $p < 0.001$).

As with the antidepressants discussed above, Lithium seems to exert its protective abilities by decreasing the gene expressions of pro-apoptotic proteins during the co-administration with glutamate. Past research found that lithium significantly increases the expression of **CREB** and **Bcl-2**, and decreases the expression of **Bax** (Nonaka *et al.*, 1998; Nonaka & Chuang, 1998; Fukumoto *et al.*, 2001; Bush & Hyson, 2006). Although increases in anti-apoptotic gene expression were not observed in the current study, a decrease was seen in **Bax** and **Caspase-8**. Based on the current data, it can be said that the dominant route for lithium-induced neuroprotection is via the inhibition of cell death inducing factors.

B.2.4 Tianeptine

Tianeptine is a novel antidepressant with a modified tricyclic structure. This drug has also proven itself to be a superior neuroprotective and antidepressant therapeutic, although the mechanisms for these characteristics are still greatly unknown. The effect of a pharmacological and high dose of tianeptine, with and without co-administration of glutamate, on mitochondrial activity, can be seen in ***Figure B-9.***

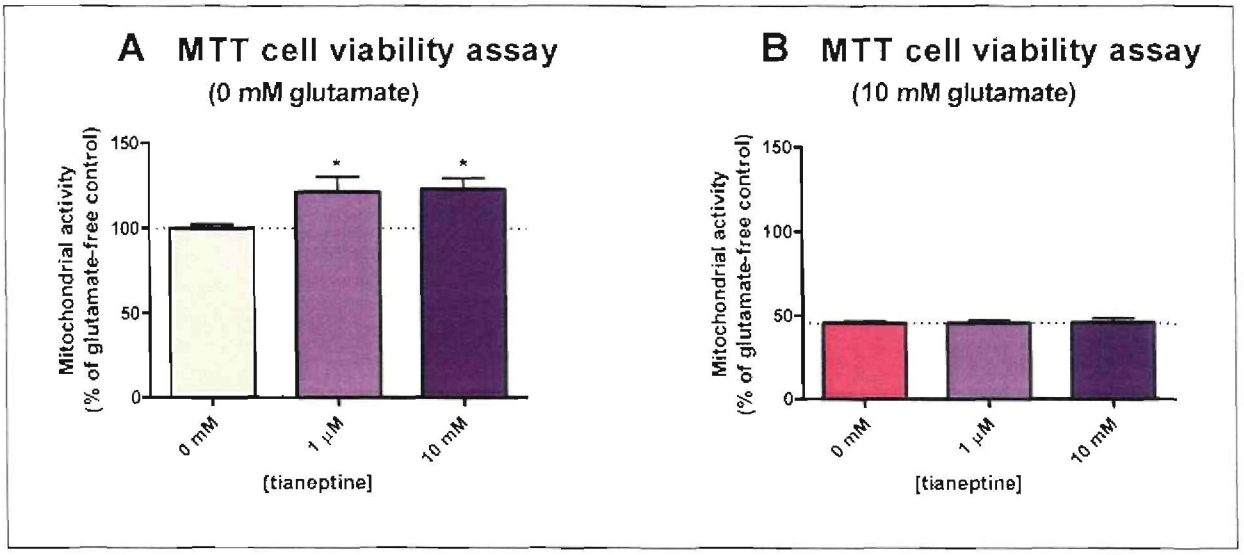

Figure B-9: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no tianeptine (control), a pharmacological and a high concentration tianeptine,* ***(A)*** *in the absence or* ***(B)*** *presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test, with statistical significance indicated as $p < 0.05$ *(*).*

In ***Figure B-9A*** it can be seen that a pharmacological and a high concentration of tianeptine significantly increase the cell viability (1 µM: 21.0 ± 27.4% increase, $p < 0.05$; 10 mM: 22.7 ± 19.1% increase, $p < 0.05$) compared to a glutamate-free control (100 ± 18.2%). However, in the presence of 10 mM glutamate, no significant effect on mitochondrial activity was noted with either 1 µM (45.52 ± 5.28%, not significant) or 10 mM (45.89 ± 7.45%, not significant) compared to the 10 mM glutamate control (45.44 ± 8.85%) (***Figure B-9B***). Therefore, tianeptine increases mitochondrial activity in cells without a stressor (i.e. no glutamate), but this property is lost in the presence of glutamate. If it is assumed that tianeptine does possess neuroprotective properties, as suggested by literature (see § 2.4.6.2), it can be postulated that tianeptine exhibits its neuroprotective characteristics via an intra-cellular mechanism that does not involve mitochondrial function as measured by the MTT test.

Figure B-10 depicts the effects of glutamate along with the pharmacological concentration tianeptine on the expression of various cell survival signals, compared to either a 0 mM or a 10 mM glutamate control.

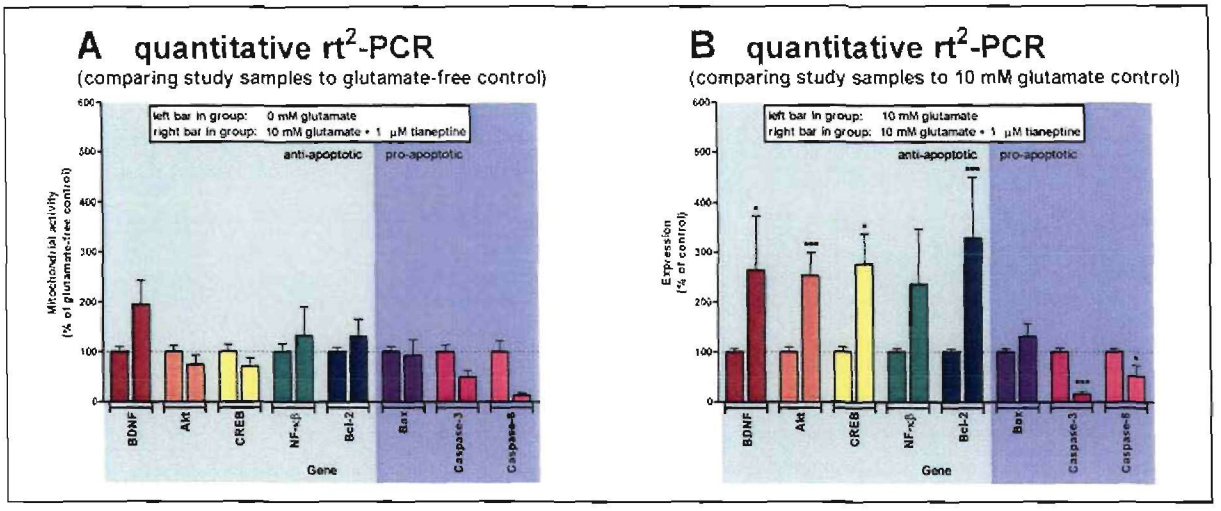

Figure B-10: *The effect of a 24-hour incubation of SH-SY5Y cells with a pharmacologic concentration (1 mM) of the antidepressant lithium, on the pro- and anti-apoptotic gene expression. Data were obtained in* ***(A)*** *the absence or* ***(B)*** *presence of 10 mM glutamate (excitotoxicity). All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical significance indicated as $p < 0.05$ (*); $p < 0.001$ (***). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison assay.*

From ***Figure B-10A*** it is clear that a pharmacological concentration of tianeptine in the absence of glutamate does not alter the expression of any of the genes evaluated. However, a tendency was observed for the gene up-regulation of anti-apoptotic factors **BDNF** (95.01 ± 48.55% increase, not significant), **NF-κβ** (32.20% ± 57.40% increase, not significant) and **Bcl-2** (30.67 ± 34.14% increase, not significant), and the gene down-regulation of the pro-apoptotic factors **Caspase-3** (50.23 ± 12.60% decrease, not significant) and **Caspase-8** (87.32 ± 4.915% decrease, not significant). The latter values might yield significant increases/decreases with analyses of a larger number of repeat samples (thus, a larger sample size; n) or with an increased number of analyses (run) repetitions. Although the results in ***Figure B-10A*** did not indicate significant changes from the control value, this drug's tendency to increase anti-apoptotic gene expression and down-regulate pro-apoptotic gene expression from normal neuronal gene expression, might contribute to the neuroprotective ability of this drug.

