The effects of sildenafil on neuroplasticity in human neuroblastoma cells

RIAAN REAY

(B.Pharm)

Dissertation submitted in fulfilment of the requirements for the degree

Magister Scientiae in Pharmacology

at the

(Potchefstroom Campus) of the North-West University

Supervisor: Prof. Christiaan B. Brink

Co-supervisor: Prof. Brian H. Harvey

Potchefstroom

2010

Abstract

The antidepressant treatment of depression, currently the most debilitating psychiatric disorder, is plagued by delayed onset of action, troublesome side-effects and treatment resistance. However, a comprehensive understanding of the biological basis and treatment of depression remains elusive, prompting extensive ongoing research. The neuroplasticity hypothesis of depression has gained support from various lines of experimental and clinical evidence, whereas chronic antidepressant treatments reverse impaired neuroplasticity. The NO/cGMP pathway is believed to play an important role in the dysregulated neuroplasticity and has been a target for novel antidepressant strategies. Our laboratory recently demonstrated antidepressant-like effects of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil when combined with the antimuscarinic drug atropine in rats. Unpublished data suggested that sildenafil may up-regulate genes encoding for the expression of anti-apoptotic proteins *in vitro*. Therefore, the primary study objective was to investigate the effects of PDE5 inhibitors and other modulators of the NO/cGMP pathway on neuroplasticity.

The human neuroblastoma (SH-SY5Y) and non-neuronal Chinese hamster ovary (CHO-K1) cell lines were subjected to various biological stressors associated with the neuropathology of major depression, including glutamate-induced excitotoxicity, H_2O_2-induced oxidative stress and serum deprivation. The latter was selected as optimal stressor in SH-SY5Y cells whereafter they were incubated for 24 hours with the antidepressant drugs imipramine, fluoxetine and tianeptine, the mood stabilizer lithium, the PDE5 inhibitors sildenafil and tadalafil, the PDE4 inhibitor, rolipram and the cGMP analogue db-cGMP under conditions of serum deprivation. Also, the incubation with sildenafil and db-cGMP was performed with and without the soluble guanylate cyclase inhibitor (ODQ) or the protein kinase G (PKG) inhibitor (RP-PET-cGMPS). Thereafter cell viability was measured with the MTT- and Trypan blue assays and DNA repair capacity was measured with the comet assay.

The results indicated that sildenafil exerts a protective effect against oxidative stress, as measured in the MTT assay. This property was shared by lithium, but not by db-cGMP or the antidepressants. In addition, ODQ and RP-PET-cGMPS reversed the protective effect of sildenafil, but with ODQ having a significantly greater effect. Results from the comet assay indicate that all antidepressant drugs, PDE inhibitors and db-cGMP significantly increased DNA repair capacity of SH-SY5Y cells. ODQ and RP-PET-cGMPS reversed the enhancing effects of sildenafil and db-cGMP on DNA repair capacity.

Main conclusions are that 24 hour serum deprivation of human neuroblastoma (SH-SY5Y) cells provides a suitable *in vitro* biological stressor. Serum deprivation in combination with DNA repair capacity as biological marker of oxidative stress, exhibit appropriate predictive validity in evaluating the effects of antidepressant drugs on neuroplasticity. Sildenafil, but not tadalafil, may possess a unique protective property against oxidative stress to increase mitochondrial function, not shared by classical antidepressants. PDE5 inhibitors share the protective property of classical antidepressants to enhance DNA repair capacity under conditions of oxidative stress. The mechanism whereby the PDE5 inhibitors exert their protective property to enhance DNA repair capacity, involve increasing levels of cGMP and is PKG mediated.

Finally, this study suggests that neuroprotective effects may contribute to the antidepressant-like activity of PDE5 inhibitors as observed in animal studies.

Uittreksel

Die antidepressantbehandeling van depressie, tans die mees ontmagtigende psigiatriese toestand, word geknel deur vertraagde aanvang van werking, kwellende newe-effekte en behandelingsweerstandigheid. 'n Grondige begrip van die biologiese basis en behandeling van depressie ontbreek egter steeds, wat uitgebreide voortgesette navorsing noodsaak. Die neuroplastisiteitshipotese van depressie het toenemende steun vanuit verskeie gronde vir eksperimentele en kliniese getuienis, terwyl die chroniese behandeling met antidepressante onderdrukte neuroplastisiteit omkeer. Die NO/cGMP sein-transduksieweg word allerweë aanvaar om 'n belangrike rol te speel in gedisreguleerde neuroplastisiteit en is 'n teiken vir nuwe antidepressantstrategieë. Ons laboratorium het onlangs antidepressant-agtige effekte van die fosfodiësterase-tipe-5- (PDE5) inhibeerder sildenafil gedemonstreer, wanneer dit met die antimuskariniese geneesmiddel atropien in rotte gekombineer word. Ongepubliseerde data suggereer dat sildenafil gene wat vir die uitdrukking anti-apoptotiese proteïene kodeer op-reguleer. Om hierdie rede was die primêre doelwit van hierdie ondersoek om die effekte van PDE5-inhibeerders en ander moduleerders van die NO/cGMP sein-transduksieweg op neuroplastisiteit te ondersoek.

Die menslike neuroblastoma- (SH-SY5Y) en die nie-neurale Sjinese hamster-ovariale- (CHO-K1) selle is onderwerp aan verskeie biologiese stressors geassosieer met die neuropatologie van major depressie, insluitend glutamaat-geïnduseerde eksitotoksisiteit, H_2O_2-geïnduseerde oksidatiewe stres en serumonthouding. Laasgenoemde was as die optimale stressor in SH-SY5Y selle geselekteer, waarna dit vir 24 uur geïnkubeer is met die antidepressant-geneesmiddels imipramien, fluoksetien en tianeptien, die gemoedstabiliseerder litium, die PDE5-inhibeerders sildenafil en tadalafil, die PDE4-inhibeerder rolipram en die cGMP-analoog db-cGMP, onder kondisies van serumonthouding. Verder is die inkubasie met sildenafil en db-cGMP uitgevoer met en sonder die oplosbare

guanilielsiklase-inhibeerder (ODQ) of die proteïenkinase-G- (PKG) inhibeerder RP-PET-cGMPS. Hierna is sellulêre lewensvatbaarheid met die MTT- en Trypan blou-toetse gemeet, asook die DNA-herstelkapasiteit met die komeetanalise.

Die resultate dui aan dat sildenafil 'n beskermende effek teen oksidatiewe stress bied, soos gemeet met die MTT-toets. Hierdie eienskap was gedeel deur litium, maar nie deur db-cGMP of die antidepressante nie. Verder het ODQ en RP-PET-cGMPS die beskermende effek van sildenafil omgekeer, maar met die effek van ODQ beduidend groter. Resultate van die komeetanalise dui aan dat al die antidepressante, PDE-inhibeerders en db-cGMP die DNA-herstelkapasiteit in SH-SY5Y selle beduidend verhoog het. ODQ en RP-PET-cGMPS het die positiewe effek van sildenafil en db-cGMP op DNA-herstelkapasiteit omgekeer.

Primêre gevolgtrekkings is dat 24 uur serumonthouding in menslike neuroblastoma (SH-SY5Y) selle 'n geskikte *in vitro* biologiese stressor verskaf. Serumonthouding in kombinasie met DNA-herstelkapasieteit as biologiese merker van oksidatiwe stress vertoon geskikte voorspellingsgeldigheid met die evaluering van die effekte van antidepressant-geneesmiddels op neuroplastisiteit. Sildenafil, maar nie tadalafil nie, beskik moontlik oor unieke beskermende eienskappe teen oksidatiewe stres om mitochondriale funksie te verhoog, wat nie deur klassieke antidepressante gedeel word nie. PDE5 inhibeerders deel die beskermende eienskap van klassieke antidepressante om die DNA-herstelkapasiteit onder kondisies van oksidatiewe stress te verhoog. Die megansime waarvolgens die PDE5-inhibeerders hul beskermende effek om DNA-herstelkapasiteit te verhoog uitoefen behels verhoogde vlakke van cGMP en is PKG-gemediëerd.

Ten slotte, dui hierdie studie aan dat neuronbeskemende effekte tot die antidepressant-agtige aktiwiteit van die PDE5-inhibeerders, soos waargeneem in dierestudies, mag bydra.

Acknowledgements

First and foremost, I thank my Heavenly Father for blessing me with a wonderfully supportive family as well as the needed mental ability and granting me with the necessary patience and perseverance throughout this study.

To my mom, my mentor and friend, for your endless love, encouragement and your always positive influence.

To my sister, Elphia, for your love, friendship and honesty.

To my study leader, Prof. C.B. Brink, my greatest appreciation for your guidance, vision, knowledge and continuous support throughout this study.

To Prof. J. Petzer for grammatical revision.

To Ms Maureen Steyn and Sharlene Lowe for their assistance in the laboratory.

To the National Research Foundation (NRF) for the necessary funding.

To my friends and colleagues (Anri, Fransie, Kevin, Marianne, Marisa, Naude and Stephan) for your friendship and making the workplace an enjoyable environment.

To my special friend, Gert, for the many hours we spent philosophising and your friendship that is bonded by brotherly love.

"The measure of a master in his success is bringing all men round to his opinion twenty years later" **Ralph Waldo Emerson**

Table of Contents

Abstract i
Uittreksel iii
Acknowledgements v
Table of contents vi
List of figures xi
List of tables xiii

Chapter 1: Introduction 1
1.1 Dissertation approach and layout 1
1.2 Problem statement 2
1.3 Study objectives 3
1.4 Study layout 4

Chapter 2: Literature review 6
2.1 Depression 6
2.1.1 Types of depression 7
2.1.1.1 Major depression 8
2.1.1.2 Dysthymic disorder 9
2.1.1.3 Double depression 9
2.1.1.4 Bipolar disorder 10
2.1.2 Hypothesis of depression 10
2.1.2.1 The monoamine hypothesis 11
2.1.2.2 The cholinergic super-sensitivity hypothesis 12
2.1.2.3 The hypothalamic-pituitary-adrenal (HPA) - axis hyperactivity hypothesis 13
2.1.2.4 The neuroplasticity hypothesis 14
2.1.3 Treatment of depression 16
2.1.3.1 The monoamine oxidase inhibitors (MAOIs) 17

2.1.3.2 The tricyclic antidepressants (TCAs) 17
2.1.3.3 The selective serotonin reuptake inhibitors (SSRIs) 18
2.1.3.4 The atypical antidepressants 19
2.2 Neuroplasticity in Depression 20
2.2.1 The brain 20
2.2.1.1 The hippocampus 21
2.2.1.2 The prefrontal cortex 22
2.2.1.3 The amygdala 22
2.2.2 Sub-cellular mechanisms of neuroplasticity 23
2.2.2.1 Excitotoxicity 23
2.2.2.2.1 N-methyl-D-aspartate (NMDA) receptor 24
2.2.2.2 Oxidative stress 25
2.2.2.3 Regulators of apoptosis 26
2.2.2.3.1 Cyclic adenosine response element binding protein 26
2.2.2.3.2 Brain derived neurotrophic factor 27
2.3 The glutamate / NO / cGMP pathway 29
2.3.1 Modulators of the glutamate / NO / cGMP pathway 30
2.3.1.1 Nitric oxide (NO) 30
2.3.1.2 Nitric oxide synthase (NOS) 31
2.3.1.3 Soluble guanylate cyclase 31
2.3.1.4 Cyclic mononucleotides 32
2.3.1.5 Phosphodiesterase (PDE) 33
2.3.1.5.1 Sildenafil 34
2.4 Synopsis 36

Chapter 3: Article 38
Title page 39
Abstract 40
Keywords 41
Introduction 42
Materials and methods 44
Results 50
Discussion 54

Conclusion 58
Acknowledgements 59
Figure caption list 60
Figures 62
References: 66

Chapter 4: Summary and conclusions 72
4.1 Summary 72
4.2 Discussion and Conclusion 74
4.3 Recommendations 78

Addendum A: Materials and methods A1
A.1 Materials A1
A.1.1 Cell lines used A1
A.1.1.1 Human neuroblastoma (SH-SY5Y) cell line A1
A.1.1.2 Chinese hamster ovary (CHO-K1) cell line A2
A.1.2 Chemicals A2
A.1.2.1 Chemicals used for cell cultures A2
A.1.2.2 Chemicals used for assays A2
A.1.3 Consumables A3
A.1.4 Apparatus A3
A.1.5 Statistical analysis A4
A.2 Methods A4
A.2.1 Cell culture preparation A4
A.2.2 Cell stressors and drug treatments A5
A.2.2.1 Cell stressors A5
A.2.2.2 Drug treatments A5
A.2.2.2.1 Sildenafil A5
A.2.2.2.2 Tadalafil A6
A.2.2.2.3 Rolipram A6
A.2.2.2.4 3-isobuthyl-1-methylxanthine (IBMX) A6
A.2.2.2.5 N^2,2’-O-Dibutyrylguanosine 3’-5’-cyclic monophosphate (db-cGMP) A7

A.2.2.2.6 1H-[1,2,4] oxadiazolol [4,3-a] quinoxallin-1-one (ODQ)....A7
A.2.2.2.7 Rp-8-bromob-phenyl-1, N^2-etheno-guanosine 3'5' cyclic monophosphorothioate (RP-PET-cGMPS) A7
A.2.2.2.8 Lithium A7
A.2.2.2.9 Imipramine A7
A.2.2.2.10 Fluoxetine..A8
A.2.2.2.11 Tianeptine A8
A.2.2.2.12 Atropine..A8
A.2.3 Assays..A9
A.2.3.1 MTT – assay..A9
A.2.3.1.1 Introduction A9
A.2.3.2.2 Assay A9
A.2.3.2 Trypan blue – assay A10
A.2.3.2.1 Introduction A10
A.2.3.2.2 Assay A11
A.2.3.3 Single cell gel electrophoresis (comet – assay)..........................A12
A.2.3.3.1 Introduction A12
A.2.3.3.2 Assay A12

Addendum B: Results and discussion B1
B.1 Pilot study..B2
B.1.1 Selection of optimal stress condition in neuronal and non- neuronal cell lines..B2
B.1.2 Selection of stress condition where sildenafil exhibited protection B6
B.1.3 Selection of optimal drug concentration of drugs used in the study......B8
B.2 Study objective experiments B12
B.2.1 MTT – assay (Results)..B12
B.2.1.1 Sildenafil vs. other PDE inhibitors and their role on neuro-protection B12
B.2.1.2 Sildenafil vs. antidepressant drugs and their role on neuro-protection B14
B.2.2 Trypan blue – assay (Results) B16

B.2.2.1 Sildenafil vs. other PDE inhibitors and their role on neuro-protection B16

B.2.2.2 Sildenafil vs. antidepressant drugs and their role on neuro-protection B18

B.3 Summary B19

Addendum C: Instructions for authors **C1**

Addendum D: Congress contribution **D1**

Addendum E: Abbreviations **E1**

Addendum F: References **F1**

List of figures

Figure 2-1: The complex intertwinement of the neuroplasticity hypothesis 16

Figure 2-2: The involvement of neurotrophins in depression 28

Figure 2-3: The mechanism whereby PDE5 exerts is effect on the NMDA/NO/cGMP pathway after excitotoxic glutamate exposure... 30

Figure 2-4: The cyclic mononucleotide signal pathway.. 34

Figure A-1: The conversion of yellow MTT to the purple formazan crystal A9

Figure B-1: The effect of excitotoxicity (glutamate exposure) and oxidative stress (presented as serum free and H_2O_2 exposure) in a neuronal (SH-SY5Y) and non-neuronal (CHO-K1) cell line using the MTT assay B4

Figure B-2: A sildenafil concentration range (0.086 – 1600 nM) on pre-selected stress conditions using the MTT assay B7

Figure B-3: The effect of 24 hour incubation with the PDE5 inhibitors, tadalafil (0-1000 nM) and zaprinast (0-1000 nM), the PDE4 inhibitor, rolipram (0-160 nM), the non-selective PDE inhibitor, IBMX (0-8400 nM), the cGMP analogue, db-cGMP (0-200000 nM) and the sGC inhibitor, ODQ (0-2000 nM) using the MTT assay B9

Figure B-4: The effect of 24 hour incubation with the SSRI, fluoxetine (0-2000 nM), the atypical antidepressant, tianeptine (0-500 nM), the TCA, imipramine

(0-710 nM) and the mood stabilizer, lithium (0-1.5 mM) using the MTT assay B11

Figure B-5: Measuring the effect of the following drug pre-treatments: the PDE5 inhibitors sildenafil, tadalafil and zaprinast, the PDE4 inhibitor rolipram and the non-selective PDE inhibitor IBMX on cell viability (mitochondrial activity) in neuroblastoma cells during 24 hour serum-free incubation .. B13

Figure B-6: The effect of 24 hour incubation with the PDE5 inhibitor, sildenafil, the mood stabilizer lithium, the SSRI fluoxetine, the TCA imipramine, the atypical antidepressant tianeptine, the anticholinergic atropine as well as a combination of sildenafil and atropine using the MTT assay during serum-free incubation B15

Figure B-7: The comparative effect of PDE5 inhibitors: sildenafil, tadalafil and zaprinast and the PDE4 inhibitor rolipram on membrane permeability after 24 hour serum-free drug pre-treatment incubation using the Trypan blue assay B17

Figure B-8: The effect of 24 hour incubation with the PDE5 inhibitor, sildenafil, the mood stabilizer lithium, the SSRI fluoxetine, the TCA imipramine, the atypical antidepressant tianeptine, the anticholinergic atropine as well as a combination of sildenafil and atropine using the Trypan blue assay during serum-free incubation B18

List of tables

Table 4-1: The effects of three different biological stress conditions, associated with the neuropathology of major depression, on cell viability in a neuronal and non-neuronal cell line, as measured in the MTT assay 72

Table 4-2: The effects of 24 hour treatment with different antidepressants, mood modulating drugs and drugs modulating the cGMP pathway under conditions of serum deprivation on viability in human neuroblastoma (SH-SY5Y) cells, as measured by the MTT, Trypan blue and DNA comet assays 73

Introduction

Chapter 1

(All abbreviations are listed in Addendum E)

This introductory chapter serves as an orientation to the dissertation and study as a whole, and is therefore very concise. A more elaborate literature study is presented in the next chapter.

1.1 Dissertation approach and layout

This dissertation is presented in an *article format*, whereby the key data is prepared as a manuscript (see Chapter 3) for publication in a chosen scientific journal. All complementary data, not included in the article, is presented in an addendum (see Addendum B). In addition, chapters with, for example, a literature review (Chapter 2) and conclusions (Chapter 4) are also included in the dissertation. The following outline serves to assist the reader where to find key elements of the study in the dissertation:

- **Problem statement, study objectives and study layout:**
  Chapter 1
- **Literature background**
  Chapter 2 (literature review) and Chapter 3 (article introduction)
- **Materials and methods**
  Chapter 3 (article methods) and Addendum A (extended materials and methods)
- **Results and discussion**
  Chapter 3 (article results and discussion) and Addendum B (additional results and discussion)
- **Summary and conclusions**
  Chapter 4 (for the study as a whole, including the article and addendum)

1.2 Problem statement

Depression affects more than 121 million people worldwide and is projected to be the second largest burden of disease by the year 2020 (Mathers et al., 2003). Depression is currently treated using drugs that exert their action based on the monoamine hypothesis (Fava et al., 2008). These antidepressant drugs are plagued *with* delayed onset of action, bothersome side-effect profile (associated with poor compliance to treatment) and treatment resistance (Fava, 2003; Katzung et al., 2002; Manji et al., 2003), suggesting an urgent need for more effective and tolerable drugs and treatment strategies. This implies also a need for extensive research on the neurobiological basis of depression and associated novel drug targets for antidepressant action.

The neuroplasticity hypothesis of the neurobiological basis of depression provides one such a framework to investigate novel drug targets, and is viewed as a unifying hypothesis in that it accommodates classical hypotheses, while also accommodating newer observations that were not explained by previous hypotheses. This hypothesis describes depression as a neuropsychiatric disease associated with impaired neuroplasticity, which is reversed by effective antidepressant treatment. Typical biomarkers of neuroplasticity include transcription factors such as cyclic adenosine monophosphate response element binding protein (CREB) and neurotrophic factors such as brain derived neurotrophic factor (BDNF), where it has been shown that decreased levels of CREB and BDNF is associated with the neuropathology of depression (Chen et al., 2001). Stress-related inhibition of neuroplasticity is also closely associated with excitotoxicity, as induced by an overactive glutamatergic neurotransmission. Glutamate acts via the NMDA receptor to activate nitric oxide (NO), which exerts its effect on higher functions that include learning and memory (Harvey, 2006), and subsequently activates soluble guanylyl cyclase to facilitate the formation of cyclic guanylate monophosphate (cGMP), a second messenger that activates several cellular pathways. cGMP is degraded by phosphodiesterases, and phosphodiesterase (PDE) inhibitors such as sildenafil (a PDE5 selective inhibitor) has been shown to increase synaptic plasticity (Andreeva et al., 2001), evoke neurogenesis (Zhang et al., 2002) and play a role in brain development (Esplugues, 2002). The novel antidepressant tianeptine has been

shown to block the release of glutamate by antagonizing the NMDA-receptor or by non-competitive NMDA-receptor modulation (McEwen et al., 2005). The role of the glutamate/NO/cGMP signal transduction pathway therefore plays an important role in the regulation of mood, anxiety and stress.

It has been demonstrated recently that sildenafil, when co-administered with atropine (but neither drug alone), produces an antidepressant-like effect in rats, comparable to that of fluoxetine (Brink et al., 2008). This finding provides further support for a role of the glutamate/NO/cGMP pathway in depression and proposes PDE5 as a novel drug target for antidepressants. In addition it has been shown recently in our laboratory that sildenafil may possess neuroprotective properties via activation of anti-apoptotic pathways in human neuroblastoma (SH-SY5Y) cells (unpublished data), thereby suggesting a role for PDE5 in the modulation of neuroplasticity.

Depression is now recognised as a multifactorial disorder, with a complex neuropathology. It is therefore likely that multiple drug targets to treat the disorder will be identified, prompting further research. In particular, there is a need to investigate the effects of the PDE5 inhibitor sildenafil, a drug with much potential as novel antidepressant strategy, on neuroplasticity.

1.3 Study objectives

The primary objective of the current study was to investigate the effects of sildenafil treatment on markers of cellular plasticity in a neuronal and non-neuronal cell line.

Utilising human neuroblastoma (SH-SY5Y) cells, this study specifically aimed to:

- investigate and select appropriate biological stress conditions that relate to neuronal stress in major depression;
- determine under the selected biological stress conditions the concentrations at which sildenafil and other drugs that modulate the NO/cGMP pathway exert optimal effects on cellular plasticity;

- evaluate under the selected biological stress conditions the effects of the various indicated drugs at the determined optimal concentrations on different markers of cellular plasticity;

- evaluate the role of the NO/cGMP signal transduction pathway in any effects observed with sildenafil on cellular plasticity.

A secondary objective of the current study was to compare the effects of sildenafil treatment on markers of cellular plasticity as observed in a neuronal to that observed in a non-neuronal cell line.

Utilising neuronal human neuroblastoma (SH-SY5Y) and non-neuronal Chinese hamster ovary (CHO-K1) cells, this study objective specifically aimed to:

- compare the effects of different biological stress conditions on the cellular plasticity of these cell lines;

- compare the effects of different concentrations of sildenafil on the stress-induced changes in cellular plasticity in these cell lines.

1.4 Study layout

All of the experiments for the current study were performed in the Laboratory for Applied Molecular Biology (LAMB) at the North-West University, Potchefstroom Campus, South Africa. For the abovementioned study objectives to be achieved the following study layout were followed:

- SH-SY5Y and CHO-K1 cells were seeded in 24-well plates and exposed to stressors (i.e. serum deprivation, H_2O_2 exposure, glutamate-induced excitotoxicity) that reduced cell viability *in vitro,* and pertaining to stressors associated with major depression. The MTT assay for cell viability was used for this selection and the 24 hour serum-free incubation was found to be the optimal stressor in both cell lines.

- Thereafter, under serum-free conditions, both cell lines were treated with sildenafil (concentrations ranging from 0 to 1600 nM). The MTT assay for cell viability was used to select the cell line and optimal sildenafil concentration where sildenafil exhibited a statistical significant increase in cell viability. It was found that sildenafil exhibited protective properties in only the neuronal cell line.
- Thereafter, under serum-free conditions, the neuroblastoma (SH-SY5Y) cells were treated for 24 hours with a concentration series of sildenafil, tadalafil, rolipram, ODQ, RP-PET-cGMPS, db-cGMP, fluoxetine, imipramine, lithium or tianeptine. Again the MTT assay for cell viability was used for this selection to obtain the optimal concentration(s) for each drug to modulate cellular plasticity.
- Thereafter, under serum-free conditions and using the optimal drug concentrations, the cells were treated for 24 hours with the indicated drugs and combinations, and the effect on cell viability measured with the MTT, Trypan blue and DNA comet assays.