Compared to a 10 mM glutamate control, a pharmacological concentration of tianeptine significantly increased expression of the anti-apoptotic genes **BDNF** (263.88 ± 108.91% vs. 100 ± 6.74% control, $p < 0.05$), **Akt** (252.60 ± 46.51% vs. 100 ± 10.34% control, $p < 0.001$), **CREB** (275.22 ± 61.74% vs. 100 ± 10.32% control, $p < 0.05$) and **Bcl-2** (329.36 ± 119.95% vs. 100 ± 6.05% control, $p < 0.001$). The expression of **NF-κβ** was not significantly increased, although, a very strong tendency for **NF-κβ** gene up-

regulation (235.31 ± 112.15% vs. 100 ± 6.58% control, not significant) can be noted (***Figure B-10B***). The pharmacological tianeptine concentration was also able to significantly decrease the expression of the pro-apoptotic genes **Caspase-3** (15.89 ± 4.58% vs. 100 ± 7.03% control, $p < 0.001$) and **Caspase-8** (51.13 ± 20.97% vs. 100 ± 6.27% control, $p < 0.05$), in the presence of glutamate (***Figure B-10B***).

From these rt^2-PCR results it can be concluded that tianeptine exerts remarkable protective properties in the presence of excitotoxicity (glutamate). Furthermore, tianeptine seems to affect a wide spectrum of both anti- and pro-apoptotic pathways.

B.2.5 *myo*-Inositol

myo-Inositol is a glucose derivative that plays a pivotal role during G-protein-coupled receptor signalling, activated/potentiated by several neurotransmitters. Recent research suggests clinical efficacy of *myo*-inositol in the treatment of anxiety-related disorders (including depression), with a favourable adverse effect profile. Studies in the literature also noted that intra-peritoneal or chronic oral administration of *myo*-inositol reversed the deleterious effects of chronic major depression on the brain. ***Figure B-11*** depicts the effect of a 24-hour pre-treatment with a physiological concentration *myo*-inositol (2 mM) on the mitochondrial activity of human SH-SY5Y cells in the presence or absence of glutamate.

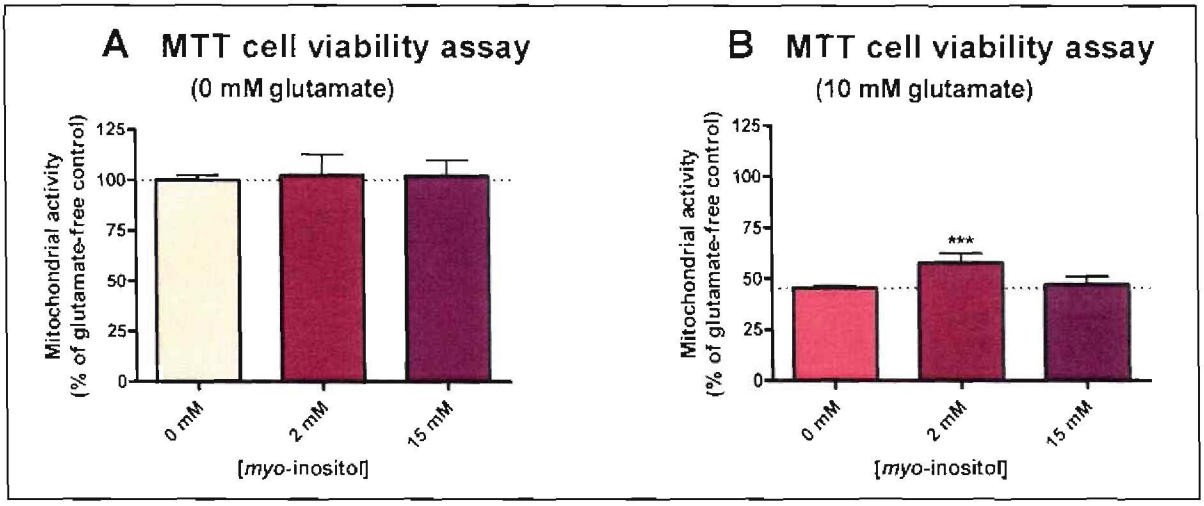

Figure B-11: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no myo-inositol (control), a pharmacological and a high concentration myo-inositol, **(A)** in the absence or **(B)** presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test, with statistical significance indicated as $p < 0.001$ (***).

The normal physiological *myo*-inositol concentration in the human brain is determined to range between 2 mM and 15 mM, therefore the lower limit of this concentration range was employed as the low *myo*-inositol study concentration (2 mM) and the higher limit as the high study concentration (15 mM). In ***Figure B-11A*** it can be seen that, in the absence of glutamate, mitochondrial activity is not altered by low (2.3 ± 31.51% increase, not significant) or high (1.9 ± 24.26% increase, not significant) concentrations of *myo*-inositol. As seen in ***Figure B-11B,*** in the presence of glutamate, the low concentration of *myo*-inositol (2 mM) significantly increases mitochondrial activity (12.4 ± 14.18% increase, $p < 0.001$), while no change is seen with a higher (10 mM) concentration (1.7 ± 11.83% increase, not significant), as compared with a 10 mM glutamate control (45.44 ± 8.85%). Based on the effect of *myo*-inositol on mitochondrial activity, it can be postulated that the protective ability of *myo*-inositol is seen only in the presence of glutamate (stressor) and only at the lower physiological concentration of *myo*-inositol (2 mM).

After a 24-hour incubation period of human SH-SY5Y cells with the physiological low *myo*-inositol concentration along with 10 mM glutamate, the following alterations in gene expression can be noted in ***Figure B-12***.

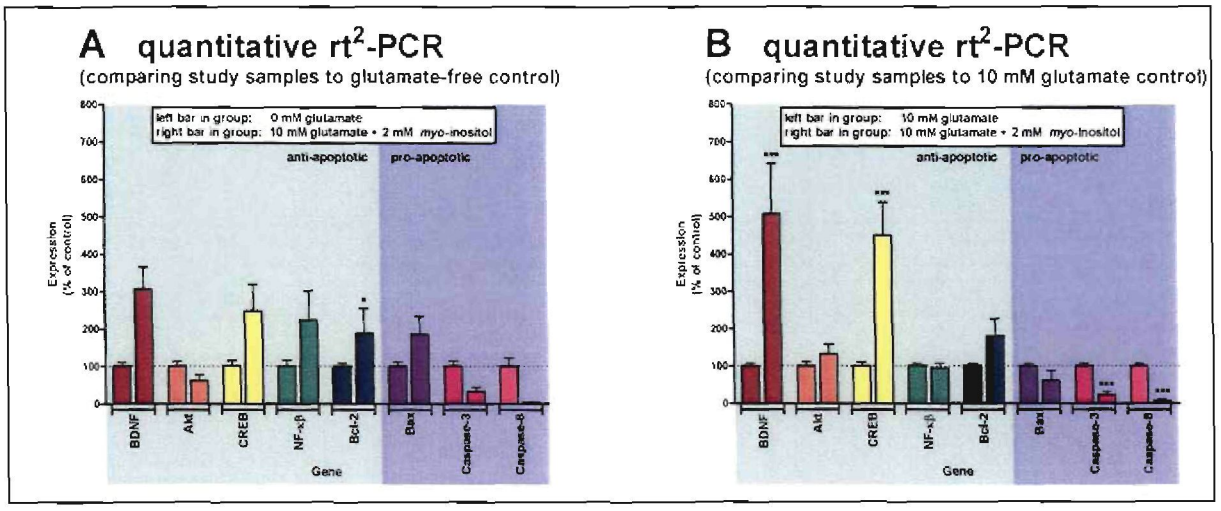

Figure B-12: *The effect of a 24-hour incubation of SH-SY5Y cells with a physiological low concentration (2 mM) of the experimental antidepressant myo-inositol, on the pro- and anti-apoptotic gene expression.*
Data were obtained in ***(A)*** *the absence or* ***(B)*** *presence of 10 mM glutamate (excitotoxicity). All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical significance indicated as $p < 0.05$ (*); $p < 0.001$ (***). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison assay.*

In ***Figure B-12A*** is can be seen that, in the absence of glutamate, a physiological concentration of *myo*-inositol caused statistically significant up-regulation only of Bcl-2, and that the tendency for up- and down-regulation for other genes did not reach statistical significance. A tendency for up-regulation of anti-apoptotic genes can be seen with **BDNF** (306.42 ± 58.34% vs. 100 ± 9.93% control, not significant), **CREB** (246.94 ± 72.51% vs. 100 ± 14.73% control, not significant) and **NF-κβ** (223.32 ± 78.51% vs. 100 ± 16.56% control, not significant). Compared to the glutamate-free control (100 ± 8.16%), a significant increase in the expression of the anti-apoptotic gene **Bcl-2** (188.13 ± 66.20%, $p < 0.05$) was observed. The pro-apoptotic genes **Caspase-3** (67.49 ± 10.75% decrease, not significant) and **Caspase-8** (96.59 ± 1.91% decrease, not significant) indicated a tendency for down-regulation, except **Bax** (85.31% ± 47.95% increase, not significant) that indicated a tendency for up-regulation compared to the 0 M glutamate control. Thus, *myo*-inositol may prove to increase the expression of anti-apoptotic genes if the number of analytical runs and duplicate samples are increased.

In ***Figure B-12B*** it can be noted that, in the presence of 10 mM glutamate (excitotoxicity), the experimental antidepressant *myo*-inositol displayed neuroprotective properties. This substance significantly increased the anti-apoptotic gene expression of **BDNF** (507.42 ± 134.95% vs. 100 ± 6.74% control, $p < 0.001$) and **CREB** (450.00 ± 88.93% vs. 100 ± 10.32% control, $p < 0.001$); and decreased the expression of pro-apoptotic **Caspase-3** (22.48 ± 8.17% vs. 100 ± 7.03% control, $p < 0.001$) and **Caspase-8** (6.55 ± 3.99% vs. 100 ± 6.27% control, $p < 0.001$) (***Figure B-12B***). The up-regulation of both **CREB** and **BDNF** correlates well with the fact that **CREB** is a transcription factor responsible for the regulation of **BDNF** expression (Tao *et al.*, 1998; Finkbeiner, 2000).

B.2.6 Gabapentin

Gabapentin was originally synthesised in 1977 for the treatment of epilepsy, however it has proven itself to be a very effective antidepressant as well. Since the discovery of this drug's antidepressant properties, much research was aimed at discovering and understanding its antidepressant and neuroprotective mechanisms. The effect of a pharmacological and a high concentration of gabapentin on cell viability in the presence/absence of glutamate are illustrated in ***Figure B-13***.

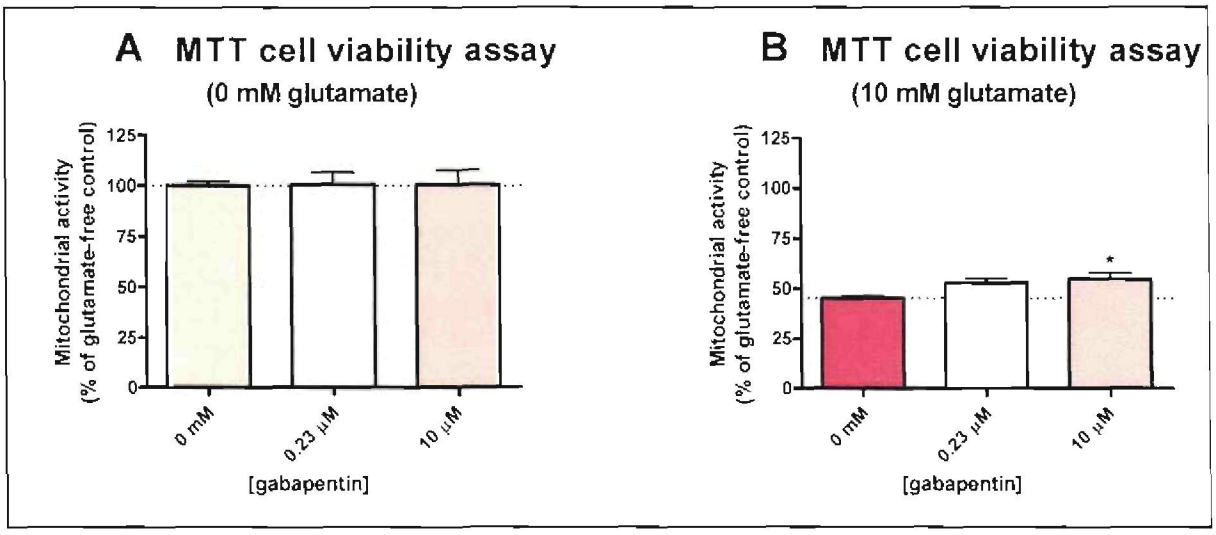

Figure B-13: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no gabapentin (control), a pharmacological and a high concentration gabapentin, **(A)** in the absence or **(B)** presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test, with statistical significance indicated as $p < 0.001$ (***).

Gabapentin has no effect on the viability of the human SH-SY5Y cells when administered without glutamate (***Figure B-13A***) after a 24-hour incubation period. In ***Figure B-13B***, it can be seen that, in the presence of glutamate, a pharmacological concentration of gabapentin (230 nM) displayed a tendency to increase cell viability (7.49 ± 6.95% increase, not significant), but it did not reach statistical significance. However, the high concentration gabapentin (10 µM) significantly increased cell viability (9.55 ± 8.19% increase, $p < 0.05$). The protective effects of gabapentin on the mitochondria can thus be said to be concentration and stressor dependent.

The apoptotic protection capacity of gabapentin was determined via rt^2-PCR analysis of the appropriately treated gabapentin samples. These samples were analysed to determine the effect of the gabapentin pre-treatment regime on selected genes which play a role in the promotion or inhibition of the apoptotic process (***Figure B-14***).

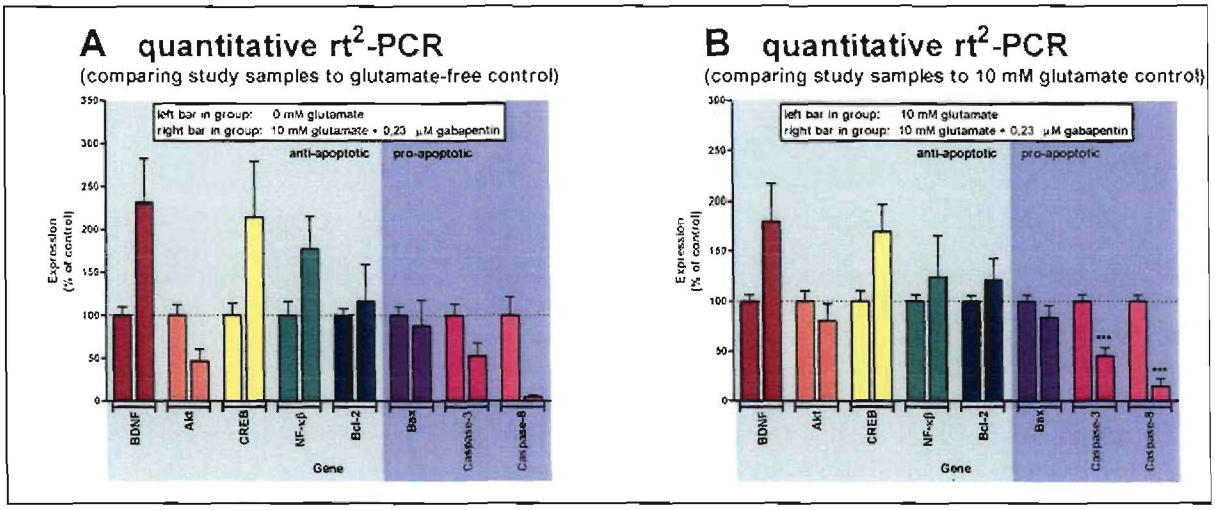

Figure B-14: *The effect of a 24-hour incubation of SH-SY5Y cells with a physiological low concentration (0.23 µM) of the experimental antidepressant gabapentin, on the pro- and anti-apoptotic gene expression.*
*Data were obtained in **(A)** the absence or **(B)** presence of 10 mM glutamate (excitotoxicity). All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical significance indicated as p < 0.001 (***). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison assay.*