Literature review

Chapter 2

(All abbreviations are listed in Addendum E)

This chapter, as a literature review, provides a background on the current understanding of neuroplasticity and its role in major depression. In this regard, there will be a specific focus on the role of the glutamate / nitric oxide (NO) / cyclic guanylyl monophosphate (cGMP) signal transduction pathway, as well as on the role of phosphodiesterase type 5 inhibitors that modulate this pathway. As a broader background to these novel developments, the chapter also describes and contextualises major depression as psychiatric disorder, discuss the various anatomical brain areas involved in major depression and neuroplasticity, the underlying neurobiology, how drugs have shown potential to reverse impaired neuroplasticity in depression, and novel approaches in drug therapy of depression.

2.1 Depression

Depression is a disease state that affects more than 121 million people worldwide at any given time. It is also estimated that depression will be the second largest burden to man by 2020 (Mathers *et al.*, 2003). This has a huge impact on global economy and also has a negative impact on overall wellbeing at the level of the individual. Depression presents with symptoms including negative mood, exhaustion, decreased pleasure, lowered libido and changes in daily dietary consumption to name only a few (American Psychiatric Association, 2000). All these symptoms can go unnoticed until normal functioning is severely and noticeably adversely effected or even until suicide seems the only way out. In South Africa it is estimated that one out of every twenty teenagers has thought of (idealised) suicide, while 7.8% of them actually attempt it and succeed, contributing to the worldwide global estimate of 850,000 suicides annually (South African Depression and Anxiety Group, 2009).

There are mainly three challenges regarding depression and its treatment: (1) insufficient attention is given to early signs of depression; (2) antidepressant drugs do not provide immediate effect, thereby hindering compliance to treatment and (3) depression involves a complex underlying neurobiology that is incompletely understood (Fava, 2003; Manji *et al.*, 2003).

From observations in earlier studies it was postulated that a reduction in monoamine neurotransmitters or neurotransmission underlies the neurobiology of depression and that the restoration of impaired monoaminergic neurotransmission underlies antidepressant action (see section 2.1.2.1 below) (Katzung *et al.*, 2002; Schildkraut, 1995). However hypotheses of the neurobiological basis of depression have developed to now also accommodate observations suggesting a more complex and multifactorial underlying neuropathology (Balu *et al.*, 2008; Blendy, 2006). Specifically, data from neuroimaging and biochemical studies suggest structural and functional changes in the brain and therefore impaired neuroplasticity associated with depression (Fuchs *et al.*, 2004; Kronenberg *et al.*, 2009; Lai *et al.*, 2000).

The following section of the literature study discusses the different types of depression, including their symptoms and aetiology, the hypotheses of depression with a reference to the brain areas involved, as well as the underlying biochemical processes where applicable. The role of stressors associated with the aetiology of depression will be mentioned briefly (and will be discussed in more detail in the section on neuroplasticity – see section 2.2.2 below), followed by the current treatment strategies for depression.

2.1.1 Types of depression

The Diagnostic and Statistical Manual of Depressive Disorders (DSM-IV) divides depression into two major classes. The first is bipolar depression, which could be either bipolar I depression (where a patient has history of mania) or bipolar II depression (where a patient has an alternation between hypomanic and brief depressive episodes). The second class of depression is denoted unipolar depression, which is a major depressive episode (without any history of hypomania

or mania) or dysthymia, in which a patient has two years of depressive symptoms without meeting the criteria of a major depressive episode. The DSM-IV has also enlisted cross sectional specifiers (melancholic, atypical and postpartum) and longitudinal specifiers (seasonal or rapidly cycling) of major depressive episode. The degree of severity (mild, moderate, severe or psychotic) has also been graded by the DSM-IV (American Psychiatric Association, 2000). Besides the DSM-IV classification, some subtypes of depression, such as depression with anger or depression with anxiety, have been identified in clinical practice and have also been studied (Swaab *et al.*, 2005). This literature review will define and briefly discuss the following four depressive conditions: (1) major depression, (2) dysthymic disorder, (3) double depression and (4) bipolar disorder.

2.1.1.1 Major depression

Major depression was estimated to be the leading cause of non-fatal burden in the world, accounting for 10.7% of total Years of Life lived with Disability (YLD). Correspondingly major depression was the fourth leading cause of total disease burden accounting for 4.4% of total Disability-adjusted life years (DALYs) for the year 2000 (Ayuso-Mateos *et al.*, 2001). The term major depressive disorder, also known as clinical depression or unipolar depression, was selected by the American Psychiatric Association in the Diagnostic and Statistical Manual of Mental Disorders in the 1980 version to designate the collection of symptoms (severely depressed mood and a loss of interest or pleasure in nearly all activities) as a mood disorder. The collection of symptoms should last for at least two weeks, for a definite diagnosis of this disorder, and include at least five other symptoms such as changes in appetite or weight, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or a loss of energy, feelings of worthlessness and guilt, a diminished ability to think or concentrate and recurrent unfocused thoughts of death or suicide (American Psychiatric Association, 2000; Belmaker & Agam, 2008). Major depressive disorder is such a multifaceted disease, including a range of both emotional (e.g. depressed mood and anxiety) and physical symptoms (e.g. sleep disruption, fatigue, loss of appetite) where physical symptoms are more frequently observed than emotional (American Psychiatric Association, 2000). An untreated occurrence of major

depressive disorder can lead to a single depressive episode lasting up to nine months (Barlow & Durand, 2002).

2.1.1.2 Dysthymic disorder

With the introduction of the third Diagnostic and Statistical Manual of Mental disorders (DSM), in 1980, dysthymic disorder was introduced to replace neurotic depression. Dysthymia is characterized by a persisting low-grade depression. While dysthymia is chronic in nature, the symptomatology often fluctuates, including frequent exacerbations that meet the criteria for a major depressive episode (Rhebergen *et al.*, 2009). Although the phenomenologies of dysthymic disorder are qualitatively similar to major depressive disorder, dysthymia persons experience fewer vegetative symptoms (Leader & Klein, 1996) and the percentage of patients with co-morbid major depressive disorder exceeds 90% (Rhebergen *et al.*, 2009). Patients with dysthymic disorder mostly have symptoms lasting for at least 2 years during which the patient is not symptom free for more than 2 months at a time (American Psychiatric Association, 2000). Symptoms presented in dysthymia are often present for up to 20 years, whereby a patient eventually experiences a major depressive episode (Barlow & Durand, 2002).

2.1.1.3 Double depression

The presence of concurrent dysthymia and major depression present in individuals is referred to as double depression (Rhebergen *et al.*, 2009). In double depression the episodes of major depression are superimposed on a more chronic depressive disorder. Studies have revealed that 25 to 66% of patients with major depression has co-morbid dysthymia. The relapse in patients with double depression is more frequent than those with major depression alone and their recovery were more rapid (Moerk & Klein, 2000), however double depressives presents a more severe symptom profile and lower recovery rates than the major depressives (Lehto *et al.*, 2008).

2.1.1.4 Bipolar disorder

Bipolar disorder is a severe, highly disabling chronic psychiatric disorder with an estimated lifetime prevalence of 1 to 3%. The increased mortality and morbidity associated with bipolar disorder is due to general medical conditions such as cardiovascular disorder, obesity and diabetes mellitus, which are not simply a result of psychiatric symptoms. This mood disorder is characterized by recurrent episodes of mania and depression, severely disturbing the quality of life. The manic episodes are characterized by an elated mood, grandiosity, flight of ideas, hyperactivity and diminished need for sleep, while the depressive episodes are characterized by depressed mood, loss of interest, psychomotor retardation, feelings of worthlessness and suicidal ideation. Bipolar I disorder can be diagnosed after one manic episode whereas bipolar II disorder are diagnosed after one hypomanic (a milder form of manic episode) and one major depressive episode. The prevalence of bipolar disorder types I and II are estimated as high as 3% of the population (Kapczinski *et al.*, 2008; Kato, 2008).

Even though bipolar disorder is associated with depressive episodes, its neurobiology and treatment differs from that of major depression, and it is strictly not included in the study objectives. It is mentioned here, however, to indicate the complex nature of the range of depressive mood disorders.

2.1.2 Hypotheses of depression

The search for cures for depressive mood can be found in the most ancient history of man. Despite the serendipitous discovery of the role of the monoamines in the regulation of mood (and hence the postulation of the classical monoamine hypothesis) and thereafter the development of several newer hypotheses, a clear understanding of the aetiology and neurobiological basis of depression still remains elusive. However, these hypotheses provide some keys to explain observations and to stimulate the development of novel treatment strategies. A few of the most prominent hypotheses of the biological basis of depression will be discussed below.

2.1.2.1 The monoamine hypothesis

The 1950's saw the dawn of a new era for depression. Researchers have found that the drug reserpine, used in the treatment of hypertension, depletes monoamine neurotransmitter stores and induces depressive-like symptoms (Coppen, 1967). This breakthrough gave birth to the classical and best studied hypothesis of depression, namely the monoamine hypothesis (Katzung *et al.*, 2002; Sapolsky, 2000; Schildkraut, 1995), suggesting that a defective monoaminergic activity occurs in the brain, leading to low levels of monoamines. Whereas at first the role of central *l*-norepinephrine (*l*-NE) was recognised, it later became clear that serotonin (5-HT) and dopamine (DA) also play a role. Several classes of antidepressants, namely monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin re-uptake inhibitors (SSRIs) and selective noradrenalin re-uptake inhibitors (NARIs) have been introduced, based on the monoamine hypothesis (Blier, 2003). The monoamine hypothesis of depression does not only propose the crucial involvement of monoamines in the therapeutic effects of these antidepressant drugs, but suggest depression to be directly related to decreased monoaminergic neurotransmission.

New developments in molecular biology enabled further investigation into the validity of the monoamine hypothesis of depression. Many recent studies provided data that fit well with the monoamine hypothesis, some of which originate from positron emission tomography (PET), where the use of selective radioligands provided evidence that pre- and postsynaptic 5-hydroxy-tryptamine type 1A (5-HT_{1A}) receptor binding is reduced in patients with depression (Drevets *et al.*, 1999). A similar reduction in 5-HT_{1A} receptor binding has been found in the pre-frontal cortex of unmedicated depressed patients relative to healthy controls (Sargent *et al.*, 2000). 5-HT_{1A} receptors are involved in the regulation of anxiety in depression (Koller *et al.*, 2006). This is also supported by observations that 5-HT_{1A} receptor-deficient mice display a notable increase in behaviour associated with increased anxiety and stress, whereas this was reversed by the 5-HT_{1A} agonist buspirone (Parks *et al.*, 1998; Ramboz *et al.*, 1998). Therefore this reduction in 5-HT_{1A} receptor binding suggest a more complex underlying mechanism involved in the neurobiology of depression, whereby the classical monoamine hypothesis that imply a mere

reduction in monoamine levels, is too simplistic. In addition, while antidepressants cause a rapid increase in brain monoamine levels, the delayed onset of clinical effect (often ten to fourteen days) also suggest that other factors are involved in therapeutic response. It is therefore believed that the alteration in monoamine neurotransmission, as induced by the antidepressants, sets off a cascade of neurobiological events that eventually culminates in response. Nevertheless, all classic antidepressants are believed to (and accordingly classified) exert their main therapeutic effect via an initial modulation of the metabolism, reuptake, or receptor signalling of serotonin, norepinephrine, or both (Katzung *et al.*, 2002; Schildkraut, 1995; Sapolsky, 2000).

2.1.2.2 The cholinergic super-sensitivity hypothesis

In 1972 Janowsky and co-workers proposed a cholinergic-adrenergic hypothesis of mania and depression (Janowsky *et al.*, 1972). They proposed that depressed individuals exhibit a cholinergic over-activity or super-sensitivity, which could be observed as a greater behavioural or hormonal response to cholinergic agonists (Janowsky *et al.*, 1994). Since then numerous lines of evidence were accumulated in support of a role for the muscarinic cholinergic system in the aetiology of depression. For instance, rats bred selectively for increased sensitivity to muscarinic receptors demonstrated behaviours that are similar to those seen in depressed patients (Daws & Overstreet, 1999). In humans, enhanced cholinergic activity induced an aggravation of symptoms in patients with unipolar depression (Janowsky *et al.*, 1972). There is also evidence to suggest that the muscarinic acetylcholine receptor (mAChR) in the nucleus accumbens may be the mediator that drives behavioural depression (Rubin & Staddon, 1999). Furthermore, neuroendocrine and pupillary responses to cholinergic activity are augmented in depressed subjects (Dilsaver, 1986). It has been found that antagonism of the mAChR is associated with reversal of depressive-like symptoms (Daws & Overstreet, 1999). Until recently, only a handful of uncontrolled studies have suggested that anticholinergic drugs might have antidepressant efficacy. The antidepressant effects of tricyclic antidepressants were believed to be partly due to their anticholinergic properties. Kasper and co-workers described the antidepressant properties of the anticholinergic

drug biperiden in 10 severely depressed inpatients (Kasper *et al.*, 1982). A more recent study found that the antimuscarinic drug scopolamine had antidepressant properties in both subjects with unipolar and bipolar depression and that the responses were rapid, occurring within three to five days (Furey & Drevets, 2006). Yet another study has shown *in vitro* that the experimental antidepressant *myo*-inositol and the therapeutically used antidepressants fluoxetine and imipramine exert a down-regulation of the mAChR (Brink *et al.*, 2004), putatively contributing to their therapeutic effects.

Because anticholinergic side-effects are bothersome and a common cause for discontinuation of tricyclic antidepressants in depressed patients, novel compounds currently in development that target the cholinergic system would need to factor in this potential problem. Further controlled short- and long-term studies are warranted to determine the efficacy, safety, and tolerability of anticholinergic compounds in mood disorders.

2.1.2.3 The hypothalamic-pituitary-adrenal (HPA) - axis hyperactivity hypothesis

The HPA-axis is regulated by corticotrophin releasing hormone (CRH), adrenocorticotrophin hormone (ACTH) and cortisol. In patients with depression there is an over-secretion of these hormones, in the presence of a defective feedback system, and leading to elevated levels of these hormones in the blood. There are studies demonstrating increased levels of CRH and cortisol in the cerebrospinal fluid of depressive patients, thereby suggesting that elevated cortisol and ACTH are involved in the neurobiology of depression and that their elevated blood levels are not merely consequences thereof. The increase in cortisol and ACTH levels is also associated with the glutamate-induced excitotoxicity (see section 2.2.2.1 below), which then results in oxidative stress (see section 2.2.2.2 below). It was observed in patients with major depression that was subjected to the dexamethasone (DEX) suppression test, that an increase in ACTH, mostly due to increased CRH levels, resulted in hypercortisolemia and non-suppression of serum

cortisol levels. The DEX suppression test measures the hyperactivity of the HPA-axis as the ability of the synthetic glucocorticoid DEX to reduce the HPA-activity in normal patients; thereby the DEX suppression test is able to detect impaired negative feedback to the pituitary gland, resulting in an endogenous hyperactivity of the HPA-axis. Increased cortisol levels have also shown to cause damage to hippocampal CA3 pyramidal neurons, as well as suppression of neurogenesis in adults (Fuchs *et al.*, 2004). In a study in rats, treatment with glucocorticoids resulted in decreased performance in object recognition tasks and structural changes to the hippocampus. However, the classical antidepressant fluoxetine has not been demonstrated to significantly control cortisol secretion (Monteleone *et al.*, 1995). Newer antidepressant strategies, such as mifepristone and metyrapone, shows promise in clinical trials in controlling the secretion of cortisol relevant to depression (DeBattista & Belanoff, 2006). Mifepristone lacks antidepressant activity (Carroll & Rubin, 2008), whereas the addition of metyrapone to antidepressant therapy enhances antidepressant effect and may also accelerate their onset of action (Young, 2005).

2.1.2.4 The neuroplasticity hypothesis

Many studies illustrate how stress (associated with depression) affects the functional and structural integrity of neuronal cells, associated with changes in markers of neuroplasticity as measured at cellular and molecular level. Cellular changes include reduced neurogenesis and cell death, while molecular changes include modified gene expression and protein synthesis and phosphorylation. These effects sketch a theoretical mechanism whereby sustained stress (such as associated with depression) may reduce neuronal plasticity and ultimately lead to selective abnormalities of limbic structures such as the hippocampus, prefrontal cortex and amygdala.

Biological stressors associated with major depression eventually induce functional and even structural changes in the brain. These biological stressors include excitotoxicity, induced by glutamate (see section 2.2.2.1 below), oxidative stress (see section 2.2.2.2 below) induced by reactive oxygen species (ROS) and neuronal

damage induced by pro-inflammatory cytokines (Myint *et al.*, 2007) and are depicted in Figure 2-1. The pro-inflammatory cytokines enhance the activity of indolamine, an enzyme leading to the excessive formation of quinolinic acid. Quinolinc acid is a neurotoxic metabolite exerting its action on the NMDA receptors (Myint & Kim, 2003). Another stressor is increased cortisol secretion as a result of a hyperactive HPA-axis. This over secretion of cortisol leads to an increased cellular glucose metabolism subsequently resulting in an increased formation of free radicals. The excess free radicals cause an imbalance in the oxidative status that leads to lipid peroxidation and DNA damage.

The sub-cellular mediators involved in regulating neuronal plasticity include the regulation of the transcription factor cyclic adenosine monophosphate (CREB) (see section 2.2.2.3.1 below) and the neurotrophic factor brain-derived neurotrophic factor (BDNF) (see section 2.2.2.3.2 below). Almost all of the current antidepressants have shown to increase CREB and BDNF. These increases are mediated via the cyclic mononucleotides which have shown to be increased after chronic antidepressant therapy.

Studies show that synaptic plasticity is very important in learning and memory, as it occurs in cerebral structures such as the hippocampus. The most studied form of synaptic plasticity is long-term-potentiation (LTP), occurring at the excitatory synapses of the hippocampus. Many studies support the hypothesis that LTP underlies memory, although the complex mechanism involved is not yet completely clarified (Constantine-Paton & Cline, 1998). Various other studies have shown that LTP and memory depend on a cellular cascade stimulated by an increase of the intracellular concentrations of cyclic adenosine monophosphate (cAMP), with subsequent protein kinase A (PKA) activation and the phosphorylation of CREB (Ahmed & Frey, 2005). Besides cAMP, a fundamental role is played by the cGMP/PKG/CREB pathway, as it seems to act in parallel with the cAMP/PKA/CREB pathway (Nguyen & Woo, 2003).

The neuroplasticity hypothesis of depression is a complex theory of intertwined mechanisms not yet completely understood which is depicted in the figure below.

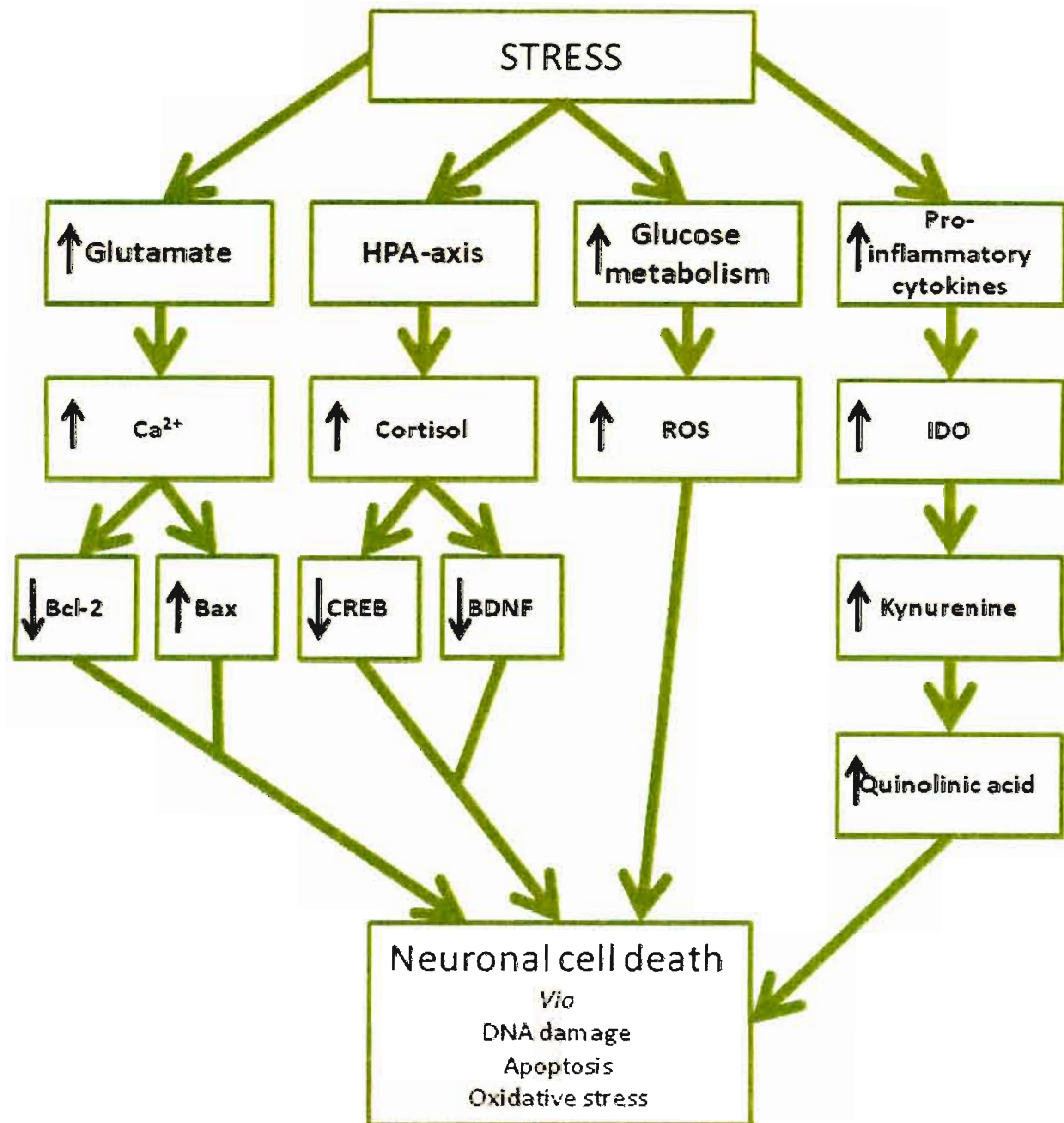


Figure 2-1 The complex intertwinement of the neuroplasticity hypothesis. Abbreviations: bcl-2 = beta cell lymphoma/leukocyte -2 gene family, Bax = bcl-2 associated protein X, BDNF = brain derived neurotrophic factor, CREB = cAMP response element binding, HPA-axis = Hypothalamic pituitary adrenal axis, ROS = reactive oxygen species, IDO = indolamine. Adapted from (Carlson *et al.*, 2006).

2.1.3 Treatment of depression

Currently there are four main drug classes in the treatment of depression which include 1) the monoamine oxidase inhibitors, 2) tricyclic antidepressants, 3) selective

serotonin reuptake inhibitors and 4) atypical antidepressants. The current basis of the treatment of depression is based on the monoamine hypothesis of depression (Katzung *et al.*, 2002; Schildkraut, 1995).

2.1.3.1 The monoamine oxidase inhibitors (MAOIs)

Monoamine oxidase is an enzyme present in the outer mitochondrial membrane of neuronal and non-neuronal cells that degrades the monoamines, including *l*-norepinephrine (*l*-NE), dopamine (DA) and serotonin (5-HT). This degradation of monoamines causes a decrease in their release from the pre-synaptic vesicle, which, according to the monoaminergic hypothesis of depression, is related to the manifestation of depression. However monoamine oxidase (MAO) inhibitors decrease the amount of monoamines degraded in the pre-synaptic neuron, thereby increasing their availability to be released into the synaptic cleft and is therefore associated with a reversal in depressive-like symptoms (Katzung *et al.*, 2002; Schildkraut, 1995). There are two isoforms of MAO, namely MAO-A and MAO-B, each with different substrate preferences, inhibitor specificity and tissue distribution. MAO-A mainly metabolises *l*-NE and 5-HT and MAO-B mainly metabolises DA. The classical and non-selective MAO-inhibitors such as phenylzine and tranylcypromine induce a hypertensive crisis when dietary tyramine (monoamine forerunner) is ingested. The selective and reversible MAO-A inhibitor moclobemide (currently marketed as Aurorix®) and the selective MAO-B inhibitor selegeline (currently marketed as Parkilyne®) are free from this potential interaction (Yamada & Yasuhara, 2004). Studies indicate that selegeline shows promise as an antidepressant drug in patients with co-morbid depression. Selegeline has also shown to be an anti-apoptotic regulator thereby indicating a possible role in neuroplasticity improvement (Rezak, 2007).