From ***Figure B-14A*** it is evident that, with a pharmacological concentration of gabapentin, only trends towards alterations of gene expressions were seen and no changes reached statistical significance, with the anti-apoptotic genes **BDNF** (230.94 ± 51.33% vs. 100 ± 9.91% control, not significant), **CREB** (214.61 ± 64.98% vs. 100 ± 14.73% control, not significant) and **NF-κβ** (177.34 ± 38.51% vs. 100 ± 16.56% control, not significant). There was also a trend for decreased expression of genes encoding for the pro-apoptotic enzymes **Caspase-3** (53.03 ± 14.65% vs. 100 ± 13.89% control, not significant) and **Caspase-8** (4.87 ± 1.98% vs. 100 ± 22.09% control, not significant).

As can be seen in ***Figure B-14B***, a similar trend was seen with tianeptine in the presence of glutamate, with the exception that a statistically significant decrease was noted with expression of genes encoding for the pro-apoptotic factors **Caspase-3** (54.62 ± 8.30% decrease, $p < 0.001$) and **Caspase-8** (85.52 ± 7.68% decrease, $p < 0.001$) in comparison with the glutamate-control (100 ± 7.03% and 100 ± 6.27% respectively) (***Figure B-14B***).

Thus, the protective characteristics of gabapentin can be attributed to the ability of this drug to significantly increase mitochondrial activity when administered at high concentrations, as well as significantly decreasing pro-apoptotic gene expression at the pharmacological concentration, in the presence of glutamate.

B.2.7 Mirtazapine

Mirtazapine belongs to a newer class of antidepressants known as NaSSAs. The pre-treatment regime of mirtazapine consisted of the 24-hour treatment of human SH-SY5Y cells with a low and a high mirtazapine concentration, with or without 10 mM glutamate. The results obtained via the standard MTT assay is depicted in ***Figure B-15*** below.

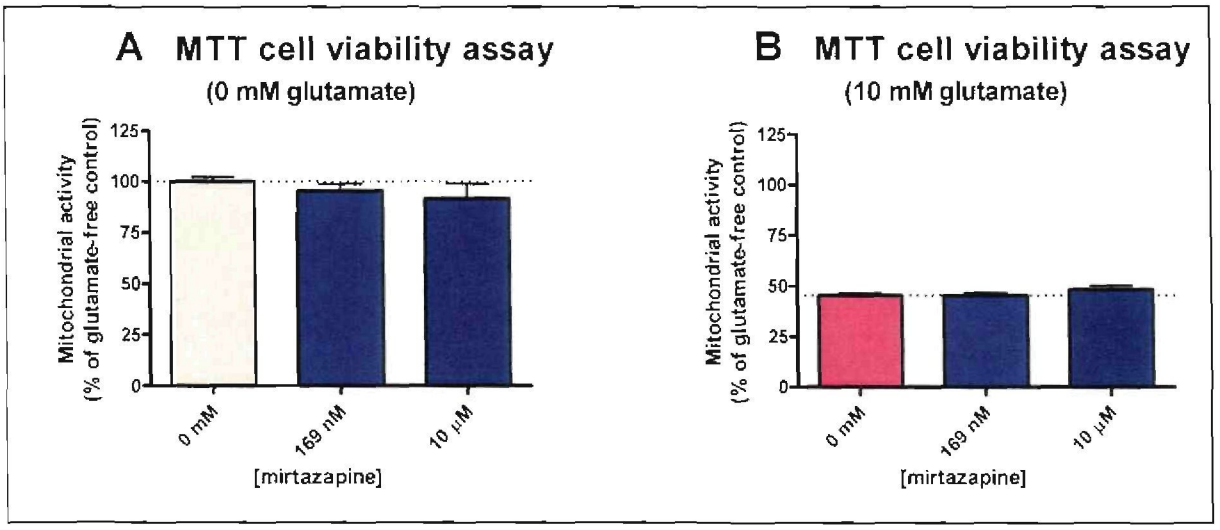

Figure B-15: *The effect on neuroplasticity of a 24-hour pre-treatment of human SH-SY5Y cells with no mirtazapine (control), a pharmacological and a high concentration mirtazapine, **(A)** in the absence or **(B)** presence of glutamate.*
Data are averages ± S.E.M. of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analysed statistically by means of a one-way ANOVA and the Dunnett's Multiple Comparison post test.

As can be seen from ***Figure B-15A and B***, mirtazapine had no effect on the mitochondrial activity (viability) of human SH-SY5Y cells, as measured by the MTT test. This was regardless the absence or presence of excitotoxicity (glutamate). Therefore, given the reports of the clinical efficacy and neuroprotective properties of mirtazapine (Rogoz *et al.*, 2005), it can be postulated that any neuroprotective property of mirtazapine does not involve altered mitochondrial activity, as measured by the MTT test.

In an attempt to further investigate potential neuroprotective properties of mirtazapine, rt^2-PCR was performed after the incubation of a pharmacological concentration of mirtazapine in the absence and presence of glutamate (***Figure B-16***).

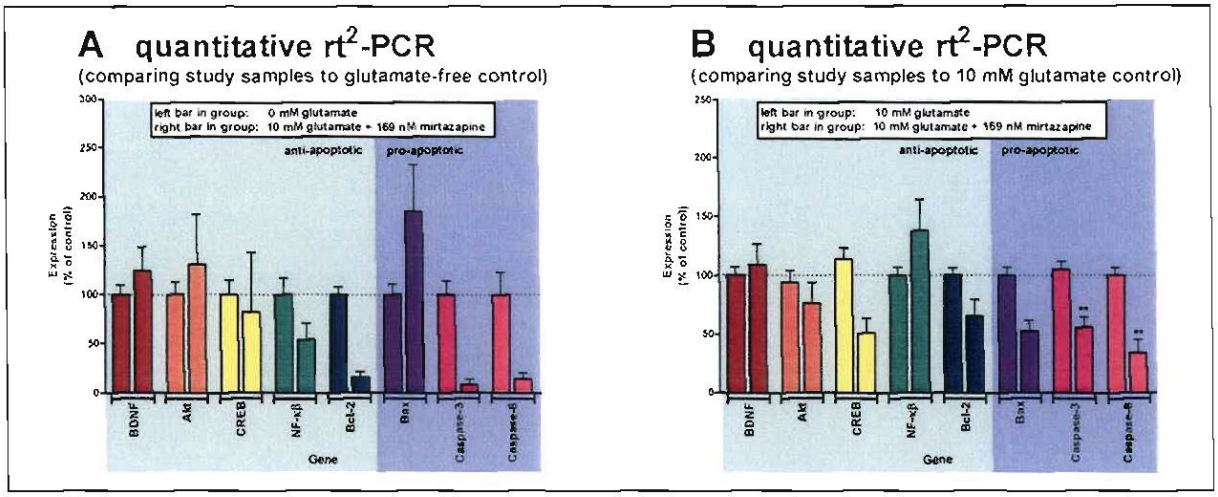

Figure B-16: *The effect of a 24-hour incubation of SH-SY5Y cells with a physiological low concentration (169 nM) of the experimental antidepressant mirtazapine, on the pro- and anti-apoptotic gene expression.*
Data were obtained in ***(A)*** *the absence or* ***(B)*** *presence of 10 mM glutamate (excitotoxicity). All data are averages of four independent experiments, each with duplicate observations (n = 8) and expressed as the mean ± SEM, with statistical significance indicated as p < 0.01 (**). Data were analysed by means of a one-way ANOVA followed by the Dunnett's multiple comparison assay.*

Compared to a glutamate-free control (***Figure B-16A***), no statistically significant changes in gene expressions were observed. A tendency can be noted for the up-regulation of pro-apoptotic **Bax** (185.31 ± 47.95% vs. 100 ± 10.49% control, not significant) and the down-regulation of anti-apoptotic **NF-κβ** (54.59 ± 16.20% vs. 100 ± 16.56% control, not significant) and **Bcl-2** (15.79 ± 5.49% vs. 100 ± 10.49% control, not significant) and pro-apoptotic **Caspase-3** (8.19 ± 5.46% vs. 100 ± 13.89% control, not significant) and **Caspase-8** (14.48 ± 6.04% vs. 100 ± 22.09% control, not significant). The tendency for **Bax** up-regulation and **Bcl-2** down-regulation corresponds well with the literature which states that the expression of these two genes is interconnected, and that an increased expression of either one will ultimately lead to a lower expression of the other (Oltvai *et al.*, 1993; Yang *et al.*, 1995).