2.1.3.2 The tricyclic antidepressants (TCAs)

Tricyclic antidepressants (TCAs) were discovered in the 1950's and subsequently introduced into treatment of depression in the 1960's. All TCAs share a basic pharmacophore consisting of three rings of atoms, hence the name. The majority of

TCAs act by inhibiting the reuptake of *l*-NE and 5-HT, thereby increasing the concentrations of these neurotransmitters and enhancing neurotransmission (Katzung *et al.*, 2002). TCAs may be tertiary or secondary amines differing significantly. Tertiary amines are metabolised in the liver by N-demethylation to the corresponding secondary amines. The secondary amines are pharmacologically active and contribute to both the therapeutic and toxic effects. The secondary amines are metabolised in the liver by aromatic hydroxylation and glucoronidization to compounds that have neither the therapeutic nor toxic effects of TCAs. Imipramine, amitriptyline and doxepin are tertiary amines and are partially metabolised to its secondary amines, desimipramine, nortriptyline and nordoxepin. Secondary amines exhibit more potent effects on *l*-NE inhibition whereas tertiary amines more potently exert their effects on 5-HT reuptake. Because tertiary amines are metabolised to secondary amines they have both actions (Rudorfer & Potter, 1999). The TCAs are structurally related to phenothiazines and therefore share most of their side-effect profile as they exhibit affinity for multiple receptors which include histamine-1, α-adrenergic receptors and muscarinic acetylcholine receptors (Leonard, 1997). Imipramine, the most frequent prescribed TCA has shown to increase the transcription factor CREB and neurotrophic factor BDNF in patients with major depression, thereby suggesting its role in enhancing neuroplasticity (Blendy, 2006).

2.1.3.3 The selective serotonin reuptake inhibitors (SSRIs)

In 1987 Eli Lilly introduced fluoxetine (Prozac®) as the first selective serotonin reuptake inhibitor (SSRI) on the market for the treatment of depression (Stokes & Holtz, 1997). Currently there are many SSRIs on the market and include: paroxetine, citalopram, escitalopram and sertraline (Furgeson, 2001; Kasper *et al.*, 2009). The SSRIs block the reuptake of serotonin into the pre-synaptic nerve terminal, thereby increasing the serotonin concentration in the synaptic cleft. During chronic treatment with fluoxetine in rats, it was found that the cell survival signals, such as CREB and BDNF, were induced, thereby promoting synaptic plasticity (Altar, 1999; Drzyzga *et al.*, 2009; Duman, 2002) and neurogenesis (Lee *et al.*, 2001). In

human subjects the increase in neurotrophic actions (specifically BDNF) of antidepressants were seen to reverse neuronal atrophy and cell loss (Duman & Monteggia, 2006).

2.1.3.4 The atypical antidepressants

The atypical antidepressants have unrelated chemical structures and their actions also vary, from maprotiline that selectively inhibits reuptake of *l*-NE to trazodone that acts as a potent 5-HT_2 receptor antagonist (Harvey, 1997). The atypical antidepressant tianeptine has mechanisms of action that differ from all previous antidepressants. Furthermore tianeptine does not affect *l*-NE or DA re-uptake and lacks affinity for these catecholamine neurotransmitter receptors (Brink *et al.*, 2006). Firstly, tianeptine enhances serotonin reuptake while also facilitating glutamate-receptor-mediated signal transduction at the hippocampal CA3 synapses, by a putative mechanism involving intracellular kinase phosphorylation and activation of transcription factors. A study has shown that long-term treatment with tianeptine reduces stress-induced increase in NMDA-receptor stimulation by glutamate (Kole *et al.*, 2002). These data add physiological support to the hypothesis that kinase phosphorylation and regulation of NMDA-receptor-mediated processes are important targets for the therapeutic treatment of major depression (Manji & Duman, 2001). Tianeptine has also shown to increase BDNF expression in the amygdala (Reagan *et al.*, 2007) (Lucassen *et al.*, 2004)as well as reduce hippocampal apoptosis in an animal model of depression (Lucassen *et al.*, 2004; Reagan *et al.*, 2007)

Agomelatine has very recently been registered in a number of countries as antidepressant and combines zeitgeber (synchronizer of circadian system) activity with neurotransmitter augmentation properties, being a $5HT_{2C}$ receptor antagonist. It has been shown to enhance the levels of dopamine and noradrenaline in the frontal cortex. Agomelatine still needs to undergo further studies to determine the efficacy over longer periods of treatment (San & Arranz, 2008).

In summary, this short overview of current antidepressant drugs available suggest that, while the monoamine hypothesis of depression still forms the basis to explain

the mechanism of action for most of these drugs, newer drugs, such as tianeptine and agomelatine, require novel hypotheses to explain their therapeutic efficacy. The neuroplasticity hypothesis may be a unifying hypothesis in this regard and may provide a platform to investigate novel targets for antidepressants.

2.2 Neuroplasticity in Depression

Neuroplasticity is a term used to describe the essential component of neuronal adaptability to environmental stressors, that primarily involves sub-cellular biochemical, rather than morphological, processes. How the neuron adapts also relates to neurotransmission and the neuron should not be seen as a fixed entity in terms of the quantity of neurotransmitter it releases. Rather, the neurotransmitter may be differentially secreted under different conditions as a result of changes in neuronal cell receptor density and affinity, thereby changing according to functional need (Leonard, 2001). The main regulator of sub-cellular processes that pertain to neuroplasticity in depression is the transcriptional factor CREB and the neurotrophic factor BDNF which will be discussed in more detail in this section and was mentioned to play a role in the hyperactive HPA-axis (see section 2.1.2.3 above) and neuroplasticity (see section 2.1.2.4 above) hypotheses of depression. Neuroplasticity is a process that occur when cells are exposed to stressors, and in this chapter stressors pertaining to neuroplasticity in major depression that will be discussed below include; glutamate excitotoxicity and oxidative stress. The brain regions mainly involved in neuroplasticity and depression, namely the hippocampus, prefrontal cortex and amygdala will also be discussed below.

2.2.1 The brain

Whereas early hypotheses of the pathophysiology of major depression focussed on aberrant intra-synaptic concentrations of neurotransmitters, more recent neuroimaging studies have demonstrated selective structural changes across limbic circuits in the brains of depressed patients. In addition, morphological studies revealed a decrease in neuronal densities in selected brain structures supporting the idea that major depression may be related to impairments of structural plasticity

(Duman *et al.*, 1999; Manji *et al.*, 2003). In a study inducing chronic stress to rats a reduction in hippocampal volume was observed, that was reversed by the atypical antidepressant tianeptine. These findings support the current theories proposing that major depression may be associated with impairment of structural plasticity and neuronal cellular resilience, and that antidepressants may act by reversing this (Fuchs *et al.*, 2004).

The brain regions most notably affected include the hippocampus, pre-frontal cortex and the amygdala, each of which will be discussed with relevance to its involvement in depression and neuroplasticity.

2.2.1.1 The hippocampus

The hippocampus function is associated with the regulation of learning and memory. Major depression is associated with both structural and functional changes in the hippocampus. Animals exposed to severe chronic stress (believed to be associated with the development of mood disorders) present with a decrease in the hippocampal activity and volume (Fuchs *et al.*, 2004). The same has been noted in humans with major depression, where immunological and neuroimaging studies revealed functional and structural damage to the hippocampus (Bremner *et al.*, 2002; Stockmeier *et al.*, 2004). In younger patients, following multiple episodes of depression, a reduction in hippocampal volume and diminished recollection memory was found (Stockmeier *et al.*, 2004), thereby suggesting a compromised primary function of the hippocampus. The cause of this structural or functional loss can be explained by the hyperactivity of HPA-axis during depression, resulting in excessive secretion of cortisol and eventually a defective negative feedback system, culminating in the excessive release of glutamate (see section 2.1.2.3 above). Chronic treatment with most antidepressants has been demonstrated to reverse the loss of hippocampal function, which is also associated with up-regulation of the neuroprotective factors CREB and BDNF (Nair & Vaidya, 2006). Evidence from stress-induced experimental models suggest that tianeptine has constructive effects on several steps of neuronal remodelling, particularly in the hippocampal CA3 region, where tianeptine has been shown to reverse stress-induced dendritic shortening in the pyramidal neurons (McEwen & Olie, 2005; Uzbay, 2008).

2.2.1.2 The prefrontal cortex

The prefrontal cortex regulates complex planning of cognitive behaviour, which has been observed to be diminished in patients with major depression (Miller & Manji, 2006). Magnetic resonance imaging (MRI) has shown a decrease in the volume of the prefrontal cortex in patients with major depression (Bremner *et al.*, 2002). The prefrontal cortex is an important target of the monoamine oxidase inhibitors such as phenelzine which has shown a 10% – 30% increase in the neuroprotective neurotrophic factor BDNF after treatment in rats (Balu *et al.*, 2008). In an animal model of chronic unpredicted stress a reduction in the proliferation rate of glial cells of the prefrontal cortex was the same as in animals exposed to glucocorticoid stress (Pittenger & Duman, 2008). Since glial cells provide neurons with metabolic support, a lower glial proliferation rate (due to stress-induced reductions as observed in MDD) could impact the function and morphology of glial cells in the prefrontal cortex. Glial cells play an important role in the synthesis and inactivation of glutamate, an excitatory amino acid, which plays a central part in many forms of neuroplasticity (Danbolt, 2001).

2.2.1.3 The amygdala

The boundaries of the amygdala are difficult to define, as it has complex interactions with more than twelve modalities (Sheline *et al.*, 2001). The amygdala is associated with fear and emotions and studies have shown an increased activity of the amygdala in major depression, suggesting a heightened sense of fear (Rosenkranz *et al.*, 2003). In human patients with major depression the amygdala showed an increase in volume as a result of emotional response (Bremner *et al.*, 2002; Pittenger & Duman, 2008). In patients with major depression hyper-arousal of the left amygdala occurs when processing a stressful situation, which is then normalized by antidepressant treatments (Blendy, 2006). Bilateral damage to the amygdale, however, results in impaired processing of fearful facial expressions (Sheline *et al.*, 2001).

2.2.2 Sub-cellular mechanisms of neuroplasticity

As alluded to above, major depression is strongly associated with impaired neuroplasticity. While strong evidence for this phenomenon in literature, and particularly its association with specific brain structures, were discussed, this part will focus on sub-cellular mechanisms involved in neuroplasticity. In particular it will discuss the role of stressors such as glutamate (causing excitotoxicity) oxidative stress (as a result of a defective antioxidant system) and regulators of apoptosis.

2.2.2.1 Excitotoxicity

The amino acid glutamate is considered to be the major mediator of excitatory signals in the central nervous system (CNS) and is involved in most aspects of normal brain function, including cognition, learning and memory (du Bois & Huang, 2007). Glutamate also plays a role in the development of the CNS, including synapse induction and elimination, as well as cell differentiation, cell migration and cell death. Glutamate occurs both intracellularly and extracellularly, with the intracellular concentration being the highest in normal brains (Danbolt, 2001). Glutamate exerts its role as signalling molecule by acting on glutamatergic surface (membrane) receptors. Therefore, extracellular glutamate concentration determines the extent of glutamate receptor stimulation, so that it is of critical importance that the extracellular glutamate levels are maintained at relatively low levels for homeostasis. Increased levels of glutamate causes a specific type of toxicity, called excitotoxicity (Muresanu, 2007). While excitotoxicity is associated mostly with excessive stimulation of ionotropic glutamate receptor proteins; the N-methyl-D-aspartate (NMDA) type glutamate receptors, it may also arise from excessive stimulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) or kainate type glutamate receptors (Cull-Candy *et al.*, 2006). In addition to glutamate the NMDA-receptor requires Ca^{2+} to regulate its function on long-term maintenance of genes encoding for the expression of proteins involved in synaptic plasticity (Rao & Finkbeiner, 2007). AMPA-type glutamate receptors mediate fast excitatory synaptic transmission. AMPA receptor-mediated depolarization of the post-synaptic membrane facilitates the activation of NMDA receptors that is triggered by the Ca^{2+} entry. The dynamic regulation of these receptors is important for normal synaptic

function in depressive disease states (Cull-Candy *et al.*, 2006; Rao & Finkbeiner, 2007).

The proposed mechanism whereby glutamate excitotoxicity occurs is briefly described here (for a detailed discussion see section 2.3 below). Glutamate binds to the NMDA receptors causing an influx of Ca^{2+} thereby causing the activation of neuronal nitric oxide synthase (nNOS) which in turn converts L-arginine to NO. The excessive NO produced then binds to free radicals from normal cellular processes to induce lipid peroxidation and cell death (Greene & Greenamyre, 1996).

As mentioned above (see section 2.2.1.1 above), studies also suggest that excessive glutamate release during stress causes structural and functional changes in the hippocampus, and is also involved in the neuropathology of depression (Manji *et al.*, 2003). In addition, it has been reported that increased levels of glutamate measured in the plasma and cerebrospinal fluid of depressed patients was significantly reduced by antidepressant therapy (imipramine) (Mitani *et al.*, 2006).

Further the effects of drugs acting on the glutamatergic neurotransmission system will be discussed in context of their effect on the NMDA receptor.

2.2.2.1.1 N-methyl-D-aspartate (NMDA) receptor

The N-methyl-D-aspartate (NMDA) receptor is an ionotropic glutamate receptor which is highly expressed in the hippocampus (Danbolt, 2001). With excessive activation of this receptor by glutamate, Ca^{2+} influx and therefore intracellular levels of Ca^{2+} will increase, eventually culminating in an increase in oxidative damage and ultimately cell death (see section 2.2.2.1 above). The glutamate / NMDA pathway is involved in the development of major depression (see section 2.3.1 below). There are an increasing number of studies that indicate that an inhibition of the NMDA-receptors has antidepressant-like activity (Sanacora *et al.*, 2008; Skolnick, 1999). In depressed human subjects an improvement in mood was reported after a ketamine (a NMDA-receptor antagonist) infusion (Berman *et al.*, 2000). Another NMDA-receptor antagonist memantine, currently on the market for the treatment of Alzheimer's dementia (a neurodegenerative disease), has also been shown to

display antidepressant-like properties in rats (Moryl *et al.*, 1993). These data confirm the importance of glutamatergic NMDA receptors in neuroplasticity and also suggest a role for receptor antagonists as antidepressant target. Modulation of the NMDA-receptor in the treatment of major depression is also found with the atypical antidepressant tianeptine (see section 2.1.3.4 above).

2.2.2.2 Oxidative stress

Oxidative stress can result from an over-production of reactive oxygen species (ROS) or an impaired antioxidant defence system that fails to repair oxidative damage. ROS are free radicals that contain an oxygen atom such as a hydroxyl radical (•OH), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). The peroxynitrite is formed as a result NO, which binds to superoxide (possibly as a result of a hyperactive HPA-axis and/or excessive glutamate concentration). Excessive ROS causes oxidative stress and cell damage, by inactivating enzymes or by inducing lipid peroxidation (forming malondialdehyde) and DNA modification (Chan, 1996; Halliwell & Gutteridge, 1992). ROS are normally metabolised into less toxic molecules by the action of antioxidant enzymes, namely, superoxide dismutase (SOD), catalase (CAT) or glutathione (GSH) peroxidase. The most important antioxidant SOD catalyses the conversion of superoxide radicals to H_2O_2 (Hermes-Lima *et al.*, 1998). Oxidative stress is well known to contribute to neurodegeneration in the CNS in the process of aging, as well as in neurodegenerative diseases such as amyotrophic lateral sclerosis (Sala *et al.*, 2005), Alzheimer's dementia (Gackowski *et al.*, 2008) and Parkinson's disease (Battisti *et al.*, 2008). Oxidative stress may also be relevant in the brain morphological changes seen in major depression. One study found evidence of oxidative damage in the cell membranes of erythrocytes of patients with major depression (Sarandol *et al.*, 2007) while another study have reported elevated levels of antioxidant enzymes, particularly SOD, in peripheral cells (erythrocytes) and fluids (plasma and saliva) of acutely depressed patients, which reduced following successful antidepressant treatment. (Gilgun-Sherki *et al.*, 2001). Another study demonstrated that plasma malondialdehyde levels and the susceptibility of erythrocytes to oxidation were significantly higher in patients with major depression compared to healthy controls and furthermore showed a significantly positive correlation between the severity of depression and the SOD activity (Michel *et al.*, 2007).

2.2.2.3 Regulators of Apoptosis

In multicellular organisms, the ability of cells to communicate with their environment is critical for ensuring appropriate functioning within the organism, including appropriate response and adaptation. For example, it is known that extracellular cues promote changes and exert long-lasting effects on cells that effect cell growth, proliferation, differentiation and survival or death (via apoptosis). These processes are largely orchestrated by stimulus-induced changes in gene expression. To date various transcription factors have been identified and include, but are not limited to, Jun, nuclear factor kappa beta (NF-κβ) and cyclic adenosine monophosphate response element binding protein (CREB) (Lonze & Ginty, 2002). For this study CREB and NF-κβ will be discussed in more detail.

2.2.2.3.1 Cyclic adenosine monophosphate response element binding protein (CREB)

Amongst the class of gene regulatory factors, cyclic adenosine monophosphate response element binding protein (CREB) is considered a prototype. This is because CREB is one of the first identified and the most widely expressed transcription factor (Lonze & Ginty, 2002). CREB is a nuclear protein that belongs to the family of leucine zipper transcription factors, consisting of activating transcription factor (ATF), cAMP response element modulator (CREM) and inducible cAMP early repressor (ICER). CREB contains a basic leucine zipper motif with which it can homodimerise or heterodimerise to either CREM or ATF, to form the functional dimer and a DNA-binding domain, with which it recognises and binds to promoter cAMP response element (CRE) sequences. The phosphorylation of a serine residue (S133) in its kinase-inducible domain recruits the co-activator proteins and activates transcription; thus CREB activation can be accomplished by a number of upstream signalling cascades (Blendy, 2006).

The canonical pathway that leads to CREB phosphorylation is the cAMP-dependent protein kinase A (PKA) pathway and this cascade of events is known to be perturbed in animal models of depression (Nair & Vaidya, 2006). Animal studies have also shown that an over-expression of CREB in the hippocampus results in an antidepressant-like effect and suggest that CREB may serve as a potential molecular

target for novel therapeutic agents (Chen *et al.*, 2001). However, clinical post-mortem studies provide the strongest support for this hypothesis. Patients diagnosed with major depression that were on antidepressant treatment at the time of death showed increased levels of CREB, whereas those without antidepressant treatment showed decreased levels of CREB in the temporal cortex (Dowlatshahi *et al.*, 1998). Reductions of CREB were also found in the orbitofrontal cortex of antidepressant-free patients with major depression (Yamada *et al.*, 2003). This suggests that CREB is an important marker of major depression.

2.2.2.3.2 Brain derived neurotrophic factor (BDNF)

Neurotrophic factors were first characterized for regulating neuronal growth and differentiation during development (Altar, 1999; Duman *et al.*, 1999) and are now known also to be potent regulators of plasticity and survival of adult neurons (Shirayama *et al.*, 2002). The best known neurotrophic factor is the brain-derived neurotrophic factor (BDNF), which forms part of the neurotrophin family, also including nerve growth factor, neurotrophin-3 and neurotrophin-6. These neurotrophic proteins are closely related in terms of sequence homology and receptor specificity and bind specifically to the tyrosine kinase receptors belonging to the Trk family of receptors, including TrkA, TrkB, TrkC and pan-neurotrophin receptor P75. There are two natural forms of TrkB: a full length TrkB and a truncated form of TrkB that does not contain the intracellular tyrosine kinase domain. The truncated TrkB functions as a dominant negative inhibitor for TrkB receptor tyrosine kinases providing another mechanism to regulate BDNF signalling in the CNS (Gonzalez *et al.*, 1999; Nair & Vaidya, 2006).

The pathological effects of stress on the hippocampus (see sections 2.1.2.3 and 2.2.1.1) proposes a role for neurotrophic factors in the aetiology and treatment of depression. Acute and chronic stress decreases the levels of BDNF in the dentate gyrus and the pyramidal cell layer of the hippocampus. Conversely, the administration of virtually all classes of antidepressant treatments, increased BDNF expression in these regions (Nibuya *et al.*, 1996; Shirayama *et al.*, 2002). There is also evidence that antidepressant treatment in humans increase BDNF expression and can prevent the stress-induced decreases in BDNF expression (Chen *et al.*,

2001). BDNF is partly regulated by CREB, so that the correlation between CREB and BDNF down-regulation in depression, and reversal thereof by antidepressants, can be expected. Taken together, these findings provide strong evidence in support of the concept that antidepressant-induced up-regulation of CREB and BDNF will repair stress-induced damage to hippocampal neurons and protect from further damage (Nestler *et al.*, 2002).

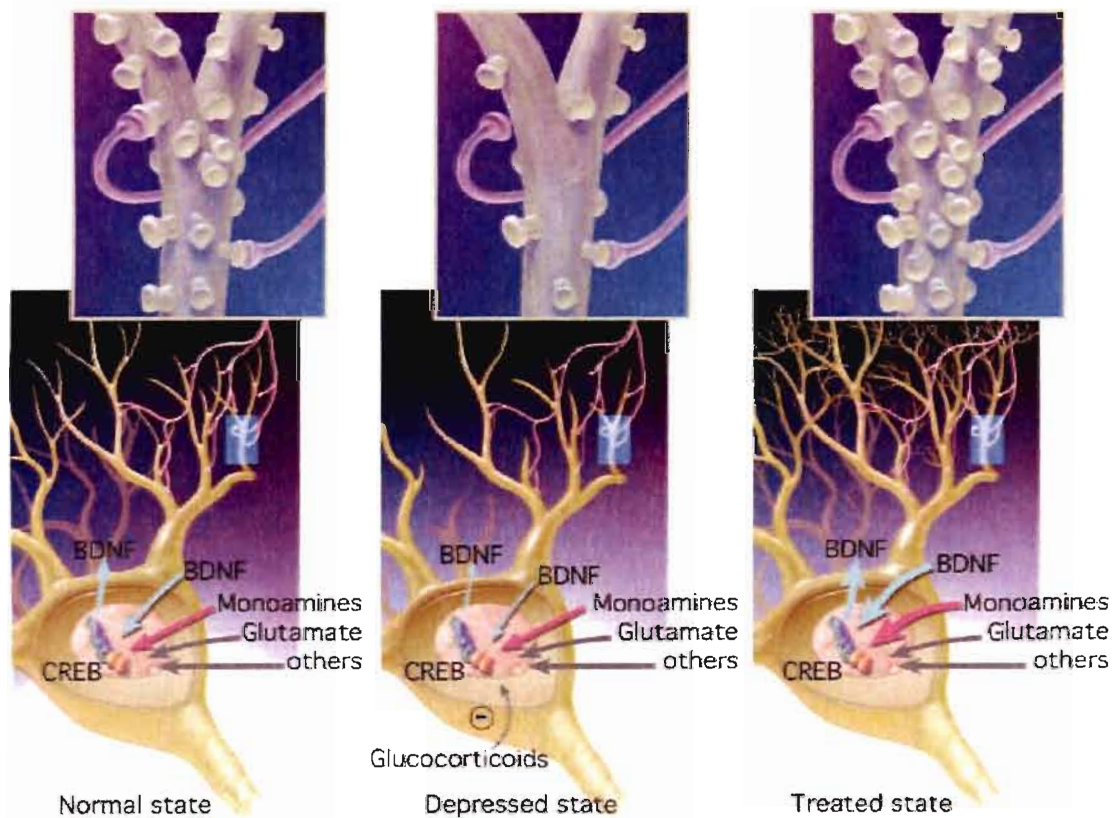


Figure 2-2 The involvement of neurotrophins in depression. On the left a normal hippocampal neuron and its innervation by glutamatergic, monoaminergic and other neurons is depicted. Its regulation by BDNF is also shown. In the middle a depressed state hippocampal neuron is depicted indicating the glucocorticoids stressor leading to decreased synapse density and reduced BDNF. On the right a treated state hippocampal neuron is shown with increased expression of BDNF and increased synapse density (Nestler *et al.*, 2002).

2.3 The glutamate / NO / cGMP pathway

As alluded to above and without dismissing the important role of monoamines in the aetiology of depression, there is now significant evidence implicating the important role of the glutamate / NO / cyclic guanosine monophosphate (cGMP) pathway in depression (Sanacora *et al.*, 2008). It was described in section 2.2.2.1 above how neuronal glutamate activates the NMDA receptor, mediating the release of NO via the activation of the Ca^{2+}-dependent neuronal NO synthase (nNOS). NO then activates soluble guanylyl cyclase (sGC) which leads to an increase in the second messenger cyclic mononucleotides namely cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Figure 2.3) (Prast & Philippu, 2001). Phosphodiesterases (PDEs) facilitate the breakdown of these cyclic mononucleotides which is important for neuronal activation of protein kinase G (PKG) (Harvey, 2006). All of these actions exert an effect on neuronal function, which will be discussed in this section below.

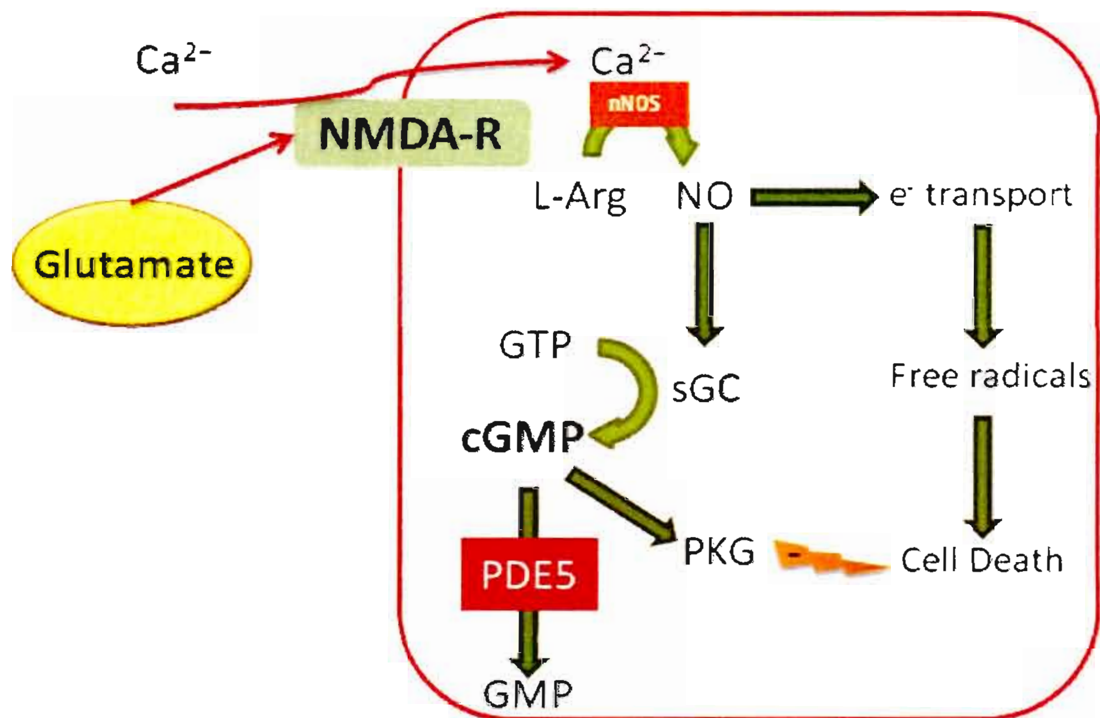


Figure 2-3: The mechanism whereby PDE5 exerts is effect on the NMDA/NO/cGMP pathway after excitotoxic glutamate exposure. Abbreviations: NMDA-R = N-methyl-D-aspartate, nNOS = neuronal nitric oxide synthetase, L-Arg = l-arginine, NO = nitric oxide, sGC = soluble guanylyl cyclase, GTP = guanosine triphosphate, GMP = guanosine monophosphate, cGMP= cyclic GMP, PKG = protein kinase G

2.3.1 Modulators of the glutamate / NO / cGMP pathway

2.3.1.1 Nitric oxide (NO)

It was found that in the peripheral nervous system endothelium-derived relaxing factor (EDRF) was responsible for blood vessel relaxation. In 1988 Furchgott was the first to deduce that EDRF was nitric oxide (NO). The drug nitro-glycerine is used to treat angina pectoris by the mechanism of NO release. NO was first recognised as a messenger molecule in the CNS in 1988, when it was identified as an unstable intracellular factor. Today NO has been recognised to have a key role in cell-to-cell communication which includes endothelium and neuronal signalling (Furchgott, 1996; Garthwaite & Boulton, 1995).

NO acts as a neurotransmitter in the peripheral nervous system, where it regulates vascular tone as well as the control of cardiac contractility, and also plays an important role in the CNS. Upon activation of glutamatergic NMDA receptors, NO increases the cyclic guanosine monophosphate (cGMP) levels via activation of soluble guanylate cyclase (Garthwaite *et al.*, 1988).

As soon as the concentration of NO exceeds what is necessary for normal physiological function, it becomes detrimental to cellular homeostasis and its role in the various pathophysiological conditions becomes evident. In the CNS NO has been implicated in neurodegenerative disorders such as Alzheimer dementia, Huntington's disease as well as psychiatric disorders that includes major depression (Harvey, 2006; Szabó, 1996).

2.3.1.2 Nitric oxide synthase (NOS)

In the human body NO is synthesized in several types of cells, such as neurons, endothelial cells and macrophages by a family of three iso-enzymes termed nitric oxide synthase (NOS). Neuronal NOS (nNOS) is present in neurons and its activity is regulated by Ca^{2+}. Endothelial NOS (eNOS), also Ca^{2+}-dependent, is present in vascular endothelial cells. The macrophages contain inducible NOS (iNOS), which is Ca^{2+} independent and is expressed upon induction by cytokines (Esplugues, 2002). All three iso-enzymes produce NO from L-arginine, following oxidation of the guanidino nitrogen of L-arginine and utilizing molecular oxygen and NADPH as co-substrates (Prast & Philippu, 2001).

2.3.1.3 Soluble guanylate cyclase (sGC)

Soluble guanylate cyclase (sGC) is the only conclusively demonstrated receptor for NO. As shown in Figure 2.3, sGC catalyses the conversion of GTP to cGMP and is one of two enzymes (the other is the membrane-bound peptide-receptor guanylate cyclase (pGC)). sGC has been shown to be intimately involved in many signal transduction pathways (Denninger & Marletta, 1999) of which the most notable are in the cardiovascular system (e.g. regulation of vascular tone) and in the nervous

system (e.g. neurotransmission, long term potentiation and depression) (Andreopoulos & Papapetropoulos, 2000).

2.3.1.4 Cyclic mononucleotides

The activation of sGC promotes the conversion of GTP to the second messenger cGMP, as a downstream signal of the NO pathway. cGMP can then activate several effectors, including cGMP-dependent protein kinase G, cGMP-gated cation channels and GMP-regulated phosphodiesterase (Denninger & Marletta, 1999).

Growing evidence suggests that cGMP signalling cascades play a critical role in major depressions pathophysiology and antidepressant mediated improvement thereof (Blendy, 2006). A real-time polymerase chain reaction (rt-PCR) in rat hippocampal slices revealed that antidepressant treatment with fluoxetine and amitriptyline increased the levels of cGMP (and not cAMP) suggesting a role for cGMP in major depression (Reierson *et al.*, 2009). Increased levels of cGMP, for example due to PDE5 inhibition by sildenafil, attenuates damage caused by glutamate excitotoxicity, as well as oxidative stress in *in vitro* cell cultures (Nakamizo *et al.*, 2003). This will be discussed in more detail in section 2.3.1.5 below.

The cyclic mononucleotides, including both cAMP and cGMP, act as second messengers that regulate signal transduction in various biological systems by responding to extracellular signals such as neurotransmitters and hormones subsequently activating intracellular targets such as ion channels, protein kinase A (PKA), protein kinase G (PKG) and transcription factors. These culminate in various cellular responses (Pilz & Casteel, 2003; Puzzo *et al.*, 2008). The signal strength and specificity of these mononucleotides are dependent on change of localization, duration of action and concentration and may contribute to alterations in neuronal function (Hebb & Robertson, 2007). There is evidence for "cross-talk" between cAMP and cGMP, thereby regulating the production of each other (see section 2.3.1.5 below). The balance between cAMP and cGMP levels are also maintained due to a balance in production carried out by adenyl cyclase (AC) and guanylyl cyclase (GC) and degradation carried out by PDEs (Corbin *et al.*, 2000; Puzzo *et al.*, 2008).

2.3.1.5 Phosphodiesterase (PDE)

PDEs are ubiquitously distributed and most of them have been found in the CNS (Bender & Beavo, 2006). The cAMP and cGMP hydrolysing phosphodiesterases (PDEs) are located in the hippocampus, where they are likely to be involved in learning and memory (Boess *et al.*, 2004). The activation of cyclic mononucleotides via PDEs indicates the importance of PDEs in the cellular process, such as cell differentiation, proliferation and apoptosis (Boswell-Smith *et al.*, 2006).

The super-family of PDEs is divided into three principle classes, each having different sequences. Mammals are included in the class I family of PDEs and these PDEs show high affinity for cAMP and cGMP molecules (Bender & Beavo, 2006). The class I PDEs is diverse and to date twenty-one (21) transcriptional variants have been identified in humans, rats and mice. They have been classified into 11 families based on their sub-cellular distribution, structural similarities, mechanisms of regulation, amino acid sequence, enzymatic properties, kinetic properties and sensitivity to endogenous regulators and inhibitors. Some PDEs are highly specific for cAMP (PDE4, PDE7 and PDE8), others are highly specific for cGMP (PDE5, PDE6 and PDE9) while others have mixed specificity (PDE1, PDE2, PDE3 and PDE10) (Boswell-Smith *et al.*, 2006; van Staveren *et al.*, 2005).

PDEs hydrolyse the phosphodiesteric bond of cyclic mononucleotides between the phosphorous and oxygen atoms at the 3' terminal, with the inversion of the phosphorous atom configuration. Consequently the formation of inactive adenosine monophosphate (AMP) and guanosine monophosphate (GMP) takes place, which are recycled as substrates for the formation of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Bender & Beavo, 2006; Corbin & Francis, 1999).

PDEs regulate the cyclic mononucleotide signal in both amplitude and duration by modulating the "cross-talk" between cAMP and cGMP. For example many effects of Ca^{2+} on cAMP and cGMP are mediated by the activation of the Ca^{2+}- Calmodulin dependent phosphorylation of PDEs (Sonnenburg *et al.*, 1995).

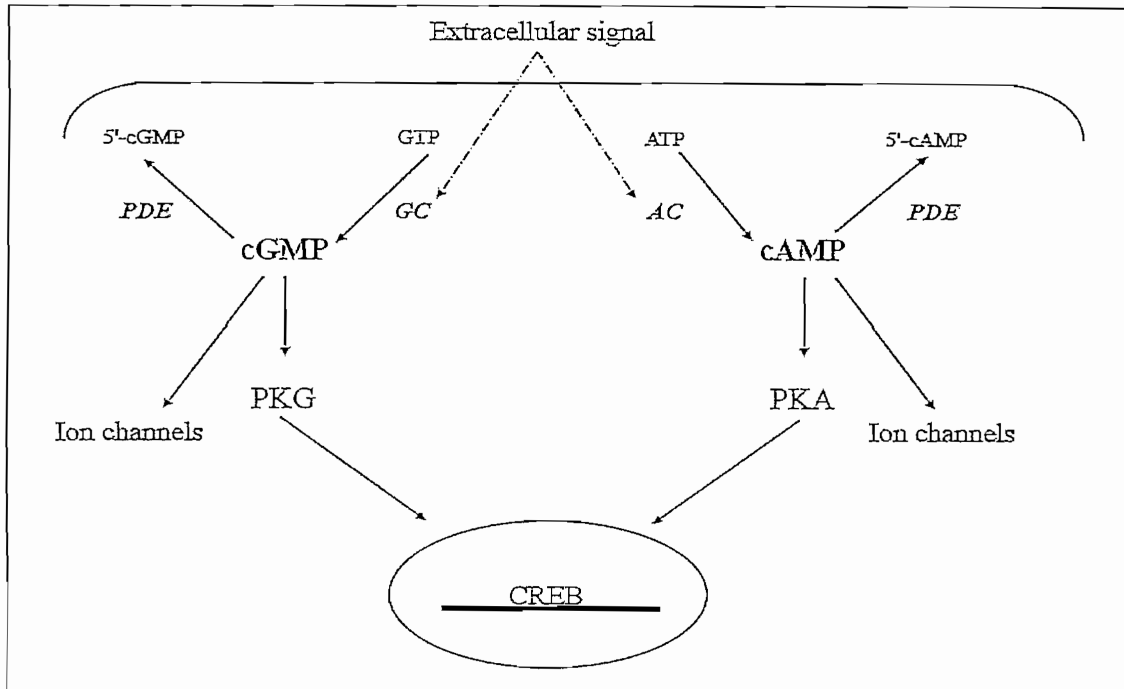


Figure 2-4 The cyclic mononucleotide signal pathway. Extracellular signals via neurotransmitters and hormones is transferred by cAMP and cGMP to an effector protein of which protein kinases (PKA and PKG) and ion channels are the most important. The protein kinases in turn phosphorylate other enzymes or transcription factors such as CREB in the nucleus. Cyclic mononucleotide levels are maintained through a balance in adenyl cyclase (AC) and guanylate cyclase (GC). Phosphodiesterases (PDEs) deactivate cAMP and cGMP to inactive 5'AMP and 5'GMP respectively. Adapted from (Puzzo *et al.*, 2008).

2.3.1.5.1 Sildenafil

The selective PDE5 inhibitor sildenafil was originally manufactured to treat angina pectoris; however in clinical trials a pronounced side-effect of sildenafil was noted, improved erectile function. This lead to the marketing of sildenafil as Viagra®, which was approved for the treatment of erectile dysfunction (ED) in 1998. The mechanism whereby sildenafil exerts its action is by increasing intracellular cGMP, which causes relaxation of the trabucular smooth muscle, dilating the arteries that result in venous construction and marked rigidity of penile erection (Corbin & Francis, 1999; Uthayathas *et al.*, 2007). In 2005 sildenafil was marketed as Revatio® to treat pulmonary arterial hypertension by causing relaxation of the arterial dilatation, also resulting from increased endothelial cGMP.

Role of sildenafil on synaptic plasticity and memory

Synaptic plasticity and its role in depression was discussed in section 2.1.2.4 above. In a study on rats, an increase in object recognition was observed after PDE5 inhibition (by sildenafil) whereafter immuno-cytochemistry studies indicated that this was associated with increased levels of cGMP in the hippocampus (Prickaerts *et al.*, 2002).

Role of sildenafil on neuroplasticity and depression

Several intracellular signalling pathways have been implicated in major depression, including the two intracellular messaging systems related to the cyclic mononucleotides. These signalling pathways represent potential targets for antidepressant treatment. By degrading cyclic mononucleotides, the PDE enzymes play an essential regulating role of this complex intracellular messaging system. The role of PDEs in psychiatric disorders, including major depression, has attracted more attention, especially because PDEs are expressed throughout the brain. Some commercially available PDE inhibitors have been demonstrated to enhance cognitive function (Blokland *et al.*, 2006).

Another study using quantitative rt-PCR and immuno-assays on rat hippocampal slices, indicated that chronic treatment fluoxetine and imipramine increased cGMP levels (Reierson *et al.*, 2009). This provides strong evidence to suggest that an underlying pathway in the treatment of depression involves the NO/cGMP pathway.

In animal studies a role for cGMP has been suggested in mood stabilization after lithium treatment (Harvey *et al.*, 1994). The effect of lithium on cGMP appears to involve enhanced cholinergic drive (Harvey *et al.*, 1990). cGMP is augmented by PDE5 inhibition by sildenafil. Pharmacogenetic studies have suggested that cGMP related PDEs may present a novel strategy in depression (Wong *et al.*, 2006) and sildenafil has seen to augment cholinergic drive in the CNS (Devan *et al.*, 2004). Janowsky suggested the role of muscarinic super-sensitivity in depression and according to this hypothesis increased cholinergic activity will be depressogenic (Janowsky *et al.*, 1972) (see section 2.1.2.2 above).

In vitro experiments in cultured human neuroblastoma cells indicated that antidepressants imipramine and fluoxetine do not increase mAChR signalling (Brink *et al.*, 2004), whereas sildenafil does (Brink *et al.*, 2008). In context of the cholinergic hypothesis of depression, the latter observation implies that sildenafil may be depressogenic. However, since some clinical studies rather suggest mild antidepressant activity in depressed patients treated for erectile dysfunction (Uthayathas *et al.*, 2007), it was postulated that sildenafil may rather possess antidepressant activity that is masked by simultaneous cholinergic activity. In support of this hypothesis, the combination of sildenafil and the antimuscarinic drug atropine presented with antidepressant-like activity in rats, comparable to that of the antidepressant fluoxetine (Brink *et al.*, 2008).

2.4 Synopsis

The current drug treatment options for depression are still based on the monoamine hypothesis. However, there is significant evidence that impaired neuroplasticity plays an important role in depression. The neuroplasticity hypothesis involves a dysfunction in transcription factors such as cyclic adenosine monophosphate response element binding protein (CREB) and neurotrophic factors such as brain derived neurotrophic factor (BDNF). It has been indicated that decreased levels of CREB and BDNF are present in the pathology of depression (Chen *et al.*, 2001).

The NO/cGMP pathway has also been associated with depression. NO exerts its effect on higher functions, including learning and memory (processed by the hippocampus) (Harvey, 2006). Intracellularly NO stimulates soluble guanylyl cyclase (sGC), that in turn catalyzes the process of guanosine triphosphate (GTP) to cyclic guanylate monophosphate (cGMP). cGMP is involved in the activation of protein kinase G (PKG), activation or inhibition of phosphodiesterases (PDEs), subsequent effects on cyclic adenosine monophosphate (cAMP) and neurotransmitter release. All of these effects significantly affect neuronal function. The effect of cGMP is terminated by PDEs, specifically phosphodiesterase 5 (PDE5) (Schwede *et al.*, 2000; van Staveren *et al.*, 2005). In the amygdala and hippocampus NO acts to conserve neural function, however during increased exposure to stressful stimuli (as

mentioned earlier), increases in the NO-producing neurons in the amygdala and hippocampus to release an excess of NO that results in cell death (Prast & Philippu, 2001). Therefore targeting glutamate and NO may play a role in neuroprotection. There are also studies indicating that an increase in cGMP levels as a result of PDE5 inhibition, increases the neurogenesis (Zhang *et al.*, 2002) and that cGMP may be involved in the treatment of depression. In our laboratory studies involving the PDE5 inhibitor sildenafil has shown sildenafil to exert antidepressant-like activity in rats when co-administered with atropine, comparable to fluoxetine (Brink *et al.*, 2008), thereby indicating the role of PDE5 inhibition in the treatment of depression. The effect of sildenafil on neuroplasticity, however, has not been thoroughly studied and needs to be elucidated.

Article: Metabolic Brain Disease

Chapter 3

This chapter presents the main data of the study as an article and is written according to the "Instructions for Authors" for "Metabolic Brain Disease" (see Addendum C). The bibliographic references pertaining to this chapter have been formatted in accordance with the "Instructions for Authors", different from that of the other chapters that is presented at the end of the dissertation. This article will discuss the modulatory effects of sildenafil on neuroplasticity in human neuroblastoma (SH-SY5Y) cells and the mechanism whereby it exerts its effects. All additional data and discussion is presented in Addendum B. A summary and conclusions of all the study results are presented in Chapter 4.

1 TITLE PAGE

Sildenafil displays protective effects against oxidative stress in human neuroblastoma (SH-SY5Y) cells

2 Running title

The neuromodulatory role of sildenafil in neuroblastoma (SH-SY5Y) cells

3 Authors

Riaan Reay [1], Christiaan B. Brink[1] and Brian H. Harvey[1]

[1]Division of Pharmacology, North-West University (PUK), Potchefstroom, South Africa

Correspondence: Christiaan B Brink, Division of Pharmacology, Box 16, North-West University (PUK), Potchefstroom, 2520, South Africa

E-mail: tiaan.brink@nwu.ac.za

Abstract

Impaired neuroplasticity plays a key role in the neuropathology of depression and is restored by antidepressants. A growing line of evidence link the NO/cGMP pathway to the regulation of neuroplasticity, while unpublished data suggest that the PDE5 inhibitor sildenafil may up-regulate genes encoding for the expression of anti-apoptotic proteins *in vitro*. Sildenafil, when co-administered with atropine, has also been demonstrated to exert antidepressant-like activity in rats. These data warrant further investigation into potential effects of PDE5 inhibitors on neuroplasticity. The human neuroblastoma cell line (SH-SY5Y) was incubated for 24 hours to drug-free control, 92 nM fluoxetine, 105 nM imipramine, 1 mM lithium, 1.6 nM sildenafil, 97 nM tadalafil, 16 nM rolipram or 200 μM db-cGMP under conditions of oxidative stress (serum deprivation), with or without the guanylate cyclase inhibitor ODQ (2 μM) or the protein kinase G (PKG) inhibitor RP-PET-cGMPS (500 nM). Results indicated that sildenafil and lithium protected SH-SY5Y cells against 24 hour serum deprivation-induced oxidative stress in the MTT assay, while all drugs improved DNA repair capacity, with the effects of sildenafil and db-cGMP being reversed by ODQ and RP-PET-cGMPS. In addition, sildenafils protective effect is related to cGMP formation and PKG mediated.

Keywords

Neuroplasticity, sildenafil, PDE5, cGMP, DNA damage, oxidative stress

Introduction

Depression is projected to be the second largest burden of disease across all age groups by 2020, and currently affects 121 million people worldwide, with more than 12% of the people in industrialized countries affected (Mathers *et al.*, 2003). Current antidepressant treatments are plagued with delayed onset of action, troublesome side-effect profiles and treatment resistance (Fava, 2003; Katzung *et al.*, 2002; Manji *et al.*, 2003). In particular, statistics showing that almost one third of patients present with treatment resistance, suggest that current antidepressants do not address the core neuropathology in these patients. All classes of clinically used antidepressants modulate central monoaminergic neurotransmission, and only for tianeptine and agomelatine is the claim for their primary mechanism of antidepressant action not related this action.

However, evidence is compounding that depression is a multifactorial disorder, with evidence strongly suggesting that restoration of neuroplasticity plays an important role in antidepressant action (Pittenger & Duman, 2008). In particular, dysfunction of the hypothalamic-pituitary-adrenal-axis (Ströhle & Holsboer, 2003) and a decreased volume of the left hippocampus (Bremner *et al.*, 2002) are typically associated with major depression. In addition it has been demonstrated that intraventricular administration of brain-derived neurotrophic factor (BDNF) exerts anti-depressant-like effects in rodents, while chronic administration of most antidepressants are associated with elevated level of BDNF (Balu *et al.*, 2008; Blendy, 2006; Shirayama *et al.*, 2002). It is believed that a dysfunction in the glutamate/nitric oxide (NO)/cyclic guanylyl monophosphate (cGMP) signal transduction pathway is strongly associated with the impaired neuroplasticity observed in depression (Almeida *et al.*, 2006).

We recently demonstrated antidepressant-like activity of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil in rats, when co-administered with the antimuscarinic drug

atropine, but not with either drug alone (Brink *et al.*, 2008a). In addition, others reported that sildenafil enhances memory in rats (Devan *et al.*, 2004; Devan *et al.*, 2007). However, it has not been demonstrated whether sildenafil display neuroprotective properties.

In the current study we investigated the effects of sildenafil, drugs acting on the NO/cGMP pathway and known antidepressant treatment on different markers of neuronal plasticity in *in vitro* human neuroblastoma (SH-SY5Y) cells that were exposed to glutamate simulating excitotoxicity, 15 min H_2O_2 incubation or serum deprivation simulating oxidative stress which are indicators of major depression.

Materials and methods

Cell culture medium

Ham's Nutrient Mixture F12, penicillin-streptomycin mixture, fungizone, foetal bovine solution (FBS) and trypsin were obtained from Celtic Molecular Diagnostics (Cape Town, South Africa).

Other chemicals

NaCl, KCl, Na_2HCO_3, formic acid and absolute alcohol, ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), NaOH, H_2O_2, low melting point agarose, high melting point agarose, NaH_2PO_4 and $NaHCO_3$ were obtained from Merck (Johannesburg, South Africa). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reagent, isopropanol (99%), Triton X100, Tris-buffer, Tris-HCl, imipramine, lithium chloride, rolipram, Rp-8-bromob-phenyl-1, N^2-etheno-guano-sine 3'5' cyclic monophosphorothioate (RP-PET-cGMPS), N^2,2′-O-dibutyrylguanosine 3′,5′-cyclic monophosphate sodium salt hydrate (db-cGMP), 1H-[1,2,4] oxadiazolol [4,3-a] quinoxallin-1-one (ODQ) and ethidium bromide were obtained from Sigma Aldrich (Johannesburg, South Africa). Trypan blue obtained from Scientific Group (Johannesburg, South Africa). Sildenafil was kindly provided by Pfizer. Tadalafil and fluoxetine was kindly provided by Eli Lilly.

Cell line

In this study we used the human neuroblastoma (SH-SY5Y) cell line, obtained from Sigma Aldrich (Johannesburg, South Africa) originally from the American Type Culture Collection (USA). SH-SY5Y cells were maintained and grown to 80% confluency in a humidified environment at 37°C with 10% CO_2 in 150cm^2 cell culture flasks with Ham's F12 medium containing 10% v/v foetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone.