From ***Figure B-16B*** it is evident that the neuroprotective characteristics of mirtazapine is at least partially attributed to this drug's ability to significantly decrease the expression of the pro-apoptotic genes **Caspase-3** (55.85 ± 8.47%, $p < 0.01$) and **Caspase-8** (33.66 ± 11.56%, $p < 0.01$) compared to the glutamate control (100 ± 6.93% and 100 ± 6.27%, respectively).

B.2.8 Primer efficiencies

The efficiencies of the study primers were determined as described in § A.2.6.2, and all the study sample results were standardised according to its respective primer efficiency value. The efficiencies of the study primers were determined as: **BDNF** 101.7%, **Akt** 105.1%, **CREB** 105.8%, **NF-κβ** 96.1%, **Bcl-2** 97.7%, **Bax** 98.8%, **Caspase-3** 106.7%, **Caspase-8** 112.6% and **UBC** 101.7%. The primer efficiency value is an indication of the accuracy and selectivity of the primer, with 100% being the optimum efficiency value. If the primer efficiency value deviates from 100% with no more than 15%, the primer can be considered as accurate and selective. Thus, it can be concluded from the study primer efficiencies that all the primers employed in this study are within the specified ranges for accuracy and selectivity.

B.2.9 Effect of the pre-treatment regimes on the house-keeping gene (Ubiquitin C) expression

In order to ensure that the quantitative rt^2-PCR data obtained from the duplicate samples as well as the independent assay runs are comparable, an internal standard is needed. Therefore a house-keeping gene was selected. A house-keeping gene is a gene naturally expressed in the study cell line which is not influenced by the different pre-treatment regimes. Thus, if no significant variation is observed in the expression levels of the house-keeping gene, the inter- and intra-run data can be considered as comparable and accurate. The house-keeping gene selected for the purpose of this study was ubiquitin C (UBC). Also, the same number of human SH-SY5Y cells was seeded from a homogenous cell suspension in each well. Consequently, the same number of homogenous cells in each well was treated with drugs and subjected to mRNA extraction and cDNA synthesis, using validated kits supplied by Quigen®. All these measures ensured satisfactory accuracy, comparability and repeatability of the study results. UBC expression was determined in each analytical run for all the implicated samples.

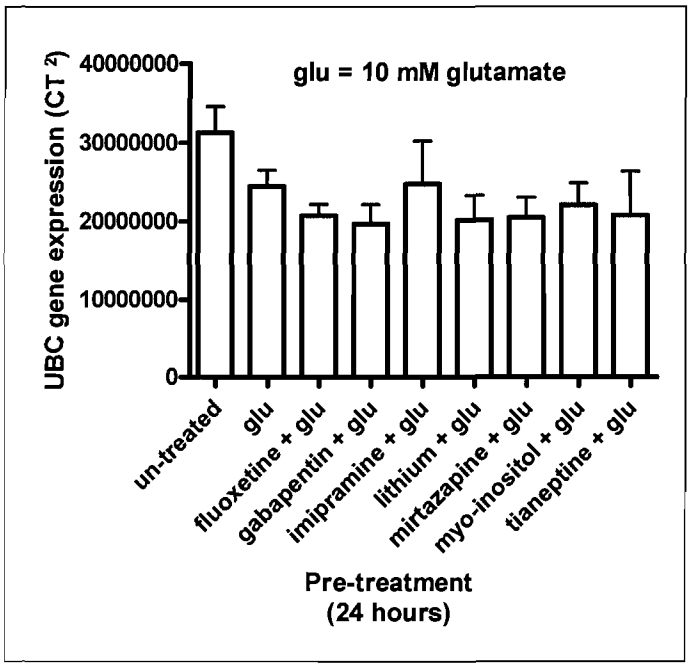

Figure B-17: *The effect of pre-treatment regimes (0 M or 10mM glutamate ± pharmacological drug concentration) on the expression of the house-keeping gene UBC.*
Data are averages of four independent experiments, each with duplicate observations and expressed as the mean ± SEM. Data were analysed by means of a one-way ANOVA followed by the Tukey post test.

As can be seen in ***Figure B-17***, no significant variation was observed in the expression of UBC in either of the study pre-treatment conditions. In particular, if all the treatment groups are measured up to the glutamate alone, the differences become negligible. Therefore, it can be concluded that the amount of analysed cDNA was constant throughout the sample analysis and the obtained results can be deemed comparable.

Instructions to authors

Appendix C

Instructions to Authors (Molecular Psychiatry)

Preparation of manuscripts

All manuscripts should be typed on paper of A4 or US Letter size and printed single-sided. All sections of the manuscript should be double-spaced with one-inch margins on all four sides. Please subdivide manuscript sections into the following sequence:

Title page
Abstract
Introduction
Materials and methods
Results
Discussion
Acknowledgments
References
Tables
Figure legends

Number each page (title page is 1). Please indicate the preferred position for each figure and table in the text (for example, "Figure 1 about here"). Because competition for space is severe, brevity is highly encouraged. Papers not following the indicated format may be returned.

Title page/keywords

The title should be as brief as possible with no abbreviations. Include each author's surname, initials and complete addresses in English including department, institution, city, postal code and country (indicating clearly which author is at which address.) A corresponding author should be indicated with telephone and fax numbers and e-mail address provided. Please also provide a running title limited to 50 characters. Please

provide 5 to 10 keywords using standard MeSH-Medline subject headings for indexing purposes. Terms not contained within the title are preferred.

Original Articles

These articles should consist of a non-structured Abstract of 150-250 words, followed by a brief Introduction that assumes the reader is knowledgeable in the field (no more than 1500 words), Materials and methods, Results, Discussion, Acknowledgments and References. There is no size limit, but papers of more than 30 typewritten pages must justify the need for a long article in their cover letter.

Abbreviations

These should be defined in parentheses after their first mention in the text, except for the use of accepted abbreviations, such as SI symbols, which need not be defined. Use generic names when referring to drugs; trade names may be given in parentheses at first mention.

Supplementary information

Supplementary information is peer-reviewed material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. It is posted on the journal's web site and linked to the article when the article is published, and may consist of data files, graphics, movies or extensive tables.

The printed article must be complete and self-explanatory, and supplemental information must ONLY enhance a reader's comprehension of the paper and not be essential to the understanding. All files must be supplied to the editorial office in final form for peer review.

References

For all papers, references should follow the Vancouver format. In the text, they should appear as numbers starting at 1. At the end of the papers they should be listed (double-spaced) in numerical order corresponding to the order of citation in the text. All authors should be quoted up to a maximum of six followed by et al. Abbreviations for titles of medical periodicals should conform to those used in the latest edition of *Index Medicus*. The journal name should be italicized, the volume number should be bold, and the first

and last page numbers for each reference should be provided. Abstracts and letters must be identified as such.

1 Barbosa MDFS, Ngyen QA, Tchernev VT, Ashley JA, Detter JC Blaydes SM *et al.* Identification of the homologous beige and Chediak-Higashi syndrome genes. *Nature* 1996; **382**: 262-265.

2 Greenberger JS. Long-term hematopoietic cultures. In Golde W. (ed) *Hematopoiesis.* Churchill Livingstone: New York, 1984, pp 203-242.

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Figures and images should be labelled sequentially, numbered and cited in the text. Figure legends should be printed and double-spaced on a separate sheet entitled 'Titles and legends to figures.' Figures should be referred to specifically in the text of the paper but should not be embedded within the text. The use of three-dimensional histograms is strongly discouraged when the addition of the third dimension gives no extra information. If a table or figure has been published before, the authors must obtain written permission to reproduce the material in both print and electronic formats from the copyright owner and submit it with the manuscript. This follows for quotes, illustrations and other materials taken from previously published works not in the public domain. The original source should be cited in the figure caption or table footnote. Colour figures can be reproduced if necessary, but the authors will be expected to contribute towards the cost of publication. A quote will be supplied upon the paper's acceptance.

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Authors are requested to follow our instructions on how to prepare their figures. For more information, see http://www.nature.com/aj/artworkguidelines.pdf. We request that at submission, ALL figures are of a high enough quality to be assessed in the peer review process. If your paper is accepted for publication and you have followed the

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6. Spaces, not commas should be used to separate thousands.
7. Abbreviations should be preceded by the words for which they stand in the first instance of use.
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File formats for manuscript files, figures and tables that are acceptable for our electronic manuscript submission process are given on the online forms. Further advice on file types is also available from the Tips webpage. Please follow our artwork guidelines for submitting figures, and use a common word-processing package (such as Microsoft Word) for the text. Submit tables either as converted images placed at the end of the

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- Select the Office Button in the upper left corner of the Word 2007 Window and choose "Save As"
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Supplementary information is peer-reviewed material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. It is posted on the journal's web site and linked to the article when the article is published. It may consist of data files, graphics, movies, or extensive tables.

The printed article must be complete and self-explanatory without the supplementary information. Supplementary information enhances a reader's understanding of the paper but is not essential to that understanding.

Supplementary information must be supplied to the editorial office in its final form for peer review.

To ensure that the contents of the supplementary information files can be viewed by the editor(s), referees, and readers, please also submit a 'read-me' file containing brief instructions on how to use the file.

Supplementary information should be cited in the text as "Figure S1, S2, etc," "Table S1, S2, etc," or "Supplementary Materials and Methods."

Supplementary information files

Authors should ensure that supplementary information is supplied in its FINAL format because it is not subedited and will appear online exactly as originally submitted. It cannot be altered, nor new supplementary information added, after the paper has been accepted for publication.

Please supply the supplementary information via the electronic manuscript submission and tracking system, in an acceptable file format (see below).

Authors should:

- Include a text summary (no more than 50 words) to describe the contents of each file.
- Identify the types of files (file formats) submitted.
- Include the text 'Supplementary information is available at the *Molecular Psychiatry* website' at the end of the article and before the References section.

Accepted file formats

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Further questions about the submission or preparation of supplementary information should be directed to the editorial office (makcline@iupui.edu).

Appendix D

Congress contributions

This appendix includes the study abstract, as well as the presented study poster, as presented at the annual congress of the South African Basic and Clinical Pharmacology Society (2007, Buffelspoort, South Africa). The congress presentation was awarded 3rd prize in the Young Scientist Awards category and the poster was awarded 1st prize for best congress poster (2007, Buffelspoort, South Africa).

The modulating effects of selected antidepressants and related drugs on markers of cellular resilience in cultured human neuroblastoma cells

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Purpose:

The neurodegenerative hypothesis of depression postulates that major depression is associated with impaired neuroplasticity in particularly the hippocampus and prefrontal cortex, and that antidepressants may be neuroprotective. Therefore the primary aim of the current study was to determine the effect of selected antidepressant drugs on the survival (mitochondrial activity) of cultured human neuroblastoma cells, before and after glutamate-induced excitotoxicity, as well as to determine the effects of these drugs on pro- and anti-apoptotic pathways.

Methods:

The free fraction of plasma protein-bound drugs were determined in Ham's F12 medium ± 10 % foetal bovine serum (FBS). Samples containing 10 % FBS along with the drug was centrifuged in microcentrifuge filters (30,000 Da pore size) to remove plasma albumin. The free and total drug concentrations were determined by means of HPLC analysis.

Cultured human neuroblastoma SH-SY-5Y cells were pre-treated for 24 hours in 24-well plates with and without 10 mM glutamate plus a pharmacological (low) and high (≥10x low) concentration of fluoxetine, mirtazapine, tianeptine, imipramine, *myo*-inositol, gabapentin and lithium. Thereafter mitochondrial activity was determined with the standard MTT cell viability assay.

In follow-up studies, currently being conducted, the effects of the antidepressants are being determined on genes encoding for the pro-apoptotic factors Bax, caspase-3 and caspase 8, and anti-apoptotic factors BDNF, NF-κβ, Akt, CREB and Bcl-2, utilising quantitative real-time RT-PCR. This will be followed by Western blot analyses of the corresponding proteins.

Results:

Results from HPLC analyses indicate that drugs are moderately bound to plasma albumin in culture medium and it is possible to calculate the free drug concentrations.

A 24-hour pre-treatment with pharmacological (low) concentrations of lithium, *myo*-inositol, imipramine and gabapentin as well as the high dose gabapentin pre-treatment, but not of fluoxetine, mirtazapine or tianeptine, protected against glutamate induced excitotoxicity, as measured with the MTT assay. However, no significant increase in mitochondrial activity was observed with fluoxetine, mirtazapine and tianeptine.

Quantitative real-time RT-PCR and Western blot analysis are being conducted to establish how these results relate to the activation and/or inhibition of pro-apoptotic and anti-apoptotic pathways. The current data supports the hypothesis that antidepressants may be neuroprotective and that this may contribute to the antidepressant action of these drugs.

The modulating effects of selected antidepressants and related drugs on makers of cellular resilience in cultured human neuroblastoma cells

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1 INTRODUCTION

Depression – a serious psychiatric disorder

Depression is the second most ubiquitous disorder in the Western world, after cardiovascular disorder, with a prevalence of 9-18% and is also among the most common causes of morbidity, disability and suicide around the world[1,2]. Current drug treatment is plagued by an unfavourable side-effect profile, delayed onset of action and high incidence of treatment resistance.

Hypotheses of Depression

Previous hypotheses were based on aberrant concentrations of monoamines in the synaptic cleft, and assumed that antidepressants exert their primary bio-chemical effects by readjusting the intrasynaptic concentrations of these neurotransmitters[3,4]. In contrast, recent neuro-imaging studies revealed selective structural changes as well as volume reductions in certain limbic and non-limbic brain regions in several stress-related neuropsychiatric illnesses, such as major depressive disorder[5,6,7]. These findings lead to the formulation of the neurodegenerative hypothesis of depression[3,4,8].

Depression & Neuroplasticity

The hippocampus, one of the major brain areas implicated in depression, has shown up to 20% reduction in volume in neuroimaging studies of depressed patients[8,9]. The high vulnerability of this brain region stimulated considerable research into its underlying causes[8]. From this emerged a central dogma centred around glutamate being the predominant neurotransmitter in the hippocampus[8].

An increase in glutamate neurotransmission, as is noted during depression, causes an excessive influx of Ca^{2+} into the neuron which ultimately leads to neuronal demise[8].

Several prototype and experimental anti-depressants has shown to prevent and/or reverse hippocampal atrophy[7]. These include tianeptine (affects structural plasticity in the hippocampus and is an effective anti-depressant[18,19,21]); fluoxetine (down-regulates neurodegenerative enzymes and is anti-apoptotic[13]); lithium (neuroprotective[11,12,17] and anti-apoptotic[10,14,15]); imipramine (increases hippocampal neurogenesis and is neuroprotectant[13,16,20]); mirtazapine (decreases corticosterone- induced gene transcription and is neuroprotectant[14]). Although some data are available on the suspected protective and antidepressant properties of these drugs, their mechanisms of action are still poorly understood[22]. Thus, determination of the intra-cellular mechanisms of these drugs might result in the discovery of new antidepressant targets as well as new and advanced treatment regimes[14].