Pre-treatments

The SH-SY5Y cells were seeded at a density of $5x10^6$ cells/well into 24-well plates in preparation for treatments and assays. Cells were allowed to attach to the bottom of the wells for 24 h where after the indicated 24 h drug treatments at the indicated concentrations were initiated in serum-free medium (oxidative stressor). The following drugs were used: Sildenafil was used at a concentration of 1.6 nM in the current study, a concentration 10 times lower than the peak free (unbound) serum concentration of 16 nM (as calculated from the peak therapeutic plasma concentration = 212 ng/ml; MM = 666.7; unbound in serum = 4%) (Hardman *et al.*, 2001). This concentration of 1.6 nM was selected from a trial dose-response series measuring resulting cell viability in the standard MTT assay (see below), indicating that sildenafil increases cell viability optimally at this concentration, with no effect at 16 nM or higher (data not shown). The K_i value of tadalafil for PDE5 has been reported as 9.7 nM (Teixeira *et al.*, 2006) and we therefore used 97 nM tadalafil in the study, which is a concentration 10 times its K_i value and sufficient to yield significant inhibition of PDE5. The K_i value of rolipram for PDE4 has been reported as 16 nM (Teixeira *et al.*, 2006), while we also indicated from a trial dose-response series measuring resulting cell viability in the standard MTT assay (see below), that rolipram displays optimal effect on cell viability at this concentration. We therefore used 16 nM rolipram in the current study. As concentration of 1 mM lithium is within therapeutic range and has been shown in previous studies not to provoke toxicity in SH-SY5Y cells (Potgieter *et al.*, 2008). We therefore used 1 mM lithium in the current study. Fluoxetine was used at a concentration of 92 nM in the current study, a concentration that represents its peak free (unbound) serum concentration (as calculated from the peak therapeutic plasma concentration = 531 ng/ml; MM = 345.79; unbound in serum = 6%) (Hardman *et al.*, 2001). Imipramine was used at a concentration of 105 nM in the current study, a concentration that represents its peak free (unbound) serum concentration (as calculated from the peak therapeutic plasma concentration = 337 ng/ml; MM = 316.87;

unbound in serum = 9.9%) (Hardman *et al.*, 2001). The cGMP analogue, db-cGMP, was used at a concentration of 200 μM, as used before to stimulate PKG which has shown to be involved in cytoprotection (Rooney *et al.*, 1996). The protein kinase-G inhibitor RP-PET-cGMPS was used at a concentration of 500 nM that was seen to inhibit pre-synaptic Ca^{2+} that causes cell damage (Schwede *et al.*, 2000).

Measurement of mitochondrial activity

The SH-SY5Y cells were seeded and treated as described above, whereafter the standard MTT assay was performed as described before (Brink *et al.*, 2008a). Briefly, additional wells were seeded to allow for negative controls (100% cell viability) and positive controls (0% cell viability). Cells designated as negative controls were incubated for 24 hours in normal serum-free growth medium, without stressor or drug, while positive controls were incubated for 24 hours as negative controls, the medium aspirated and then incubated with 0.33% formic acid in $_{dd}H_2O$ for 30 minutes to induce 100% cell death. After the 24 hour drug treatments, and following the 30 minute incubation with formic acid for positive controls, the medium was aspirated and the cells rinsed twice with 1 ml phosphate buffer solution (PBS). Thereafter 200 μl of a 0.5% MTT solution in PBS was added to each well and incubated at 37°C in 10% CO_2 for 2 hours. Due to the light sensitivity of the MTT-reagent all steps with MTT were executed in the dark with only a limited exposure to light for visibility when necessary. After the 2 hour incubation period the MTT-reagent was aspirated from the wells and 250 μl isopropanol added to each well. The 24-well plates were then incubated at room temperature for 5 minutes to dissolve the purple formazan crystals, whereafter 100 μl of the formazan-containing isopropanol from each well was transferred to a 96-well plate and the absorbance (Abs) read spectrophotometrically at 560 nm. The mitochondrial activity was calculated as a percentage of the negative control by means of the following equation:

$$\text{Cell Viability (\%)} = \frac{\text{Abs}_{(\text{sample})} - \text{Abs}_{(+\text{control})}}{\text{Abs}_{(-\text{control})} - \text{Abs}_{(+\text{control})}} \times 100$$

where $\text{Abs}_{(\text{sample})}$ is the absorbance of the study sample, $\text{Abs}_{(\text{positive control})}$ is the absorbance of the formic acid (representing 0% cell viability) and $\text{Abs}_{(\text{negative control})}$ is the absorbance of the untreated cells (representing 100% cell viability).

Measurement of membrane integrity

The SH-SY5Y cells were seeded and pre-treated as described above and the standard Trypan blue assay was performed as described before (Brink *et al.*, 2008a). Briefly, the medium from each well (from the 24-well plates) was aspirated and 200 µl of trypsin was added to each well and incubated for a further 5 minutes at 37°C in 10% CO_2 to loosen the cells. Thereafter 800 µl of serum-free medium was added into each well and pipetted up and down to ensure that cell clusters are broken. The contents of each well was transferred to an Eppendorf tube and centrifuged for 10 min at 5000 rpm at 4°C. The supernatant was aspirated and the pellet re-suspended in 1 ml of serum-free medium. The Eppendorf tubes with cells were kept at 4°C on ice. Thereafter 50 µl of 0.4% Trypan blue was added to the cells and 20 µl of the mixture containing the cells were added to an eppendorf tube containing 180 µl of 0.4% Trypan blue (for dilution). The cells were re-suspend by vortexing the Eppendorf tubes. The viable cells were counted using a 0.025 cm^2 haemocytometer with a depth of 0.1 mm and an inverted Nikon TMS microscope. The percentage viable cells were calculated as the number of white cells after drug treatment divided by the number of cells in the serum-free control and is presented in the equation below:

$$\text{Viable cells (\%)} = 100 \times \frac{N_{(\text{white cells after drug treatment})}}{N_{(\text{white cells of serum free control})}}$$

where $N_{(\text{white cells of serum free control})}$ is the number of white (unstained, intact) cells of the serum-free control and $N_{(\text{white cells after drug treatment})}$ is the number of white cells after drug treatments.

Measurement of DNA fragmentation

The SH-SY5Y cells were seeded and pre-treated as described above and the standard Comet assay was performed as originally described by Singh and co-workers (Singh *et al.*, 1998) and modified by Brink and co-workers (Brink *et al.*, 2008a). Briefly, slides were covered with a 1.3 mm layer of 1% v/v high melting point agarose (HMPA) in 0.1 M EDTA, by pipetting 350 µl of preheated (80°C) HMPA on the slide and evenly distributing it across the slide with a heated steel scraper and then allowing it to air dry for 15 min. Thereafter 0.5% v/v low melting point agarose (LMPA) was prepared and kept at 37°C until loaded with cells as described below. After 24 hour serum-free incubation, the cells were detached from the well bottoms by trypsinization and ± 500,000 cells transferred to microcentrifuge tubes. The cells were centrifuged at 5500 *g* for 5 min and resuspended in 1 ml PBS. 20 µl of the suspension was transferred to 150 µl LMPA at 37°C and immediately transferred onto the centre of a HMPA pre-coated slide. Similar to the HMPA, the cell-containing LMPA was evenly distributed over half of the slide in a 0.3 mm layer, to yield a monolayer of cells. These slides were placed on moist paper towels to cool down to room temperature and set. Immediately thereafter, the cell-containing slides were lysed in a lysis buffer (2.5 M NaCl, 10 mM Trizma base, 0.1 mM EDTA, 1% Triton-X-100, 10% v/v DMSO, 0.2 M NaOH with pH adjusted to 10) at 4°C, while protected from light. Thereafter slides were rinsed in $_{dd}H_2O$, incubated in the electrophoresis buffer (0.3 M NaOH, 1mM EDTA and pH adjusted to 13) at 4°C for 30 min and then electrophoresed (37 V and 400 mA) at 4°C for 20 min. The slides were rinsed with $_{dd}H_2O$ and incubated in 0.4 M Trizma-HCl buffer for 15 min at 4°C and rinsed again with $_{dd}H_2O$, then further incubated in 10 µM ethidium bromide solution for 15 min at 4°C, then rinsed again with $_{dd}H_2O$. Within 6 hours the image of the resulting DNA

comets of 50 cells per treatment condition was captured and the resulting comets analysed using the Comet IV® computer software program. The analyses computed the amount of DNA in the head (intact DNA) with the amount of DNA in the tail (DNA fragment). DNA integrity was expressed as percentage DNA in tail and calculated as follows:

$$\text{DNA in tail (\%)} = 100 \times \frac{F_{(tail)}}{F_{(tail)} + F_{(head)}}$$

where $F_{(tail)}$ = fluorescence in comet tail, $F_{(head)}$ = fluorescence in comet head. When DNA repair capacity (measuring cellular resilience) was to be analysed, an additional step was introduced as before (Brink *et al.*, 2008b) just before the cells were suspended in LMPA, where 10 μM H_2O_2 was added to the cell suspension and incubated at 37°C for 40 min whereafter the samples were centrifuged for 5 min at 5500 *g*, and the supernatants removed and the pellet resuspended in 1 ml PBS, centrifuged for another 5 min at 5500 *g*, and resuspended in Ham's F12 growth medium. From the resulting cell suspension 20 μl was immediately mixed with 150 μl LMPA in a microcentrifuge tube and loaded onto the slide (as described above), while the remainder of the cell suspension was incubated for 40 min (allowing DNA repair), whereafter another 20 μl of cell suspension was mixed with LMPA and loaded onto the slide. The assay was thereafter continued as described above.

Data analysis

Data from all assays were obtained as triplicate measurements from at least three separate experiments (i.e. $n \geq 9$), and expressed as mean ± S.E.M, and a value of $p < 0.05$ was considered to be statistically significant. GraphPad Prism® version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used to process the experimental data. For comparison of two values, the Student's t-test (one or two tailed, as indicated) was implemented. For multiple comparisons the one-way ANOVA comparison

was performed followed by either the Dunnett's post-test (for comparing groups to the control) or the Tukey-Kramer post-test (for comparing all groups to one another). For all reported statistical probability values, $P < 0.05$ was regarded as statistically significant.

Results

Determination of the optimum duration of serum deprivation for oxidative stress and its impact on various parameters of cell viability

Figure 1A depicts the cell viability of SH-SY5Y cells, as measured in the MTT assay, following different durations of serum-deprivation. It can be seen that the mitochondrial activity (cell viability) is not statistically significantly reduced relative to the control after 16 hour incubation in serum-free medium, but significantly reduced after 8 hours (85% ± 4%, $p < 0.01$) and most robustly after 24 hours (68% ± 3%, $p < 0.001$). ***Figures 1B & C*** compares reduced cell viability before and after 24 hours serum deprivation, as measured by the MTT assay (68 ± 3%, $p < 0.001$) and the Trypan blue assay (65 ± 1%, $p < 0.001$), respectively. ***Figures 1D*** depicts DNA damage before and after 24 hours serum deprivation, superimposed by H_2O_2-induced DNA damage and 40 minutes DNA repair (14 ± 3% vs. 34 ± 4%, $p < 0.001$).

Determination of the optimum sildenafil concentration that displays a protective effect against oxidative stress

Figure 2 depicts the viability of SH-SY5Y cells following 24 hours incubation with different concentrations of sildenafil under the simultaneous oxidative stress condition of 24 hours serum-deprivation, as measured in the MTT assay. It can be seen that the mitochondrial activity (cell viability) is statistically significantly increased at 1.6 nM (114% ± 3%), with a trend at 0.16 nM and 16 nM (not significant), and significantly reduced at 1600 nM (52% ± 5%) relative to the control.

Evaluation of the effects of select classical antidepressants, PDE inhibitors and a cGMP analogue on various parameters of cell viability, during oxidative stress

Figure 3A depicts the effect of 24 hour incubation with various drugs on cell viability under conditions of oxidative stress induced by simultaneous serum deprivation, as measured by the standard MTT assay (i.e. mitochondrial activity). Only lithium and sildenafil cell viability significantly (114% ± 3%, $p < 0.001$ and 109% ± 3%, $p < 0.01$, respectively) relative to control. A significant decrease in cell viability can be seen with imipramine (76% ± 3%, $p < 0.001$) and tadalafil (77% ± 2%, $p < 0.001$) relative to control. ***Figure 3B*** depicts the effect of 24 hour incubation with various drugs on cell viability under conditions of oxidative stress induced by simultaneous serum deprivation, as measured by the standard Trypan blue assay (i.e. membrane integrity). Only tadalafil and rolipram increased cell viability significantly (116% ± 6%, $p < 0.001$ and 127% ± 4%, $p < 0.001$, respectively) relative to control. ***Figure 3C*** depicts the effect of a 24 hour incubation with various drugs on cell viability under conditions of oxidative stress induced by simultaneous serum deprivation, as measured by the DNA comet assay (i.e. DNA repair capacity following H_2O_2-induced damage, followed by 40 minutes incubation in growth medium to allow repair). All the drugs significantly enhanced DNA repair capacity, with the resulting significantly reduced DNA damage being measured for sildenafil (11% ± 1%, $p < 0.01$), tadalafil (12% ± 3%, $p < 0.01$), rolipram (15% ± 3%, $p < 0.05$), lithium (12% ± 2%, $p < 0.01$), fluoxetine (15% ± 5%, $p < 0.05$) and imipramine (16% ± 5%, $p < 0.05$) relative to the control (34 ± 4%).

Evaluating the role of cGMP in the mechanism whereby sildenafil enhances cell viability

Figure 4A depicts the effect of 24 hour incubation with control, sildenafil or db-cGMP on cell viability under conditions of oxidative stress induced by simultaneous serum deprivation, in the presence and absence of the guanylate cyclase inhibitor (ODQ) or the protein kinase G

inhibitor (RP-PET-cGMPS), as measured by the standard MTT assay (i.e. mitochondrial activity). As before, sildenafil alone significantly increased (114% ± 3%, $p < 0.001$) cell viability relative to the control, whereas db-cGMP alone significantly decreased cell viability (84% ± 3%, $p < 0.001$). The guanylate cyclase inhibitor ODQ alone decreased cell viability relative to control (55% ± 4%, $p < 0.001$). Furthermore, ODQ reversed the protective effect of sildenafil alone, and cell viability after ODQ + sildenafil was less than control, after sildenafil alone or after ODQ alone (27% ± 1%, $p < 0.001$ compared to all three aforementioned treatment values). Similar to ODQ, the protein kinase G inhibitor RP-PET-cGMPS alone decreased cell viability relative to control (34% ± 5%, $p < 0.001$). However, RP-PET-cGMPS did not reverse the protective effect of sildenafil as was seen with ODQ, and cell viability after RP-PET-cGMPS + sildenafil was unchanged relative to RP-PET-cGMPS alone, but still reduced relative to control (25 ± 1%, $p < 0.001$). Since db-cGMP alone did not yield any protective effects, it was not evaluated in combination with ODQ or RP-PET-cGMPS.

Figure 4B depicts the effect of 24 hour incubation with control, sildenafil or db-cGMP on cell viability under conditions of oxidative stress induced by simultaneous serum deprivation, in the presence and absence of the guanylate cyclase inhibitor (ODQ) or the protein kinase G inhibitor (RP-PET-cGMPS), as measured by the standard Trypan blue assay (i.e. membrane integrity). Neither sildenafil alone nor db-cGMP alone altered cell viability relative to control as measured in the Trypan blue test. As in the MTT test, both ODQ alone (45% ± 5%, $p < 0.001$) and RP-PET-cGMPS alone (41% ± 4%, $p < 0.001$) reduced cell viability significantly relative to control. However, different from the results in the MTT test, ODQ did not reverse the effect of sildenafil and cell viability after ODQ alone was not statistically different from after ODQ + sildenafil (58% ± 4%, $p < 0.001$). No other combinations of

sildenafil or db-cGMP with ODQ or RP-PET-cGMPS were evaluated, since neither sildenafil alone nor db-cGMP alone yielded protective effects.

Figure 4C depicts the effect of a 24 hour incubation with control, sildenafil or db-cGMP on cell viability under conditions of oxidative stress induced by simultaneous serum deprivation, in the presence and absence of the guanylate cyclase inhibitor (ODQ) or the protein kinase G inhibitor (RP-PET-cGMPS), as measured by the DNA comet assay (i.e. DNA repair capacity following H_2O_2-induced damage, followed by 40 minutes incubation in growth medium to allow repair). A two-way ANOVA analysis of the data confirmed the absence of an interaction between the treatments that stimulate (sildenafil/cGMP analogue) and inhibit (GC/PKG antagonist) the NO/cGMP pathway ($F [4, 21] = 2.647$. $p = 0.0622$), therefore we could perform a one-way ANOVA followed by a Dunnett's post-test (comparing to control). It was found that only sildenafil alone and db-cGMP alone significantly increased DNA repair capacity, indicated respectively as 11% ± 1% ($p < 0.05$) and 15% ± 5% ($p < 0.05$) DNA damage relative to the control. Due to the small number of observations with this experiment ($n = 3$), the statistical power of the data was too weak to allow for the Tukey-Kramer post-test to reveal any statistically significant differences, as in ***Figures 4A&B***.

Discussion

In the study we were able to determine an optimal duration of serum deprivation to induce a suitable degree of oxidative stress in human neuroblastoma (SH-SY5Y) cells, for the evaluation of the modulatory effect of drugs on cell viability. The data of the current study suggest that a 24 hour serum deprivation induces sufficient oxidative stress to reduce cell viability, as measured by the MTT and Trypan blue assays (***Figure 1B&C***), between 25 to 50%, and to reduce DNA repair capacity such that the resulting DNA damage is just more than double (***Figure 1D***). Therefore, according to these non-specific tests of cell viability, the cellular damage induced by 24 hours serum deprivation is sufficient to enable evaluation of any protective effects of a drug, yet not excessive, so that any augmentation of stress by a drug can be observed as well. Serum deprivation has been demonstrated to induce oxidative stress *in vitro* (Jämsä *et al.*, 2006) as other studies indicate that excessive release of NO occurs when cells are exposed to oxidative stress. The excess NO binds to the free radicals to form the reactive oxygen species (ROS) peroxynitrate (Prast & Philippu, 2001). An increased level in ROS has shown to lead to lipid peroxidation and DNA damage (Emerit *et al.*, 2004; Foster *et al.*, 2006).

We therefore implemented a 24 hour serum deprivation as oxidative stressor in all further experiments. From a concentration series it was evident that only a relatively low concentration of 1.6 nM of sildenafil exhibited any neuroprotective properties against oxidative stress, as measured in the MTT assay (***Figure 2***). This was unexpected, since the K_D of sildenafil for PDE5 has been reported as 3.7 ± 0.29 nM (Blount *et al.*, 2004), so that insufficient inhibition of PDE5 is expected at a concentration of 1.6 nM. These data suggest that the protective effect of sildenafil against oxidative stress may not be fully dependent on its inhibition of PDE5. The augmentation of the oxidative stress, as seen at a relatively high concentration of 1600 nM sildenafil, was expected, as similar effects have been observed in

another study (Erdogan *et al.*, 2007) on a non-neuronal cell line, which suggests excessive NO release that leads to cell death due to ROS production (Andoh *et al.*, 2000).

Further analyses of the data (***Figure 3***) suggest that the various antidepressants, as well as phosphodiesterase inhibitors have different effects on mitochondrial activity (MTT assay) and membrane integrity (Trypan blue assay), whereas all the drugs enhanced DNA repair capacity (DNA comet assay). Neither of the two antidepressants, fluoxetine and imipramine, increase cell viability as measured by the MTT and Trypan blue assays (***Figure 3A&B***), suggesting that these *in vitro* assays do not predict neuroprotective effects of antidepressants. That sildenafil at a low concentration enhanced cell viability in the MTT assay and tadalafil in the Trypan blue assay, suggest that these PDE 5 inhibitors may possess unique but mutually distinct protective properties. Lithium has been shown to exert neuroprotective properties (Cleary *et al.*, 2008). However, the significance of the protective effects by lithium in the MTT, but not the Trypan blue assay, in the current study is not known. That the PDE4 selective inhibitor rolipram displayed protective properties in the Trypan blue, but not in the MTT assay, may potentially be related to its effects on cyclic adenosine monophosphate (cAMP), where cAMP has been associated with increased expression of the neuroprotective transcription factor cAMP response element binding (CREB) protein. CREB has shown to have increased expression in cells undergoing stress (Valera *et al.*, 2008), to be increased in patients receiving antidepressant treatment (Dowlatshahi *et al.*, 1998) as well as an involvement in antidepressive-like effect seen reported in animal models of depression (Chen *et al.*, 2001).

A very important observation of the current study was that all of the drugs enhanced DNA reparative capacity (***Figure 3C***). Since this effect is seen with the antidepressants fluoxetine and imipramine, as well as with the mood stabilizer lithium, this may suggest that this *in vitro* assay may be predictive of/related to the neuroprotective effects of antidepressants. Both

sildenafil (a structurally unrelated PDE5 inhibitor) and tadalafil, displays protective properties, suggesting that this may be related to their PDE5 inhibitory action. Furthermore, this effect is mimicked by the cGMP analogue db-cGMP, which further provides additional support for a role of PDE5 inhibition in the protective effects of sildenafil and tadalafil. The presence of PDE5 in the CNS and the ubiquitous nature of the second messenger on which they act indicate the potential of PDE5 inhibitors in the CNS disorders (Blokland *et al.*, 2006). The second messenger cGMP has been found to play a role in synaptic plasticity and neurogenesis (Zhang *et al.*, 2002).

The role of cGMP in the mechanism whereby sildenafil exerts its protective properties was also examined (***Figure 4***). Since the guanylate cyclase inhibitor ODQ reverses the protective effects of sildenafil as measured in the MTT assay (mitochondrial activity - ***Figure 4A***), this suggests that the formation of cGMP is important for the protective effect of sildenafil to be exerted. It furthermore suggests that the ability of sildenafil to inhibit PDE5, thereby to increase cGMP under normal condition may be important in this regard. However, since the protein kinase G inhibitor RP-PET-cGMPS did not also reverse the effects of sildenafil in the MTT assay, the data suggest that this protective effect of sildenafil is not associated with signalling of cGMP via protein kinase G. Under normal physiological baseline conditions the intracellular concentration of cGMP is approximately 0.1 times that of cAMP (Gibson, 2001). When the concentration of cGMP is elevated to 10 times the baseline concentration, it has been shown to activate protein kinase A (PKA) (Cornwell *et al.*, 1994), suggesting that PKA may become an effector of cGMP when its levels are elevated by, for example, inhibition of PDE5. The pharmacological profile of db-cGMP is not yet fully characterised and it is a mere assumption that db-cGMP (a membrane permeable analogue of cGMP) will model the physiological effects of cGMP. It is however possible, or even likely, that the pharmacological profiles of cGMP and db-cGMP may differ at some point, and therefore that

PKA will not be activated by the PKG activator db-cGMP, which will provide a possible explanation for the lack of protective effect of db-cGMP in the MTT assay. In addition, PKA is not inhibited by the PKG antagonist RP-PET-cGMPS, providing an explanation for the inability of RP-PET-cGMPS to reverse the response of sildenafil. Furthermore, that these protective effects of sildenafil was not observed in the Trypan blue assay (***Figure 4B***), suggest that the protective effects of sildenafil is not via mechanisms that involve membrane integrity.

Importantly, the protective effects of both sildenafil and db-cGMP to improve DNA repair capacity (***Figure 4C***). Both the guanylate cyclase inhibitor ODQ and the protein kinase G antagonist RP-PET-cGMPS reversed the protective effects of sildenafil and of db-cGMP. Together these data provide two lines of evidence to suggest that the protective effect of sildenafil is dependent on the formation of cGMP and that it is mediated via protein kinase G. From the data, however, there was a trend that the reversal of the protective effect by sildenafil was only partially reversed by RP-PET-cGMPS, but the data did not have the statistical power to allow for such analyses. Previous studies have also demonstrated that cGMP levels in the hippocampus of rats increase after 8 week chronic treatment with antidepressants fluoxetine and amitriptyline (Reierson *et al.*, 2009). The data therefore suggest that neuroprotective effect via a cGMP-dependent mechanism may underlie the reported antidepressant-like effects observed after the treatment of rats with sildenafil + atropine (Brink *et al.*, 2008a).

Conclusion

The current study suggests that 24 hour serum deprivation of cultured human neuroblastoma (SH-SY5Y) cells to induce oxidative stress (stressor) is sufficient to evaluate the effects of drugs on cell viability, either augmenting or attenuating the effect of the stressors. Sildenafil displays in the MTT assay a protective effect against oxidative stress at a relatively low concentration of 1.6 nM (lower than the K_D value for PDE5). This observation, as well as that the cGMP analogue db-cGMP does not share the protective effect of sildenafil in the MTT assay, suggest that the protective effect of sildenafil may not be fully dependent on PDE5 inhibition and cGMP levels. However, we cannot omit a role for cGMP, as the guanylate cyclase inhibitor was able to reverse the effect of sildenafil, although this effect is not mediated via protein kinase G. Also, this effect seems to be unique to sildenafil and lithium, and was not observed with other antidepressants tested. Furthermore, whereas the protective effect of sildenafil seems to be associated with mechanisms that relate to mitochondrial activity, it does not relate to membrane integrity. Regarding the effect on DNA repair capacity, the protective effect of sildenafil is clearly related to the formation of cGMP and protein kinase G mediated, while the effect is also shared by other antidepressants and the mood stabilizer lithium. Further studies are now needed to investigate what the effects of sildenafil are on markers of neuroplasticity, for example brain-derived neurotrophic factor (BDNF), and also to investigate what the *in vivo* relevance of the current findings will be in the brain tissue of animals treated with sildenafil.

Acknowledgements

The authors would like to acknowledge Ms. Maureen Steyn and Sharlene Lowe of the Laboratory for Applied Molecular Biology (LAMB), North-West University, Potchefstroom Campus, for their assistance with some of the experimental procedures. This project was funded by a grant from the National Research Foundation of South Africa (grant no. 47695).

Figure caption list

Figure 1: Determination of the optimum serum free incubation time on neuroblastoma (SH-SY5Y) cells by exposing cells to serum free conditions for (A) 6, 12, 18 and 24 hours and (B) 24 hours using the MTT cell viability assay. The effect of 24 hour serum free incubation using the (C) Trypan blue assay and (D) Comet assay respectively. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments where (A & B) mitochondrial activity is expressed as percentage of control, (C) membrane permeability is expressed as percentage of control and (D) DNA damage is expressed as percentage in comet tail. Data were analyzed (A) using the one-way ANOVA and Tukey-Kramer Multiple-Comparison post-test and (B, C & D) unpaired student's t-test with statistical significance indicated as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Figure 2: Determination of the optimum sildenafil concentration pre-treatment (0.086 to 1600 nM) in neuroblastoma (SH-SY5Y) cells during 24 hour serum deprivation using the MTT assay. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments where mitochondrial activity is expressed as percentage of control. Data were analyzed using the one-way ANOVA and Tukey-Kramer Multiple-Comparison post-test with statistical significance indicated as $p < 0.05$ (*) and $p < 0.001$ (***).

Figure 3: Determination of cellular plasticity of the following drug pre-treatments: Sildenafil (indicated as Sil representing 1.6 nM), tadalafil (indicated as Tad representing 97 nM), rolipram (indicated as Rol representing 16 nM), lithium (indicated as Li representing 1 mM), fluoxetine (indicated as Flx representing 92 nM), imipramine (indicated as Imi representing 105 nM) and the cGMP analogue (indicated as db-cGMP representing 200 nM) in neuroblastoma (SH-SY5Y) cells using the (A) MTT, (B) Trypan blue and (C) the comet assays. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments where (A) mitochondrial activity is expressed as percentage of

control, (B) membrane permeability is expressed as percentage of control and (C) DNA damage is expressed as percentage in comet tail. Data were analyzed using the one-way ANOVA and Tukey-Kramer Multiple-Comparison post-test with statistical significance indicated as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Figure 4: Determination of the mechanism whereby sildenafil exerts cellular plasticity by using the following drug pre-treatments: Sildenafil (indicated as Sil representing 1.6 nM), a cGMP analogue (indicated as db-cGMP representing 200 nM) and a soluble guanylate cyclase inhibitor (indicated as ODQ representing 2 μM) in neuroblastoma (SH-SY5Y) cells using the (A) MTT, (B) Trypan blue and (C) the comet assays. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments where (A) mitochondrial activity is expressed as percentage of control, (B) membrane permeability is expressed as percentage of control and (C) DNA damage is expressed as percentage in comet tail. Data (in Figure 4A & 4B) were analyzed using the one-way ANOVA followed by a Tukey-Kramer post-test (i.e. comparing all values) where $p < 0.001$ (***) relative to control and (###) relative to indicated value, or (in Figure 4C) a Dunnett's Multiple-Comparison post-test (i.e. comparing to control) where $p < 0.05$ (*) and $p < 0.01$ (**).

Figures

Figure 1

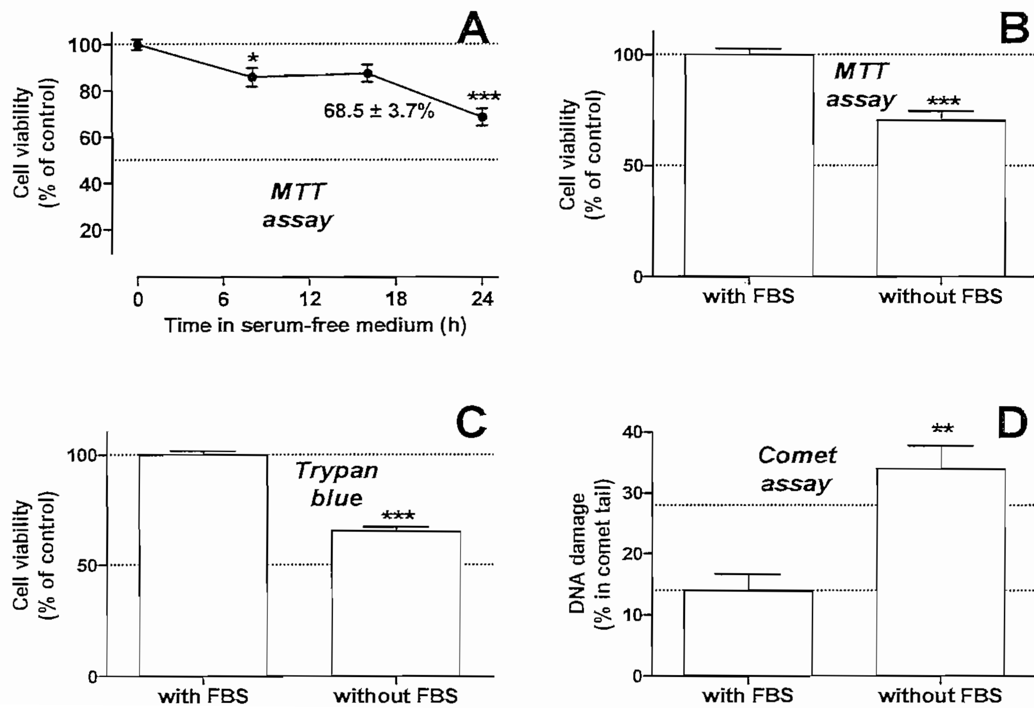


Figure 2

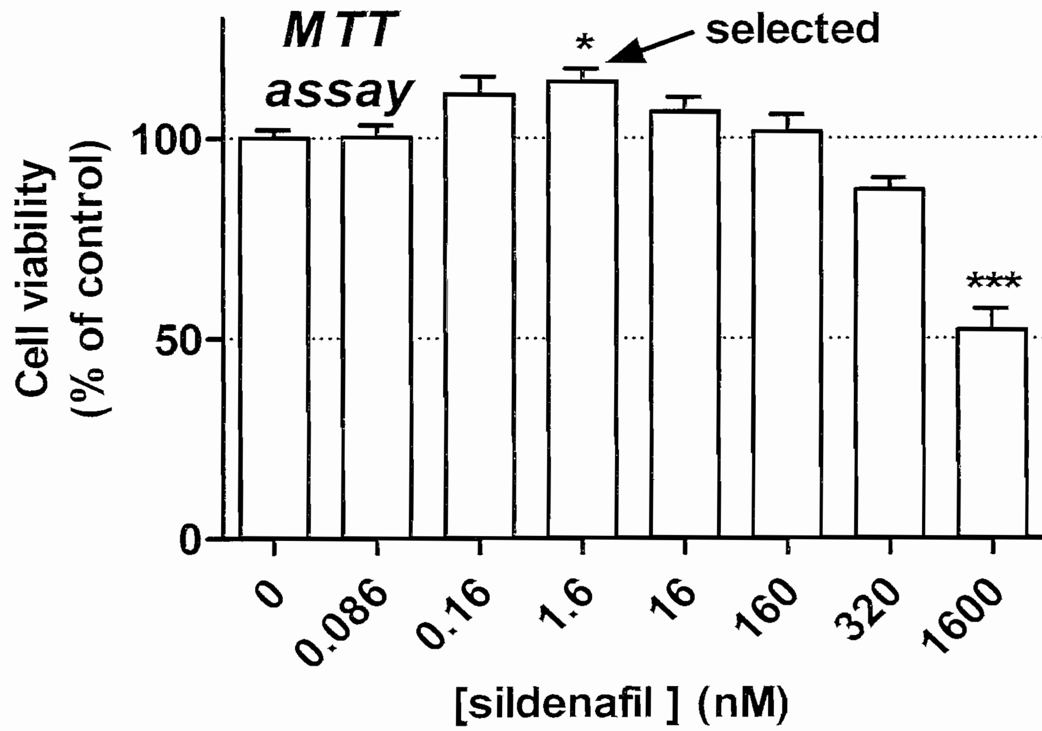


Figure 3

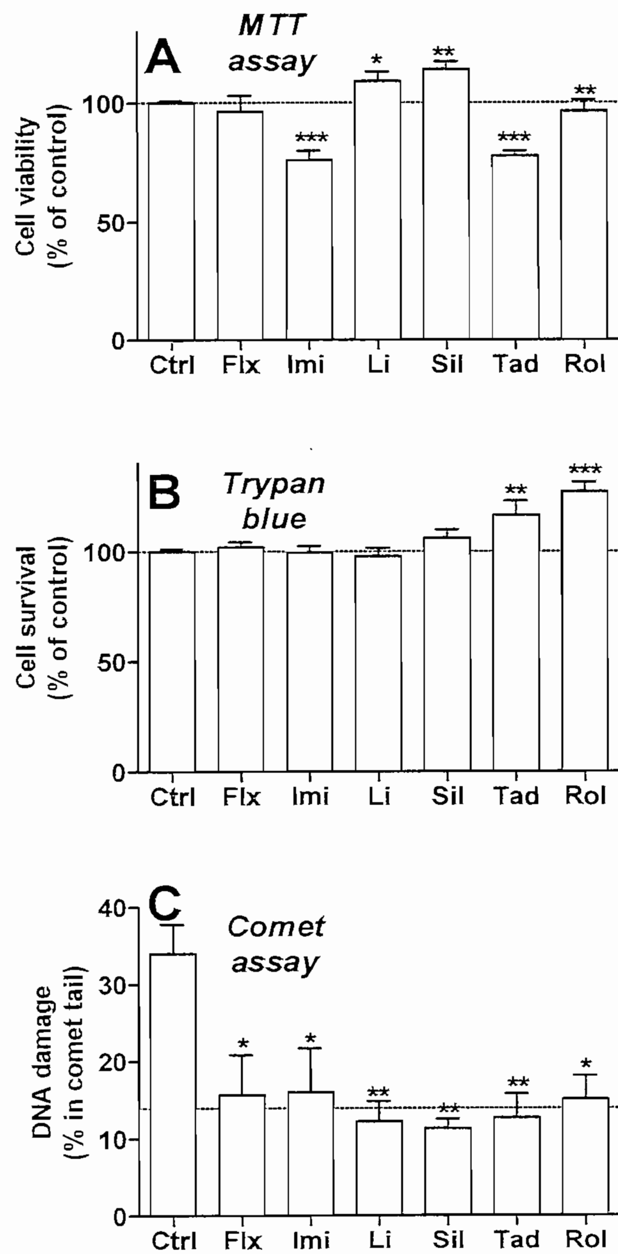
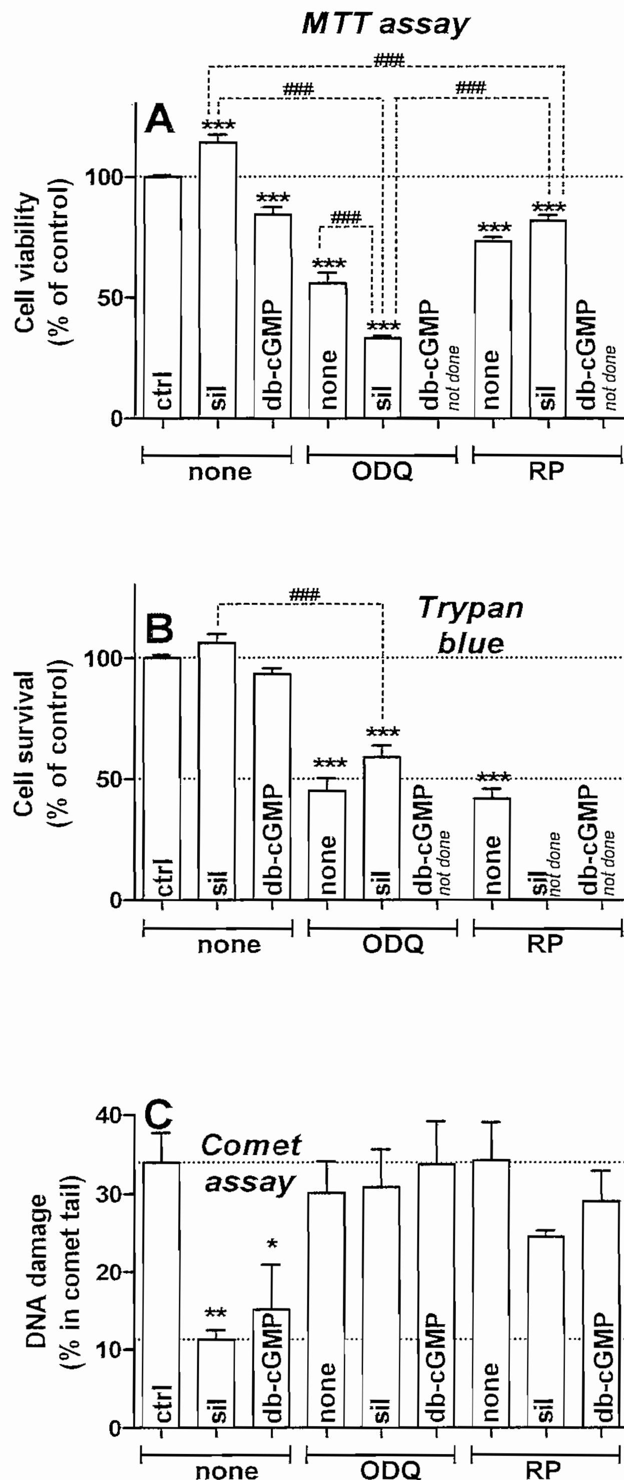


Figure 4



References:

Almeida, R.C., Felisbino, C.S., López, M.G., Rodrigues, A.L.S. and Gabilan, N.H. (2006). Evidence for the involvement of l-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like effect of memantine in mice. *Behavioral Brain Reseasch.* 168:318-322.

Andoh, T., Lee, S.G. and Chiueh, C.C. (2000). Preconditioning regulation ofbcl-2 and p66shc by human NOS1 enhances tolerance to oxidative stress. *Federation of American Societies for Experimental Biology.* 14:2144-2146.

Balu, D.T., Hoshaw, B.A., Malberg, J.E., Rosenzweig-Lipson, S., Schechter, L.E. and Lucki, I. (2008). Differential regulation of central BDNF protein levels by antidepressant and non-antidepressant drug treatments. *Brain Research.* 1211:37-43.

Blendy, J.A. (2006). The role of CREB in depression and antidepressant treatment. *Biological Psychiatry.* 59:1144-1150.

Blokland, A., Schreiber, R. and Prickaerts, J. (2006). Improving memory: A role for phosphodiesterases. *Current Pharmaceutical Design.* 12:2511-2523.

Blount, M.S., Beasley, A., Roya, Z., Konjeti, R.S., Bessay, E.P., Francis, S.H. and Corbin, J.D. (2004). Binding of tritiated sildenafil, tadalafil, vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogenicity, and cGMP stimulation. *Molecular Pharmacology.* 66:152.

Bremner, J.D., Vythilingam, M., Vermetten, E., Nazeer, A., Adil, J., Khan, S., Staib, L.H. and Charney, D.S. (2002). Reduced volume of orbitofrontal cortex in major depression. *Biological Psychiatry.* 51:273-279.

Brink, C.B., Clapton, J.D., Eagar, B.E. and Harvey, B.H. (2008a). Appearance of antidepressant-like effect by sildenafil in rats after central muscarinic receptor blockade: Evidence from behavioural and neuro-receptor studies. *Journal of Neural Transmission.* 115:117-125.

Brink, C.B., Pretorius, A., Van Niekerk, B.P.J., Oliver, D.P. and Venter, D.P. (2008b). Studies on cellular resilience and adaptation following acute and repetitive exposure to ozone in cultured human epithelial (HeLa) cells. *Redox Report.* 13:87-100.

Chen, A.C.-., Shirayama, Y., Shin, K., Neve, R.L. and Duman, R.S. (2001). Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. *Biological Psychiatry.* 49:753-762.

Cleary, C., Linde, J.A.S., Hiscock, K.M., Hadas, I., Belmaker, R.H., Agam, G., Flaisher-Grinberg, S. and Einat, H. (2008). Antidepressive-like effects of rapamycin in animal models: Implications for mTOR inhibition as a new target for treatment of affective disorders. *Brain Research Bulletin.* 76:469-473.

Cornwell, T.L., Arnold, E., Boerth, N.J. and Lincolin, T.M. (1994). Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *American Journal of Physiology - Cell Physiology.* 36:C1405-C1413.

Devan, B.D., Pistell, P.J., Daffin, J.,Lee W., Nelson, C.M., Duffy, K.B., Bowker, J.L., Bharati, I.S., Sierra-Mercado, D., Spangler, E.L. and Ingram, D.K. (2007). Sildenafil citrate attenuates a complex maze impairment induced by intracerebroventricular infusion of the NOS inhibitor Nω-nitro-L-arginine methyl ester. *European Journal of Pharmacology.* 563:134-140.

Devan, B.D., Sierra-Mercado, J., Demetrio, Jimenez, M., Bowker, J.L., Duffy, K.B., Spangler, E.L. and Ingram, D.K. (2004). Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats. *Pharmacology Biochemistry and Behavior.* 79:691-699.

Dowlatshahi, D., MacQueen, G.M., Wang, J.F. and Young, L.T. (1998). Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. *The Lancet.* 352:1754-1755.

Emerit, J., Edeas, M. and Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. *Biomedecine & Pharmacotherapy.* 58:39-46.

Erdogan, A., Wiebke Luedders, D., Muenz, B.M., Schaefer, C.A., Tillmanns, H., Wiecha, J. and Kuhlmann, C.R.W. (2007). Sildenafil inhibits the proliferation of cultured human endothelial cells. *International journal of Biomedical science.* 3:94-97.

Fava, M. (2003). Diagnosis and definition of treatment-resistant depression. *Biological Psychiatry.* 53:649-659.

Foster, K.A., Galeffi, F., Gerich, F.J., Turner, D.A. and Müller, M. (2006). Optical and pharmacological tools to investigate the role of mitochondria during oxidative stress and neurodegeneration. *Progress in Neurobiology.* 79:136-171.

Gibson, A. (2001). Phosphodiesterase 5 inhibitors and nitrergic transmission—from zaprinast to sildenafil. *European Journal of Pharmacology.* 411:1-10.

Hardman, J.G., Limbird, L.E. and Gilman, A.E. (2001). Design and optimization of dosage regimas: Pharmacokinetic data, (In Anon. 10th edn no., GOODMAN & GILMAN: The pharmacological basis of therapeutics, pp. 1917-2023.).

Jämsä, A., Bäckström, A., Gustafsson, E., Dehvari, N., Hiller, G., Cowburn, R.F. and Vasänge, M. (2006). Glutamate treatment and p25 transfection increase Cdk5 mediated tau phosphorylation in SH-SY5Y cells. *Biochemical and Biophysical Research Community.* 345:324-331.

Katzung, B.G., Trevor, A.J. and Masters, S.B. (2002). Antidepressants, (In Foltin, J., Lebowitz, H. and Boyle, P.B., eds.), sixth edn no., Katzung & Trevor's pharmacology: Examination & board review, McGraw-Hill Medical Publishing Division, pp. 269-278.).

Manji, H.K., Quiroz, J.A., Sporn, J., Payne, J.L., Denicoff, K., A. Gray, N., Zarate, C.A. and Charney, D.S. (2003). Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biological Psychiatry.* 53:707-742.

MATHERS, C., Stein, C. and Shibuja, K., (2003). *The Global Burden of Disease: A response to the need for comprehensive, consistent and comparable global information on diseases and injuries.* www.who.int: World Health Organization.

Pittenger, C. and Duman, R.S. (2008). Stress, depression, and neuroplasticity: A convergence of mechanisms. *Neuropsychopharmacology.* 33:88-109.

L. Potgieter, B. H. Harvey and C. B. Brink. (2008). North-West Universtiy, Potchefstroom, MSc, Potchefstroom.

Prast, H. and Philippu, A. (2001). Nitric oxide as modulator of neuronal function. *Progression in Neurobiology.* 64:51-68.

Reierson, G.W., Mastronardi, C.A., Licinio, J. and Wong, M. (2009). Repeated antidepressant therapy increases cyclic GMP signaling in rat hippocampus. *Neuroscience Letters.* In Press, Uncorrected Proof:.

Rooney, T.A., Joseph, S.K., Queen, C. and Thomas, A.P. (1996). Cyclic GMP induces oscillatory calcium signals in rat hepatocytes. *The Journal of Biological Chemistry.* 271:19817-19825.

Schwede, F., Maronde, E., Genieser, H. and Jastorff, B. (2000). Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacology and Therapeutics.* 87:199-226.

Shirayama, Y., Chen, A.C.-., Nakagawa, S., Russell, D.S. and Duman, R.S. (2002). Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *Journal of Neuroscience.* 22:3251-3261.

Singh, N.P., McCoy, M.T., Tice, R. and Schreiber, E.L. (1998). A simple technique for quantification of low levels of DNA damage in individual cells. *Experimental Cell Research.* 175:184-191.

Ströhle, A. and Holsboer, F. (2003). Stress responsive neurohormones in depression and anxiety. *Pharmacopsychiatry.* 36:207-214.

Teixeira, C.E., Priviero, F.B.M. and Webb, R.C. (2006). Differential effects of the phosphodiesterase type 5 inhibitors sildenafil, tadalafil and vardenafil in rat aorta. *The Journal of Pharmacology and Experimental Therapeutics.* 316:654-661.

Valera, E., Sánchez-Martín, F.J., Ferrer-Montiel, A.V., Messeguer, Á. and Merino, J.M. (2008). NMDA-induced neuroprotection in hippocampal neurons is mediated through the protein kinase A and CREB (cAMP-response element-binding protein) pathway. *Neurochemistry International.* 53:148-154.

Zhang, R., Wang, Y., Zhang, L., Zhang, Z., Tsang, W., Lu, M., Zhang, L. and Chopp, M. (2002). Sildenafil (viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke*. 33:2675-2680.

Summary and Conclusion

Chapter 4

(All abbreviations are listed in Addendum E)

4.1 Summary

Since the results of the current study are presented in two separate sections, namely Chapter 3 (article) and Addendum B (supplementary and additional data), it was necessary to provide a concise summary of all the results combined. Such a summary of the results is presented in the two tables below.

In the current study neuronal (human neuroblastoma, SH-SY5Y) and non-neuronal (Chinese hamster ovary, CHO-K1) cell lines were used to investigate protective properties of the phosphodiesterase type 5 (PDE5) inhibitor, sildenafil, against biological stressors. As a first specific aim, the study set out to determine an appropriate *in vitro* biological stressor (associated with the neuropathology of major depression), and a concise summary of the results are presented in Table 4-1.

Table 4-1 The effects of three different biological stress conditions, associated with the neuropathology of major depression, on cell viability in a neuronal and non-neuronal cell line, as measured in the MTT assay. These stress conditions yielded a reduction in cell viability of 25-50%.

Optimal biological stress condition selected		
Glutamate	H_2O_2 exposure	Serum deprivation
• 12.5 mM in SH-SY5Y • 17.5 mM in CHO-K1	• 1.75 µM for 15 min (both cell lines)	• 24 h (both cell lines)

Since further data obtained from this study indicated that sildenafil did not display protective properties on the CHO-K1 cells (see § B.1.2) this cell line was not used throughout further experiments in the study. However further data indicated that sildenafil displayed a protective effect only human neuroblastoma (SH-SY5Y) cells

and only after 24 hours serum deprivation, as measured in the MTT assay, therefore this cell line and biological stressor was used for all further studies.

Human neuroblastoma (SH-SY5Y) cells were treated for 24 hours with drug-free control, fluoxetine, imipramine, lithium, sildenafil, tadalafil, rolipram or db-cGMP under conditions of serum-deprivation, with or without ODQ or RP-PET-cGMPS (when indicated). The effects of these treatments on cell viability, as measured by the MTT-, Trypan blue and comet assays are summarized in Table 4-2.

Table 4-2 The effects of 24 hour treatment with different antidepressants, mood modulating drugs and drugs modulating the cGMP pathway under conditions of serum deprivation on viability in human neuroblastoma (SH-SY5Y) cells, as measured by the MTT, Trypan blue and DNA comet assays. In the table ↑ = significant increase in cell viability; - = no significant effect; ↓ =significant decrease in cell viability; "Not done" = experiment was not performed and no results are available.