Study objective

The objective of the current study was to determine the effect of selected antidepressant drugs on neuroplasticity during neuronal stress (i.e. glutamate-induced excitotoxcicity), measuring mitochondrial activity and the expression of genes involved in neuronal survival and growth.

2 EXPERIMENTAL PROCEDURES

High performance liquid chromatography (HPLC) determination of (free drug)

The free fraction of plasma protein bound drugs were determined by spiking known concentrations of these drugs into Ham's F12 medium + foetal bovine serum (FBS). The samples containing FBS were centrifuged in microcentrifuge tubes with a pore size of 30kDa, to remove the protein-bound drug. The drug concentration of all samples were analysed by HPLC, using a acetonetrile/0.005 M heptane sulphonic acid sodium salt in water mobile phase (pH 3.5), flow rate 1.0 ml/min, 150 x 4.6 mm Luna C18-2 column (100 Å pores), at a UV detection wavelength of 228 nm.

Study drugs

Fluoxetine (97 nM), gabapentin (0.23 µM), imipramine (71 nM), lithium (1 mM), mirtazapine (169 nM), *myo*-inositol (2 mM) and tianeptine (1 µM).

Cell seeding

SH-SY5Y human neuroblastoma cells were maintained in Ham's F12 medium, 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone in culture flasks at 37°C in 5% CO_2 and grown to 90% confluency. Monolayer cells were detached from culture flasks by means of trypsination and seeded into 24-well plates at a

MTT cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT - 0.25 mg/ml) was added to each well and incubated at 37°C in 5% CO_2 for 2 hours. The formazan crystals synthesised in viable cells (with mitochondrial reductase activity) were dissolved in iso-propanol and the colour intensity determined spectrophotometrically at 560 nm[23].

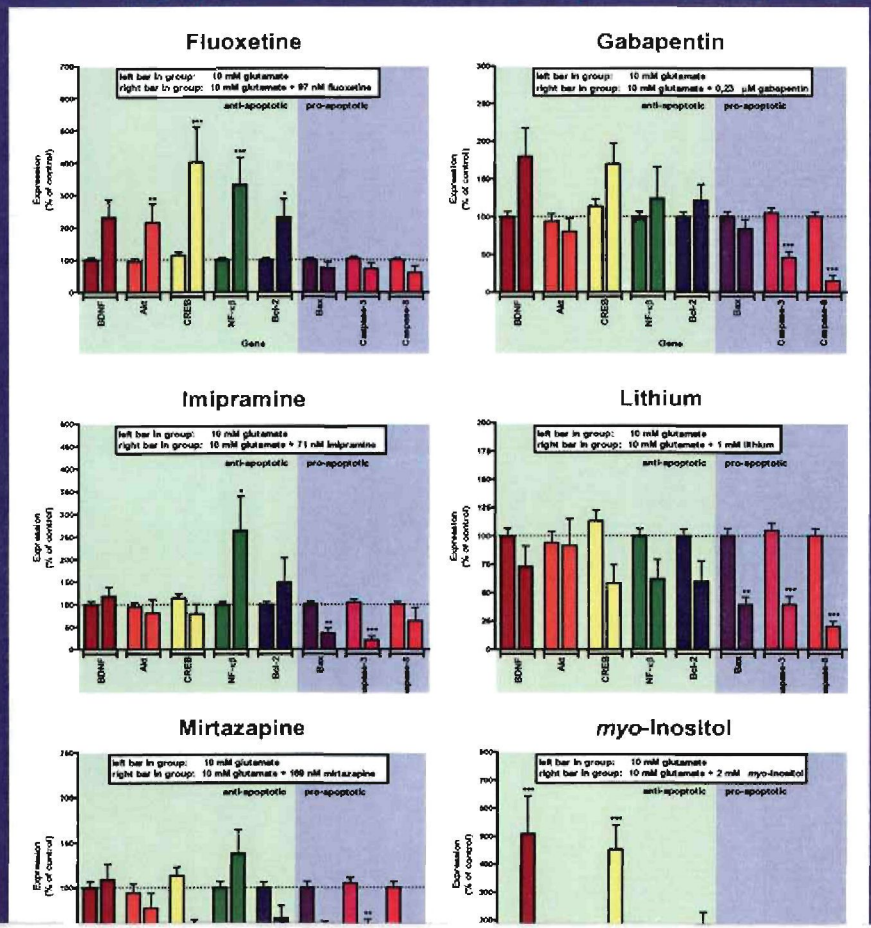
Real-time reverse transcriptase-polymerase chain reaction

Real-time reverse transcriptase polymerase chain reaction (rt²-PCR) was employed to determine gene expression (mRNA) before and after drug treatments. Genes investigated included pro- and anti-apoptotic genes (pro-apoptotic: Bax, Caspase-3 & 8; anti-apoptotic: BDNF, NF-κβ, Akt, CREB & Bcl-2). The Quigen Fastlane cDNA kit® was used for mRNA extraction and cDNA synthesis. BioRad SYBR Green® and QuantiTec Primer assays® (specific for each study gene) was added for cDNA amplification during the rt²-PCR process. The rt²-PCR samples were analysed using 25µl reaction volumes and the run-time conditions supplied by Quigen®.

Statistical analysis

The computer software, Microsoft Excel® 2002® and GraphPad Prism 5.01®, were used to

Pharmacological concentrations of fluoxetine, imipramine, tianeptine and *myo*-inositol significantly increases anti-apoptotic gene expression in human neuroblastoma cells after glutamate induced excitotoxicity, while pro-apoptotic gene expression is significantly decreased with pharmacological concentrations of gabapentin, imipramine, lithium, mirtazapine, *myo*-inositol and tianeptine.



density of ± 5 x 10^5 cells/well.

Drug pre-treatment

After 48 hours of incubation the culture medium was aspirated and the cells were pre-treated with a pharmacological concentration of the study drug ± 10 mM glutamate in Ham's F12 medium + 10% FBS and incubated for 24 hours at 37°C in 5% CO_2. Thereafter the medium with drug was aspirated and the cells rinsed before applying the appropriate assay.

process the experimental data. All study data were analysed using a one-way analysis of variance (ANOVA), followed by the Dunnett's Multiple Comparison test. Data are expressed as the mean ± SEM with statistical significance expressed as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

3 RESULTS

Pharmacological concentrations of the prototype antidepressants imipramine and lithium, and the experimental antidepressant *myo*-inositol, protect against glutamate-induced neurotoxicity (excitotoxcicity) in human neuroblastoma cells.

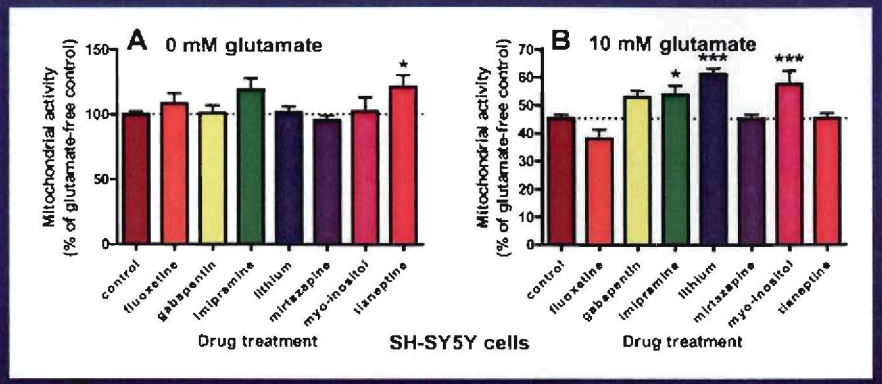


Fig 1: Effect of prototype and experimental antidepressants on mitochondrial activity of human neuroblastoma (SH-SY5Y) cells, either (A) without glutamate or (B) in the presence of 10 mM glutamate. Data are averages of three independent experiments, each with triplicate observations and expressed as the mean ± SEM, with statistical significance indicated as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

5 CONCLUSION

The obtained study results supports the hypothesis that the efficiency of antidepressant treatments may be partially dependable on the degree in which they exert neuroprotective characteristics. The current data also indicates that all the study drugs (prototype and experimental antidepressants) induce intra-cellular alterations, which might ultimately lead to enhanced neuronal resilience.