Effect on cell viability			
Drug	**MTT**	**Trypan blue**	**Comet assay**
Fluoxetine	–	–	↑
Imipramine	–	↓	↑
Tianeptine	–	↓	↑
Lithium	↑	–	↑
Sildenafil + atropine	↓	↓	Not done
Sildenafil	↑	–	↑
Tadalafil	–	↑	↑
Rolipram	–	↑	↑
db-cGMP	–	–	↑
ODQ	↓	↓	↓
RP-PET-cGMPS	↓	↓	↓
Sildenafil + ODQ	↓	↓	↓
Sildenafil + RP-PET-cGMPS	↓	–	↓
db-cGMP + ODQ	Not done	Not done	↓
db-cGMP + RP-PET-cGMPS	Not done	Not done	↓

The results in Table 4-2 reveal that only sildenafil and lithium exhibited an increase in cytoprotection (as measured by the MTT assay), which was not shared by the antidepressant drugs or tadalafil. However, in the Trypan blue assay only tadalafil and the rolipram displayed an increase in cell survival, which was not shared by the antidepressant drugs or sildenafil. The classical antidepressants fluoxetine and

imipramine therefore did not display protective properties in MTT assay (mitochondrial activity) and Trypan blue assay (membrane integrity), while the structurally unrelated PDE5 inhibitors sildenafil and tadalafil displayed their protective effects differently in these assays. Importantly, all the antidepressant drugs (fluoxetine, imipramine and tianeptine), the mood stabilizer lithium, the PDE5 inhibitors sildenafil and tadalafil, the PDE4 inhibitor rolipram as well as the cGMP analogue db-cGMP increased the DNA repair capacity of SH-SY5Y cells (as measured by the comet assay). The results also indicated that, when the sGC inhibitor ODQ or the PKG antagonist RP-PET-cGMPS was added to sildenafil, the protective effect of sildenafil was reversed. This reversal was significantly more pronounced with ODQ that with RP-PET-cGMPS.

4.2 Discussion and Conclusion

Appropriate in vitro biological stressor

In this study the evaluation of various *in vitro* biological stress conditions, associated with the neuropathology of major depressive disorder, suggested that oxidative stress caused by 24 hour serum-free incubation was the optimal stressor. Serum-free incubation of SH-SY5Y cells has also been shown in another study to exhibit decreased cell viability (Andoh *et al.*, 2002). The increased cell death caused by oxidative stress result from an increase in nitric oxide (NO) that subsequently binds to the hydroxyl (·OH) free radical to form the reactive oxygen species (ROS) peroxynitrite ($ONOO^-$) (Andoh *et al.*, 2002; Chan, 1996). Lipid peroxidation is caused by increased levels of ROS, which has been associated with major depression (Bilici *et al.*, 2001; Halliwell & Gutteridge, 1992), Alzheimer's dementia (Gackowski *et al.*, 2008), as well as amyotrophic lateral sclerosis (Sala *et al.*, 2005). Cell damage, as induced by oxidative stress, has been demonstrated to be attenuated by cyclic guanosine monophosphate (cGMP) (Andoh *et al.*, 2000). Moreover, the role of the NO/cGMP pathway in stress disorders, including major depression, are increasingly recognised (Blendy, 2006).

Since serum-deprivation of SH-SY5Y cells were the only biological stress condition and cell line where sildenafil displayed a protective effect (as measured in the MTT assay), this provided further support for selecting this condition and cell line.

Furthermore, the antidepressants fluoxetine and imipramine and the mood stabilizer lithium increased DNA repair capacity (as measured in the comet assay) following serum deprivation (oxidative stress). Such protective effects by antidepressants and lithium, as positive controls, are to be expected. In this regard, yet another study indicated that antidepressants attenuate cell death induced by oxidative stress in rats (Zafir & Banu, 2007). Therefore, these results from the comet assay suggest that the measurement of DNA repair capacity may have good predictive validity to identify protective effects of antidepressants (i.e. an appropriate *in vitro* biological marker in SH-SY5Y cells), rendering it superior to the MTT assay or the Trypan blue assay in this regard.

The effects of selected drugs on oxidative stress

The effects of antidepressant drugs on oxidative stress

The results obtained from treating the SH-SY5Y cells with antidepressant drugs by measuring the mitochondrial activity (as measured by the MTT assay) and membrane integrity (as measured by the Trypan blue assay) found that none of the drugs exhibited protection. These data suggest that the MTT and Trypan blue assays may not have good predictive validity to identify protective effects of antidepressants (i.e. not appropriate *in vitro* biological markers in SH-SY5Y cells). Other studies also indicated that the antidepressants fluoxetine and amitriptyline did not protect neuronal cells from damage caused by oxidative stress as measured by the MTT- and Trypan blue assays respectively (Han & Lee, 2009; Kolla *et al.*, 2005). Furthermore, the PDE5 inhibitor sildenafil has been shown to display antidepressant-like activity in rats when co-administered with atropine, comparable to the prototype antidepressant fluoxetine (Brink *et al.*, 2008). However, that this combination of sildenafil plus atropine did not display any protective effect in the MTT or Trypan blue assays therefore was unexpected. Unfortunately this combination was not evaluated with the DNA comet assay. However the results from the comet assay revealed that all the antidepressant drugs exhibited protection by increasing the DNA repair capacity. As alluded to above, these results suggest that the mechanism of protection by the antidepressants against serum deprivation-induced oxidative stress in SH-SY5Y cells involve enhancement of DNA repair capacity.

The effects of PDE inhibitor drugs on oxidative stress and the role of cGMP

The results obtained by the MTT assay suggest that sildenafil has a unique property in neuroprotection occurring at a dose 10 times lower (1.6 nM) than its peak serum-free therapeutic level of 16 nM. However in the Trypan blue assay tadalafil exhibited a significant increase in cell viability at a concentration representing its peak serum-free therapeutic level of 97 nM, as well as rolipram at a concentration equalling its K_i value for PDE4. When considering the abovementioned protective effect of the antidepressant drugs on DNA repair capacity, it is worth mentioning that the PDE inhibitors used (sildenafil, tadalafil and rolipram) all exhibited the same significant increase in DNA repair capacity, as measured by the comet assay. In addition, recent studies also suggest that increased levels of cGMP result from chronic antidepressant treatment with fluoxetine and amitriptyline (Reierson *et al.*, 2009).

Taking into consideration that the cGMP analogue (db-cGMP) also increased DNA repair capacity it may suggest that cGMP plays an important role in the observed protective effects. In addition, studies have shown sildenafil to increase cGMP levels and promote neurogenesis (Zhang *et al.*, 2002) while increased cGMP levels has also shown to play a role in the NO/cGMP pathway that is important in regulating neuroplasticity in depression (Dhir & Kulkarni, 2007). The presence of cGMP intracellularly occurs at concentrations 10x lower than cAMP and the role of the cross-talk interaction between cAMP and cGMP as well as their effect on CREB activation needs further investigation as studies suggest that CREB activation (as direct result of increased cGMP levels) may be relevant in the neuropathology of depression (Blendy, 2006; Chen *et al.*, 2001; Dowlatshahi *et al.*, 1998; Puzzo *et al.*, 2008).

The effects of NO/cGMP pathway inhibitors on the oxidative stress modulating effects of sildenafil and db-cGMP

The NO/cGMP pathway is involved in the neuropathology of depression (Sanacora *et al.*, 2008), and as mentioned, increased levels of cGMP contributes to neurogenesis (Zhang *et al.*, 2002), neuroplasticity (Andreeva *et al.*, 2001) and improved depressive symptoms (Blendy, 2006). In addition, previous studies suggested that when the inhibition of sGC results in a sufficient reduction of the catalization of guanosine triphosphate (GTP) to cGMP, this will also diminish the

downstream protective effect of cGMP (Schwede *et al.*, 2000). The results of the current study, showing that ODQ reverses the protective effect of sildenafil and db-cGMP, are supportive of this finding. In this regard, the data of the current study also indicated that the PKG antagonist RP-PET-cGMPS is able to reverse the protective effect of sildenafil and db-cGMP, which is in support of previous reports that the protective effect of cGMP is inhibited by RP-PET-cGMPS (Yang *et al.*, 2005). These results suggest that the protective effect of sildenafil on DNA integrity is dependent on cGMP and on PKG signalling.

Final conclusions

This study reveals that 24 hour serum deprivation-induced oxidative stress is sufficient to cause a significant decrease in cell viability. It was found that a low concentration sildenafil (at a concentration lower than the K_D value for PDE5 inhibition) significantly increases cell viability after 24 hour serum-deprivation in the neuronal SH-SY5Y cell line, suggesting that the protective effect of sildenafil may not rely mainly on PDE5 inhibition. Mitochondrial activity (MTT assay) and membrane integrity (Trypan blue assay) were not found to be the optimal bio-markers for measuring the protective effects of antidepressant drugs or PDE inhibitors. However the comet assay (measuring the DNA repair capacity) suggests that all the antidepressants (fluoxetine, imipramine and tianeptine), as well as the mood stabilizer lithium, the PDE5 inhibitors sildenafil and tadalafil, the PDE4 inhibitor rolipram and the cGMP analogue db-cGMP increase DNA repair capacity of the SH-SY5Y cells. In addition the sGC inhibitor ODQ and the PKG antagonist RP-PET-cGMPS reversed the effect of sildenafil and db-cGMP to enhance DNA repair capacity, thereby suggesting that cGMP and PKG-mediated signalling plays an important role in their cytoprotective properties.

In the final analysis, impairments in the NO/cGMP signal transduction pathway has been implicated as a possible mechanism of reduced neuronal plasticity and neuronal survival in major depression. The data of the current study provide additional evidence to suggest that the NO/cGMP/PKG signal transduction pathway may be a promising target for the development of novel neuroprotective drugs for the treatment of anxiety-related disorders. Whereas other modulators of this pathway,

for example NMDA antagonists evaluated pre-clinically, have been associated with serious side-effects, the PDE5 inhibitors employed in the current study are already used clinically for other indications. Therefore, PDE5 inhibitors may provide a viable tool to positively modulate the NO/cGMP/PKG signal transduction pathway in order to reverse the effects of neuronal stress. Furthermore they may possess neuroprotective properties comparable to those of classical antidepressants. These characteristics of the PDE5 inhibitors, together with the behavioural animal data and a host of other collateral clinical and pre-clinical data mentioned before, suggest that these drugs may become valuable antidepressant strategies of the future.

4.3 Recommendations

In the current study there are questions that remain unanswered. These are presented below as listed shortcomings of the current study (following critical reflection), and subsequently some recommendations for further studies.

Shortcomings of the current study include:

- This study measured the effect of various concentrations (including the peak therapeutic serum-free concentrations of sildenafil) only in the MTT assay (a screening test that is easy to perform). However, since it then continued to evaluate only the protective lower concentration (0.16 nM) of sildenafil (as measured in the MTT assay) in other assays, the effect of sildenafil on DNA repair capacity in the comet assay at its peak therapeutic serum-free concentration was not evaluated.
- Similarly, since tadalafil did not display protective properties at concentrations lower than its K_D value for PDE5 (as sildenafil) in the MTT assay, the effects of tadalafil on DNA repair capacity in the comet assay at such lower concentrations were not evaluated.
- The combination of sildenafil and atropine did not display protective properties in the MTT assay. Consequently this combination was not evaluated in the comet assay. In previous studies in rats this combination has been shown to

display antidepressant-like activity (Brink *et al.,* 2008) and, in hindsight, this combination may yield different results in, for example, the comet assay. It may be worth re-evaluating this combination.

- The MTT assay and Trypan blue assay measure non-specific markers of cytoplasticity while the DNA comet assay is only one non-specific marker of apoptosis. The study therefore lacks the measurement of specific markers of cytoplasticity, and particularly those associated with the neuropathology of major depression (e.g. CREB, NF-κβ or BDNF).
- The current study evaluated the effects of PDE5 inhibitors *in vitro* only, so that extrapolation of these results to the *in vivo,* or even more so to the clinical situation, is challenging.

Recommendations include:

- The concentration-response relationship of sildenafil and tadalafil on DNA repair capacity should be performed with the DNA comet assay. This will indicate whether protective the effects on DNA repair capacity observed at a low concentration of sildenafil is unique to sildenafil, or whether this may be shared by other PDE5 inhibitors.
- The effects of the combination of sildenafil and atropine on DNA repair capacity should be determined with the DNA comet assay.
- The effect of PDE5 inhibitors (e.g. sildenafil and tadalafil) on other markers of neuroplasticity should be investigated, including on the expression and/or activity of bcl-2, Bax, caspase 3, 8, 9 and 10 as well as CREB, NF-κβ and BDNF. These evaluations can be done *in vitro*, or *ex vivo*, utilising brain tissue of animals following chronic treatment with the drugs.
- The role of potential cGMP-cGMP cross-talk, including the potential activation of CREB, in the mechanism of protective effects of sildenafil can be investigated. This phenomenon seems to be concentration dependent (Wen *et al.*, 2007; Zsombok *et al.*, 2005).

Materials and Methods

Addendum A

(All abbreviations are listed in Addendum E)

A.1 Materials

A.1.1 Cell lines used

The human neuroblastoma (SH-SY5Y) cell line was obtained from Sigma Aldrich – Johannesburg (catalogue no: 94030304) and the Chinese hamster ovary (CHO-K1) cell line was obtained from BD-Bioscience – Scientific group – Midrand – South Africa.

A.1.1.1 Human neuroblastoma (SH-SY5Y) cell line

The SH-SY5Y cell line is a neuronal cell line cloned thrice from the sub-line of bone marrow biopsy-derived SK-N-SH (SK-N-SH → SH-SY → SH-SY-5 → SH-SY5Y) and was established in 1970.

The SH-SY5Y cells were maintained under aseptic conditions in Ham's F12 solution with 10% enriched foetal bovine 100 µg/ml streptomycin, 25 µg/ml fungizone and 100 units/ml penicillin. The cell culture was incubated in 150 cm^3 culture flasks (Corning®) at 37°C in 10% CO_2 and humidified environment. The cell duplication time under these conditions was approximately 48 hours.

A.1.1.2 Chinese hamster ovary (CHO-K1) cell line

The Chinese Hamster Ovary cell line was obtained from BD Biosciences – Scientific Group – Midrand – South Africa. The CHO-K1 cell line is a non-neuronal mammalian cell line and was used to compare the effects of sildenafil and other drug pre-treatments on cell viability to that of the neuroblastoma (SH-SY5Y), neuronal cell line. The CHO-K1 cells were maintained under the same conditions as the SH-SY5Y cells. The CHO-K1 cells had a duplication time of 24 hours under these conditions (as mentioned above).

A.1.2 Chemicals

A.1.2.1 Chemicals used for cell cultures

- **From Celtic Molecular Diagnostics (Cape Town):**
  Foetal bovine serum (FBS), penicillin, streptomycin, fungizone, trypsin and HAMS F12 medium.

A.1.2.2 Chemicals used for assays

- **From Merck (Johannesburg, South Africa)**
  NaCl, KCl, Na_2HCO_3, formic acid and absolute alcohol, EDTA, DMSO, NaOH, H_2O_2, low melting point agarose, high melting point agarose, NaH_2PO_4 and $NaHCO_3$.

- **From Sigma Aldrich (Johannesburg, South Africa)**
  MTT reagent, isopropanol (99%), Triton X100, Tris buffer, Tris HCl, imipramine, lithium chloride, zaprinast, IBMX, rolipram, RP-PET-cGMPS, db-cGMP, ODQ and ethidium bromide.

- **From Scientific group (Johannesburg, South Africa)**
  Trypan blue stain (0.4%)

- **From Pfizer**
  Sildenafil citrate

- **From Eli Lilly**
  Tadalafil and fluoxetine

- **From Servier**
  Tianeptine

A.1.3 Consumables

- **From Corning® (New York, U.S.A)**
  Culture flasks (150cm^3), 24-well plates, 96-well plates, 15 ml sterile conical tubes, 50 ml sterile conical tubes and serological pipettes.

A.1.4 Apparatus

The following apparatus were used during the experiments:

- Eppendorf micropipettes (10 – 100 µl and 100 – 1000 µl) were used for analytical measurements.
- For performing the Trypan blue assay, a haemocytometer (0.1 mm depth, 0.0025 cm^2), a Nikon TMS inverted microscope and bench top centrifuge were used.
- For spectrophotometric analyses employed in the MTT assay, a 96-well plate reader with 560 nm filter (Labsystems, Multiscan, RC) were used.

- During electrophoresis in the DNA comet assay a Power Pac (from Biorad) was used.
- For visualization of the DNA comets a Olympus 1X70 inverted system microscope was used.
- The analysis® program was used for image capturing of the DNA comets visualized on the microscope.
- The Comet IV® program software was used for quantifying the comets during capturing.

A.1.5 Statistical analysis

GraphPad Prism® version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used to process the experimental data. All data were analyzed using a one-way analysis of variance (ANOVA) followed by either a Dunnet or Tukey-Kramer multiple comparison test, as indicated. Experiments were performed with triplicate observations in at least in three independent experiments (i.e. $n \geq 9$), as indicated. Data are expressed as the mean ± S.E.M., and a value of $p < 0.05$ was considered to be statistically significant.

A.2 Methods

A.2.1 Cell culture preparation

Human neuroblastoma (SH-SY5Y) and Chinese Hamster ovary (CHO-K1) cells were maintained in culture medium as described above and grown to 80% confluency before seeding. Cells were detached from the culture flasks by means of trypsination and seeded into 24-well plates at a density of 5×10^5 cells/well, then incubated for 24 hours at 37°C in 10% CO_2 allowing cells to attach to the bottom of the wells. Cell multiplication during the 24 hours resulted in a cell quantity of about 7.5×10^5 cells/well before the 24-hour stressor with or without experimental drug treatments (see below) were initiated.

A.2.2 Cell stressors and drug treatments

Cells attached as mono-layers in 24-well plates (see above) were subjected to certain selected stressors (see § A.2.2.1 and § B.1.1).

A.2.2.1 Cell stressors

There was three conditions selected that according to the literature simulate stressors seen in major depression. The stressors used to induce *in vitro* stress in the cells lines were glutamate (used to induce excitotoxicity), H_2O_2 exposure and serum-free incubation (used to induce oxidative stress) (Jämsä *et al.*, 2006). The stress condition selected was one where roughly 50% reduction in cell viability would occur, so that it would be possible to test for any intervention (such as a drug) to either augment (enhanced reduction in viability) or protect (reversal of reduced viability) against the stressor-induced reduction in cell viability. The selected stressor for glutamate was 12.5 mM in the SH-SY5Y cell line and 17.5 mM in the CHO-K1 cell line, for both cell lines 1.75 µM H_2O_2 as well as a 24 hour serum free incubation revealed this stressor-induced reduction (see § B.1.1).

A.2.2.2 Drug treatments

Drugs were introduced simultaneous to cell stressors that reduced cell viability with more or less 50%, as determined using the MTT assay (*see § A.2.4.1*). The optimal concentration for some drugs were determined from a dose-response series (*see § B.1.3*) or by using the reported maximal therapeutic free plasma concentration. As employed by many others before, Brink and co-workers (Brink *et al.*, 2004) proposed that 24 hour incubation with antidepressants under artificial *in vitro* conditions could be sufficient to model cellular responses as seen with chronic *in vivo* drug treatments.

A.2.2.2.1 Sildenafil

The selective PDE5 inhibitor, sildenafil, has a therapeutic peak concentration of 212 ± 59 ng/ml (Hardman *et al.*, 2001) *in vivo* and is 96% bound to plasma protein (i.e. 4% unbound/free), yielding a free concentration of 10.84 ng/ml. Calculated from its molecular weight (MM 666.7), the therapeutic peak free plasma concentration is

therefore 16 nM. While we have demonstrated from a concentration series, where we selected concentrations lower and higher than the 16 nM plasma free concentration (*see § B.1.2*), that sildenafil exert neuroprotective properties *in vitro* in the MTT assay at concentrations of 0.16 nM and 1.6 nM sildenafil respectively.

A.2.2.2.2 Tadalafil

The selective PDE5 inhibitor, tadalafil, has a therapeutic peak plasma concentration of 378 ng/ml *in vivo* and is 94% bound to plasma protein (i.e. 6% unbound/free), yielding a free concentration of 22.68 ng/ml. Calculated from its molecular weight (MM 389.41), the therapeutic peak free plasma concentration is 58 nM. However the K_i value of tadalafil for PDE5 has been reported as 9.7 nM in one study (Teixeira *et al.*, 2006) and 10 nM in another study (Maurice *et al.*, 2003). In the Trypan blue 97 nM tadalafil (10 times the Ki) exhibited neuroprotection and was used throughout the study (*see § A.2.2.1*)

A.2.2.2.3 Rolipram

The selective PDE4 inhibitor, rolipram, is not used therapeutically, so that therapeutic plasma concentrations are not available as for sildenafil and tadalafil. The K_i value of rolipram for PDE4 has been determined as 16 nM (ZHAO *et al.*, 2003). While we have demonstrated from a concentration series, that no concentration exhibited neuroprotection (*see § B.1.2*) we have selected 16 nM rolipram and was used throughout this study.

A.2.2.2.4 3-isobuthyl-1-methylxanthine (IBMX)

The non-selective PDE inhibitor, IBMX, is not used therapeutically, so that therapeutic plasma concentrations are not available as for sildenafil and tadalafil. The K_i value of IBMX for PDE5 has been determined as 8.4 µM (Zhao *et al.*, 2003). However we have demonstrated from a concentration series that 8.4 µM exhibit antiproliferative effects, while the lower concentration of 840 nM did not (*see § B.1.2*). We therefore selected 840 nM IBMX throughout this study.

A.2.2.2.5 N^2,2'-O-Dibutyrylguanosine 3'-5'-cyclic monophosphate (db-cGMP)

The cGMP analogue, db-cGMP, is not therapeutically used so that therapeutic plasma concentrations are not available as for sildenafil and tadalafil. Another study indicated that 200 µM is sufficient to reverse the effect of a PKG blocker (Rooney *et al.*, 1996). We therefore used a concentration of 200 µM db-cGMP throughout this study.

A.2.2.2.6 1H-[1,2,4] oxadiazolol [4,3-a] quinoxallin-1-one (ODQ)

The soluble guanylyl cyclase inhibitor, ODQ, is not therapeutically used. Another study indicated that 2 µM is sufficient to block the activity of soluble guanylyl cyclase (Yang *et al.*, 2005). We therefore used a concentration of 2 µM ODQ throughout this study.

A.2.2.2.7 Rp-8-bromob-phenyl-1, N^2-etheno-guanosine 3'5' cyclic monophosphorothioate (Rp-PET-cGMPS)

The PKG antagonist, RP-PET-cGMPS, is not therapeutically used. Another study indicated that 500 nM is sufficient to block the activity of protein kinase G (Schwede *et al.*, 2000). We therefore used a concentration of 500 nM RP-PET-cGMPS throughout this study.

A.2.2.2.8 Lithium

The mood stabilizer, lithium, has a therapeutic peak concentration of 1 mol/l *in vivo* and is 0% bound to plasma protein (i.e. 100% unbound/free), yielding a free concentration of 1 mM. Because of the narrow therapeutic window and toxic effects, we did not test higher concentrations and a concentration of 1 mM was used throughout the study which corresponds to the concentration used in previous studies on SH-SY5Y cells in our laboratory (Potgieter *et al.*, 2008).

A.2.2.2.9 Imipramine

The tricyclic antidepressant, imipramine, has a therapeutic peak concentration of 200 ± 137 ng/ml (Hardman *et al.*, 2001) *in vivo* and is 90.1% bound to plasma protein

(i.e. 9.9% unbound/free), yielding a free concentration of 33.36 ng/ml. Calculated from its molecular weight (MM 316.87), the therapeutic peak free plasma concentration is therefore 105 nM. The therapeutic free plasma concentration of 105 nM imipramine was therefore be used throughout this study.

A.2.2.2.10 Fluoxetine

The selective serotonin reuptake inhibitor, fluoxetine, has a therapeutic peak concentration of 531 ng/ml (Hardman *et al.*, 2001) in vivo and is 94% bound to plasma protein (i.e. 6% unbound/free), yielding a free concentration of 31.86 ng/ml. Calculated from its molecular weight (MM 345.79), the therapeutic peak free plasma concentration is therefore 92 nM. The therapeutic free plasma concentration of 92 nM fluoxetine was therefore be used throughout this study.

A.2.2.2.11 Tianeptine

The atypical antidepressant, tianeptine, has a therapeutic peak concentration of 458 ng/ml (ZINI *et al.*, 1990) in vivo and is 95% bound to plasma protein (i.e. 5% unbound/free), yielding a free concentration of 22.9 ng/ml. Calculated from its molecular weight (MM 458.93), the therapeutic peak free plasma concentration is therefore 50 nM. The therapeutic free plasma concentration of 50 nM tianeptine was therefore used throughout this study.

A.2.2.2.12 Atropine

The prototype anticholinergic drug, atropine, has a K_D value of 1 nM for muscarinic acetylcholine (mACh) receptors. In rat spinal neurons (*in vitro*) a pre-treatment concentration of 10 nM atropine (10x the K_D value) has been shown to be protective (Warnick *et al.*, 1993) and therefore a concentration of 10 nM atropine was used throughout this study.