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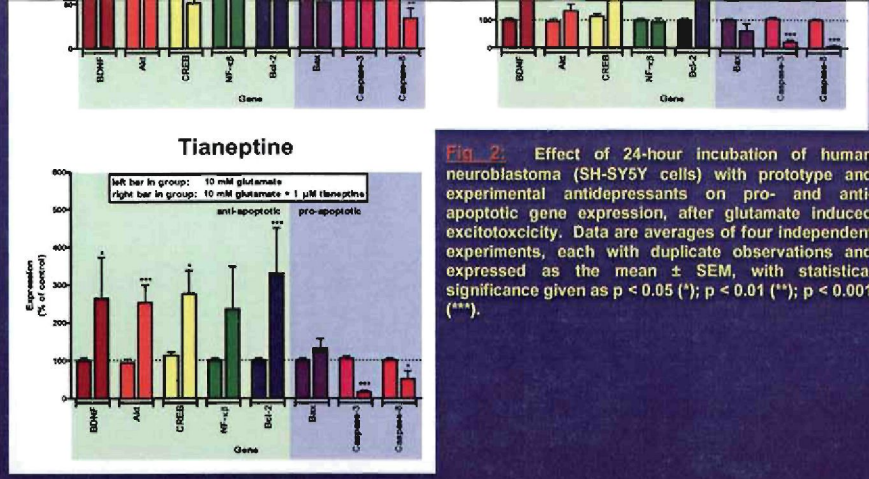

Fig 2: Effect of 24-hour incubation of human neuroblastoma (SH-SY5Y cells) with prototype and experimental antidepressants on pro- and anti-apoptotic gene expression, after glutamate induced excitotoxcicity. Data are averages of four independent experiments, each with duplicate observations and expressed as the mean ± SEM, with statistical significance given as $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

4 SUMMARY AND DISCUSSION

Mitochondrial Activity

The prototype antidepressants imipramine and lithium, as well as *myo*-inositol, protected against the glutamate-induced reduction of mitochondrial activity, as depicted in Figure 1B. These results suggest that these drugs may possess neuroprotective properties during neuronal stress. However, these drugs have no significant effect on neuronal viability in the absence of glutamate (Figure 1A), suggesting that their protective abilities are stress-dependant. Tianeptine, on the other hand, increase the viability of the cells in the absence of glutamate, but has no significant effect on mitochondrial activity in the presence of glutamate. Therefore it can be postulated that tianeptine exerts its neuroprotective characteristics via an alternative intra-cellular mechanism.

Gene Expression

The rt^2-PCR results indicated an overall tendency for neuroprotection by all antidepressants (Fig. 2). Fluoxetine increases the expression of CREB, which correlates with results from previous studies[13,25]. Previous research found that fluoxetine significantly increases the expression of BDNF, although the results from this study only indicated a tendency for BDNF gene up-regulation[13]. The genes encoding for the anti-apoptotic transcription factors Akt and NF-κβ also significantly increases with fluoxetine pre-treatment.

Gabapentin, lithium and mirtazapine seems to exert their protective abilities by decreasing the gene expressions of pro-apoptotic proteins. Past research found that lithium significantly increase the expression of CREB and Bcl-2, and decrease the expression of Bax[10,15,17]. Although the increases in anti-apoptotic gene expression were not observed, the decrease seen in Bax expression correlates with previous research[17]. The imipramine data for Akt expression correlates well with previous studies, which found that no significant changes in overall Akt expression is brought about by imipramine pre-treatment[24]. CREB, BDNF and Bcl-2 gene up-regulation were also noted in previous imipramine studies however, these results are not confirmed by this study though a tendency for Bcl-2 up-regulation is observed[10,20,25]. The experimental antidepressant *myo*-inositol demonstrates an excellent potential as neuroprotective agent, by increasing anti- and decreasing pro-apoptotic gene expression. Tianeptine has previously been found to be a superior neuroprotective agent, as is evident from the results regarding this drugs ability to significantly increase genes encoding for neuronal survival as well as decreasing the genes encoding for neuronal demise[18,19].

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Abbreviations

Appendix E

Numerical

5-HIAA	5-Hydroxyindoloacetic acid
5-HT	Serotonin (5-hydroxytryptamine)
5-HT_1	Serotonin 1 receptor
5-HT_{1A}	Serotonin 1A receptor
5-HT_{1B}	Serotonin 1B receptor
5-HT_{1D}	Serotonin 1D receptor
5-HT_2	Serotonin 2 receptor

A

Abs	Absorbance
ADP	Adenosine diphospate
AIF	Apoptosis-inducing factor
Akt	Protein kinase B
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionate
ATF1	Activating transcription factor 1
ATP	Adenosine triphosphate

B

Bax	Bcl2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
BDNF	Brain derived neurotrophic factor

C	
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
Caspases	Cysteinyl-aspertate-specific proteinases
CBP	CREB-binding protein
cDNA	Coding deoxyribonucleic acid
CNS	Central nervous system
CO_2	Carbon dioxide
CRE	cAMP-response elements
CREB	Cyclic adenosine monophosphate response element binding protein
CREM	cAMP response modulatory protein
D	
DA	Dopamine
DA	Dopamine
DAG	Diacylglycerol
ddH_2O	Double distilled water
DED	Death effector domain
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
F	
FADD	Fas-associated death domain protein
FasL	Fas ligand
FBS	Foetal bovine serum

	G
GABA	Gamma-amino butyric acid
	H
HDVC	Hoë-druk-vloeistof-chromatografie
HPA-axis	Hypothalamus-pituitary-adrenal axis
	I
IAP	Inhibitory apoptotic proteins
IDO	Indoleamine-2,3-dioxygenase
IKK	Inhibitory kappa beta kinases
IL-10	Interleukin-10
IL-1Ra	Interleukin-1Ra
IL-1β	Interleukin-1beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IMPase	*myo*-Inositol Monophasphatase
INFα	Interferon-alpha
INFγ	Interferon-gamma
IP_3	Inositol trisphosphate
it^2-PKR	Intydse inverse transkripsie-polimerase ketting reaksie
Iκβα	Inhibitory kappa beta alpha
	L
l-NE	Norepinephrine
	M
MAO	Monoamine oxidase

MAO-A	Monoamine oxidase A enzyme
MAO-B	Monoamine oxidase B enzyme
*m*Ins	*myo*-Inositol
Mn-SOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
	N
NaSSA	Noradrenergic and serotonin-specific antagonist
NF-κβ	Nuclear factor kappa beta
NGF	Nerve growth factor
NLS	Nuclear localization signal
NMDA	*N*-methyl-*D*-aspartate
NO	Nitric oxide
	P
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol bisphosphate
PNS	Peripheral nervous system
PTSD	Post-traumatic stress disorder
	R
RHD	Relationship homology domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rt^2-PCR	Real time reverse-transcriptase polymerase chain reaction
rt-PCR	Reverse-transcriptase polymerase chain reaction
	S
SF	Survival factor

SMAC	Second mitochondria-derived activator of caspases
SSRI	Selective serotonin re-uptake inhibitors
	T
TBARS	Thiobarbituric acid reacting substances
TCA	Tricyclic antidepressants
TGFβ1	Tumor growth factor-beta1
TNF	Tumor necrosis factor
TNF-R1	Tumor necrosis factor receptor 1
TNF-R2	Tumor necrosis factor receptor 2
TNFα	Tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis inducing ligand
	U
UBC	Udiquitin C gene

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Appendix F

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