A.2.3 Assays

A.2.3.1 MTT – assay

A.2.3.1.1 Introduction

The mitochondria provide cells with energy to maintain cellular metabolic function and cellular growth. The classical and standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay is on the ability of the mitochondria in viable cells to cleave the tetrazolium rings of the yellow MTT and produce purple insoluble formazan crystals (Figure A-1). As cell viability diminishes (for example following a stressor), mitochondrial activity also declines. The MTT assay is therefore a non-specific, general measure of cell viability. Importantly, however, this assay may underestimate the mitochondrial damage, because cell death is detected only at late stage apoptosis when cellular metabolic activities are reduced.

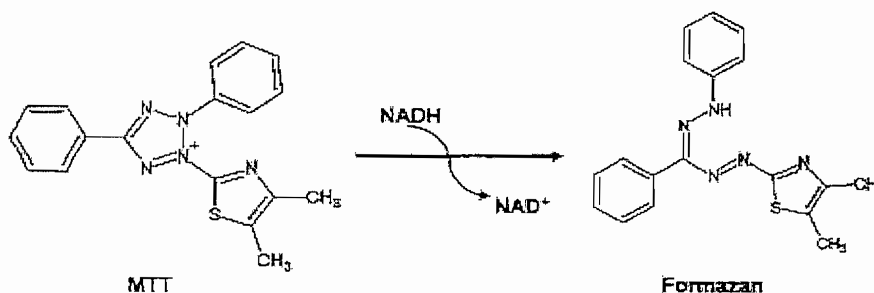


Figure A-1 The conversion of yellow MTT to the purple formazan crystal (Berridge & Tan, 1993).

This assay was implemented in the current study to determine the modulating effect of the stressors (see § A.2.2.1) on cell viability, and of the various test drugs to modulate (e.g. reverse) the effects of the stressors.

A.2.3.1.2 Assay

The SH-SY5Y and CHO-K1 cells were seeded and pre-treated as described in § A.2.1. Additional wells were seeded to allow for negative controls (100% cell viability) and positive controls (0% cell viability). Cells designated as negative

controls were incubated in normal serum-free growth medium, without stressor or drug, while positive controls were incubated with 0.33% formic acid in $_{dd}H_2O$ for 30 minutes to induce 100% cell death. After the 24 hour drug pre-treatments, and following the 30 minute incubation with formic acid for positive controls, the medium was aspirated and the cells rinsed twice with 1 ml phosphate buffer solution (PBS). Thereafter 200 µl of a 0.5% MTT solution in PBS was added to each well and incubated at 37°C in 10% CO_2 for 2 hours. Due to the light sensitivity of the MTT-reagent all steps with MTT were executed in the dark with only minimal light for visibility when necessary. After the 2 hour incubation period the MTT-reagent was aspirated from the wells and 250 µl isopropanol added to each well. The 24-well plates were then incubated at room temperature for 5 minutes to dissolve the purple formazan crystals, whereafter 100 µl of the formazan-containing isopropanol from each well was transferred to a 96-well plate and the absorbance (Abs) read spectrophotometrically at 560 nm. The mitochondrial activity was calculated as a percentage of the negative control by means of the following equation:

$$\text{Cell Viability (\%)} = \frac{\text{Abs}_{\text{(sample)}} - \text{Abs}_{\text{(+control)}}}{\text{Abs}_{\text{(-control)}} - \text{Abs}_{\text{(+control)}}} \times 100$$

Where $\text{Abs}_{\text{(sample)}}$ is the absorbance of the study sample, $\text{Abs}_{\text{(positive control)}}$ is the absorbance of the formic acid (representing 0% cell viability) and $\text{Abs}_{\text{(negative control)}}$ is the absorbance of the untreated cells (representing 100% cell viability).

A.2.3.2 Trypan blue – assay

A.2.3.2.1 Introduction

The Trypan blue assay assesses the permeability of cell membranes to the Trypan blue dye, thereby being a non-specific measure of membrane integrity, or cell viability. However, this assay cannot distinguish cell death due to apoptosis and will also not detect early stages of apoptosis. Trypan blue is a negatively charged dye that does not cross the intact cell membrane of viable cells. However, the cell membranes of non-viable cells become permeable, allowing the dye to enter and

stain the cells. Therefore, viable cells (with intact membranes) appear under the microscope as white, unstained cells, whereas non-viable cells (with damaged membranes) appear as blue, stained cells.

A.2.3.2.2 Assay

The SH-SY5Y and CHO-K1 cells were seeded and pre-treated as described in § A.2.1 and the Trypan blue assay was performed. The medium from each well (from the 24-well plates) was aspirated and 200 µl of trypsin was added to each well and incubated for a further 5 minutes at 37°C in 10% CO_2. Thereafter 800 µl of serum-free medium was added into each well and pipetted up and down to ensure that cell clusters are broken. The contents of each well was transferred to an Eppendorf tube and centrifuged for 10 min at 5000 rpm at 4°C. The supernatant was aspirated and the pellet re-suspended in 1 ml of serum-free medium. The Eppendorf tubes with cells were kept at 4°Con ice. Thereafter 50 µl of 0.4% Trypan blue was added to the cells and 20 µl of the mixture containing the cells were added to an eppendorf tube containing 180 µl of 0.4% Trypan blue (for dilution). The cells were re-suspend by vortexing the Eppendorf tubes. The viable cells were counted using a 0.025 cm^2 haemocytometer with a depth of 0.1 mm and an inverted Nikon TMS microscope. The percentage viable cells were calculated as the number of white cells after drug treatment divided by the number of cells in the serum-free control and is presented in the equation below:

$$\text{Viable cells (\%)} = 100 \times \frac{N_{\text{(white cells after drug treatment)}}}{N_{\text{(white cells of serum free control)}}}$$

where $N_{\text{(white cells of serum free control)}}$ is the number of white (unstained, intact) cells of the serum-free control and $N_{\text{(white cells after drug treatment)}}$ is the number of white cells after drug treatments.

A.2.3.3 Single cell gel electrophoresis (Comet – assay)

A.2.3.3.1 Introduction

The aim of the comet assay was to determine whether oxidative stress can induce DNA damage in neuroblastoma (SH-SY5Y) cells and to determine whether drugs acting on the NO/cGMP pathway as well as antidepressants have an effect on cellular plasticity. This microelectrophoretic technique for measuring DNA damage was first described by Ostling and Johanson in 1984, to visualize the direct effect of DNA damage in individual cells. The DNA damage was named for their appearance as "comets". The experimental procedure causes breakage of large duplex DNA molecules and the electric current causes the DNA to migrate from the original location (comet nucleus) further through the agarose gel (forming a so-called comet tail) (Ostling & Johanson, 1984).

A.2.3.3.1 Assay

The cells for treatment were incubated as discussed in § A.2.1. In preparation of the assay, microscope slides were prepared with a modification of the technique described by Singh (1998) to detect DNA fragmentation (Singh *et al.*, 1998). Slides were covered with a 1.3 mm layer of 1% v/v high melting point agarose (HMPA) in 0.1 M EDTA, by pipetting 350 µl of preheated (80°C) HMPA on the slide and evenly distributing it across the slide with a heated steel scraper and then allowing it to air dry for 15 min. Thereafter 0.5% v/v low melting point agarose (LMPA) was prepared and kept at 37°C until loaded with cells as described below. After 24 hour serum-free incubation, the cells were detached from the well bottoms by trypsinization and ± 500,000 cells transferred to microcentrifuge tubes. The cells were centrifuged at 5500 *g* for 5 min and resuspended in 1 ml PBS. 20 µl of the suspension was transferred to 150 µl LMPA at 37°C and immediately transferred onto the centre of a HMPA pre-coated slide. Similar to the HMPA, the cell-containing LMPA was evenly distributed over half of the slide in a 0.3 mm layer, to yield a monolayer of cells. These slides were placed on moist paper towels to cool down to room temperature and set. Immediately thereafter, the cell-containing slides were lysed in a lysis buffer (2.5 M NaCl, 10 mM Trizma base, 0.1 mM EDTA, 1% Triton-X-100, 10% v/v DMSO, 0.2 M NaOH with pH adjusted to 10) at 4°C, while protected form light. Thereafter slides were rinsed in $_{dd}H_2O$, incubated in the electrophoresis buffer (0.3 M NaOH,

1mM EDTA and pH adjusted to 13) at 4°C for 30 min and then electrophoresed (37 V and 400 mA) at 4°C for 20 min. The slides were rinsed with $_{dd}H_2O$ and incubated in 0.4 M Trizma-HCl buffer for 15 min at 4°C and rinsed again with $_{dd}H_2O$, then further incubated in 10 µM ethidium bromide solution for 15 min at 4°C, then rinsed again with $_{dd}H_2O$. Within 6 hours the image of the resulting DNA comets of 50 cells per treatment condition was captured and the resulting comets analysed using the Comet IV® computer software program. The analyses computed the amount of DNA in the head (intact DNA) with the amount of DNA in the tail (DNA fragment). DNA integrity was expressed as percentage DNA in tail and calculated as follows:

$$\text{DNA in tail (\%)} = 100 \times \frac{F_{(tail)}}{F_{(tail)} + F_{(head)}}$$

Where $F_{(tail)}$ – fluorescence in comet tail, $F_{(head)}$ = fluorescence in comet head.

When DNA repair capacity (measuring cellular resilience) was to be analysed, an additional step was introduced as done by Brink (Brink *et al.*, 2008) just before the cells were suspended in LMPA, where 10 µM H_2O_2 was added to the cell suspension and incubated at 37°C for 40 min whereafter the samples were centrifuged for 5 min at 5500 *g,* and the supernatants removed and the pellet resuspended in 1 ml PBS, centrifuged for another 5 min at 5500 *g*, and resuspended in Ham's F12 growth medium. From the resulting cell suspension 20 µl was immediately mixed with 150 µl LMPA in a microcentrifuge tube and loaded onto the slide (as described above), while the remainder of the cell suspension was incubated for 40 min (allowing DNA repair), whereafter another 20 µl of cell suspension was mixed with LMPA and loaded onto the slide. The assay was thereafter continued as described above.

Results and Discussion

Addendum B

(All abbreviations are listed in Addendum E)

This addendum contains all the experimental data that were not included in the article (Chapter 3). In some instances, where appropriate and necessary, results from Chapter 3 were repeated here to provide a complete picture of the data. This addendum also contains data obtained from the selection of optimal *in vitro* stress conditions for cultured cells, different dose-dependent responses of drugs, as well as responses in both a neuronal (SH-SY5Y) and non-neuronal (CHO-K1) cell line.

As mentioned in Chapter 1, the primary aim of the study was to investigate the role of sildenafil on neuroplasticity in cultured human neuroblastoma (SH-SY5Y) cells. All the results presented here therefore revolve around understanding this phenomenon.

The results gained from experiments performed will be discussed according to the following layout:

- **Pilot study (Phase 1)**
  § B.1.1 (Selection of optimal stress condition)
  § B.1.2 (Selection of optimal sildenafil concentration)

- **Study objectives (Phase 2)**
  § B.2.1 (Results of MTT assay)
  § B.2.2 (Results of Trypan blue assay)

- **Summary**
  § B.3

B.1 Pilot study

The pilot study (phase 1) contains the results that were obtained from experiments that were intended to determine the optimal cellular stress conditions in a neuronal (SH-SY5Y) and a non-neuronal (CHO-K1) cell line. After the stress condition was selected, the cell lines were exposed to the stress condition as well as a concentration series of the PDE5 inhibitor (sildenafil). The obtained results will be discussed below.

B.1.1 Selection of optimal stress condition in neuronal and non-neuronal cell lines

We selected a human neuronal (neuroblastoma SH-SY5Y) and a mammalian non-neuronal (Chinese hamster ovary CHO-K1) cell line to evaluate the effects of the indicated drugs of cellular plasticity. Thereby any observed drug effects could be evaluated for tissue selectivity (neuronal versus non-neuronal) and species selectivity (human versus hamster).

Secondly, a suitable *in vitro* stress condition, to model neuronal stress associated with major depression, had to be investigated, thereby to induce a stress condition under which the drugs under investigation may demonstrate the ability to protect (i.e. improve cellular plasticity). We investigated three *in vitro* stress conditions, namely glutamate-induced excitotoxicity, as well as peroxide (H_2O_2)-induced and serum deprivation-induced oxidative stress. Importantly, we had to determine the optimal dose (concentration/duration) for application of each of these stressors for both the SH-SY5Y and CHO-K1 cell lines for the purpose of the study. An optimal dose would induce roughly 50% reduction in cell viability, so that it would be possible to test for any intervention (such as a drug) to either augment (enhanced reduction in viability) or protect (reversal of reduced viability) against the stressor-induced reduction in cell viability.

Figure B-1 depicts the results obtained from stress conditions representing excitotoxicity and oxidative stress in SH-SY5Y and CHO-K1 cell lines. We used the MTT assay (a non-specific cell viability assay, measuring mitochondrial activity) as screening test to determine the conditions that would induce close to 50% inhibition of cell viability.

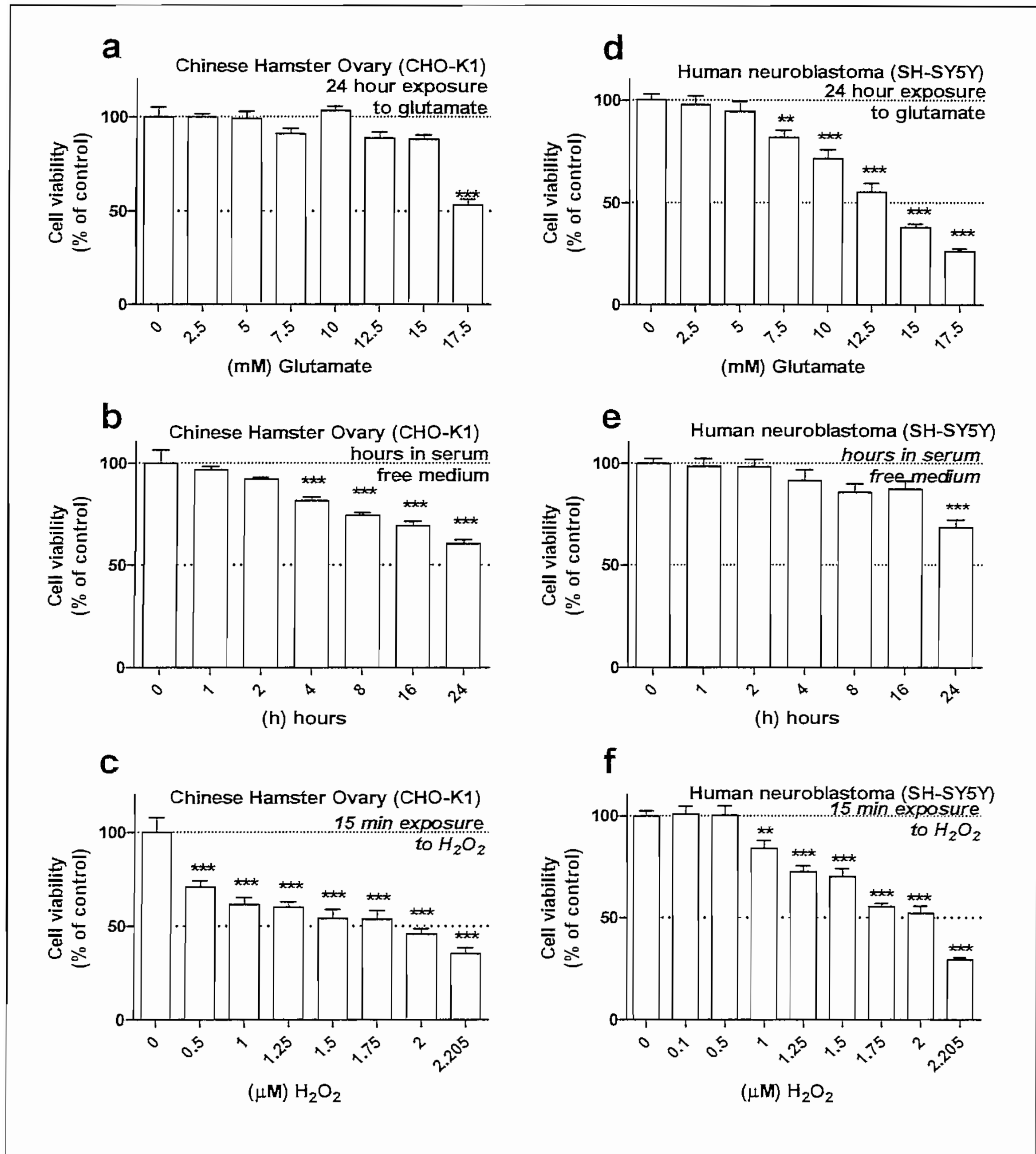


Figure B-1 The effect of excitotoxicity (glutamate exposure) and oxidative stress (presented as serum free and H_2O_2 exposure) in a neuronal (SH-SY5Y) and non-neuronal (CHO-K1) cell line using the MTT assay. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data presented was analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test, with statistical significance indicated as $p < 0.01$ (**) and $p < 0.0001$ (***).

Figure B-1(a-c) depicts the effects of the stressors on the non-neuronal cell line. In **Figure B-1a** it can be seen that a 24 hour exposure to none of the concentrations below 17.5 mM glutamate caused a statistically significant decrease in cell viability, whereas a concentration of 17.5 mM glutamate decreased cell viability to 53% ± 3.% relative to the control. In **Figure B-1b** it can be seen that serum-deprivation caused a time-dependent decrease in cell viability over a 24 hour period, with the cell viability decreasing to 61% ± 2% relative to the control at 24 hours. In **Figure B-1c** it can be seen that a 15 minute exposure to H_2O_2caused a concentration-dependent decrease in cell viability, with 1.75 µM H_2O_2 rendering a decrease in cell viability of 53% ± 4% relative to the control.

In Figure B-1(d-f) the effects of stressors on the neuronal cell line can be seen. In **Figure B-1d** it can be seen that 24 hour exposure to 12.5 mM glutamate caused a significant decrease in cell viability to 55% ± 4% relative to the control. In **Figure B-1e** it can be seen that 24 hour serum deprivation caused a cell viability of 68% ± 3% relative to the control. In **Figure B-1f** it can be seen that a 15 minute exposure to H_2O_2 caused a significant decrease in cell viability with 1.75 µM H_2O_2 rendering a decrease in cell viability of 55% ± 1% relative to the control.

While these results indicate that the non-neuronal and neuronal cell lines displayed similar broad trends with regard to their responses to the different stressors, there were also some subtle differences. For example, the non-neuronal CHO-K1 cell line had a slightly greater resilience towards glutamate exposure than the neuronal cell line (i.e. the CHO-K1 cell line responded to a slightly higher concentration of glutamate). For further experiments with SH-SY5Y cells 12.5 mM glutamate, and for CHO-K1 cells 17.5 mM glutamate for 24 hours was selected as excitotoxic stressor.

Both cell lines represent similar effects to both oxidative stress inducers and a 24 hour serum deprivation or 1.75 µM H_2O_2 for 15 min was sufficient for both cell lines to induce sufficient reduction in cell viability for further experiments.

The next phase of experiments was aimed to establish whether sildenafil would be protective against any of these selected stressors, and also at which concentration.

B.1.2 Selection of stress condition where sildenafil exhibited cytoprotection

In Figure B-2 the neuronal and non-neuronal cells were exposed to a dose range of sildenafil as to indicate which stressor (as selected in § B.1, Figure B-1) and at which concentration sildenafil exhibited cytoprotection.

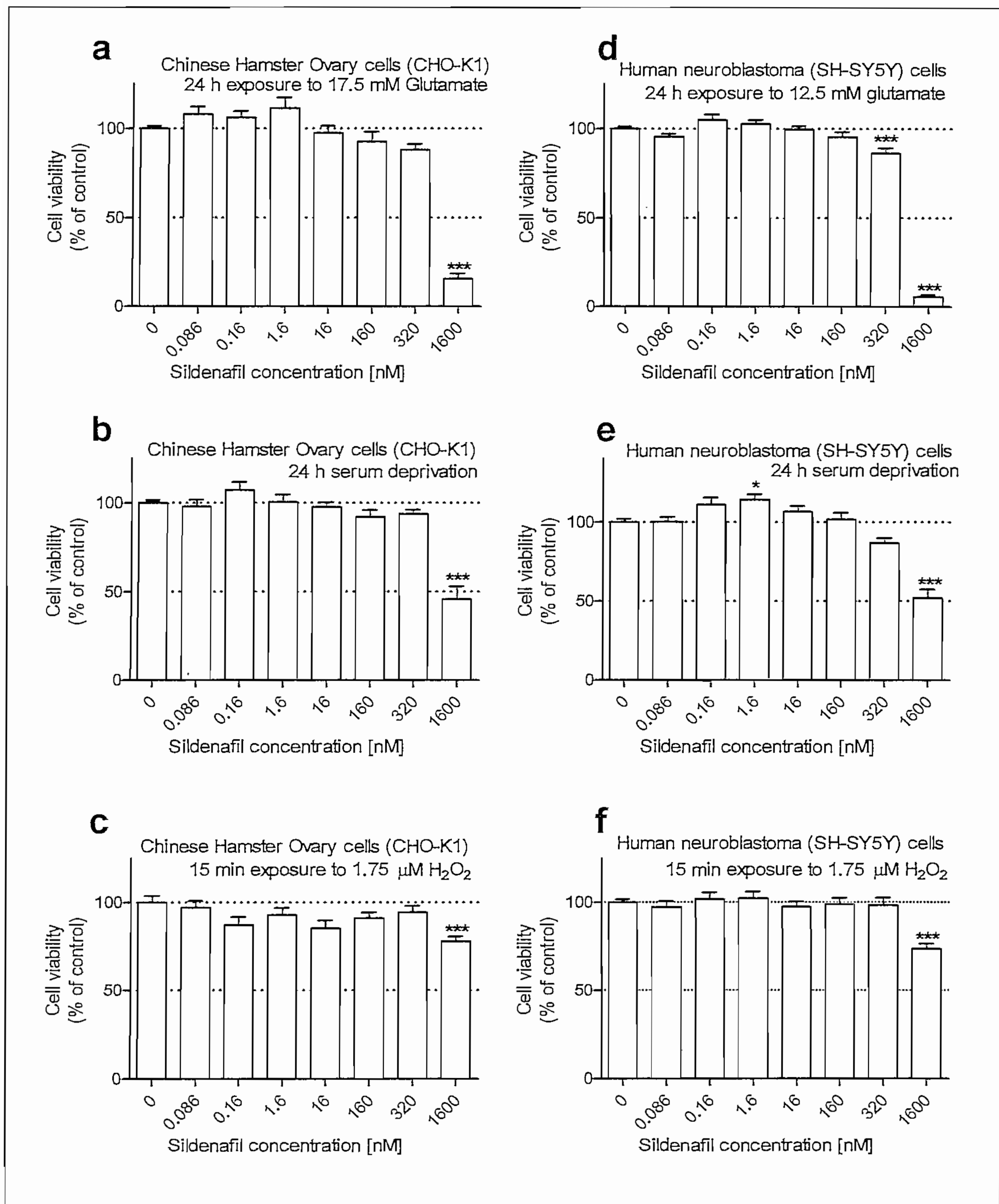


Figure B-2 A sildenafil concentration range (0.086 – 1600 nM) on pre-selected stress conditions using the MTT assay. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test, with statistical significance indicated as $p < 0.01$ (*) and $p < 0.0001$ (***).

Figure B-2 depicts the results obtained from sildenafil pre-treatment on a neuronal and non-neuronal cell line with the stressors selected in Figure B-1 using the MTT assay. As can be seen in **Figure B-2(a-f)** the highest concentration of sildenafil (1600 nM) constantly decreased cell viability regardless of the stressor. Similar cytotoxic effects at high concentrations of sildenafil were also reported by Erdogan in an endothelial cell line (Erdogan et al. 2007). In **Figure B-2e** it can be seen that only 1.6 nM sildenafil in SH-SY5Y cells after 24-hour serum deprivation caused a significant increase in cell viability of 114% ± 3% relative to the control.

No other concentration or stress condition gave similar results, although a trend for a neuroprotective effect was also apparent at 10-times lower concentration of 0.16 nM of sildenafil under the same conditions (see similar trend at 0.16 nM sildenafil also in CHO-K1 cells, Figure B-2b). The absence of statistical significance here was likely due to the lack of statistical power after multiple comparison. No statistically significant cytoprotective effects were observed in CHO-K1 cells. For all further studies we therefore selected 0.16 and 1.6 nM sildenafil in SH-SY5Y cells, with 24 hour serum deprivation as stressor.

B.1.3 Selection of optimal drug concentration of drugs used in the study

The aim of this phase of the study was to determine the optimal drug concentration of drugs acting on the NO/cGMP pathway as well as antidepressant drugs. As mentioned in § A.2.2.2 a drug concentration series was performed using concentrations based on literature findings.

In Figure B-3 the results of drugs acting on the NO/cGMP pathway are presented, and in Figure B-4 the results of antidepressant drugs are presented.

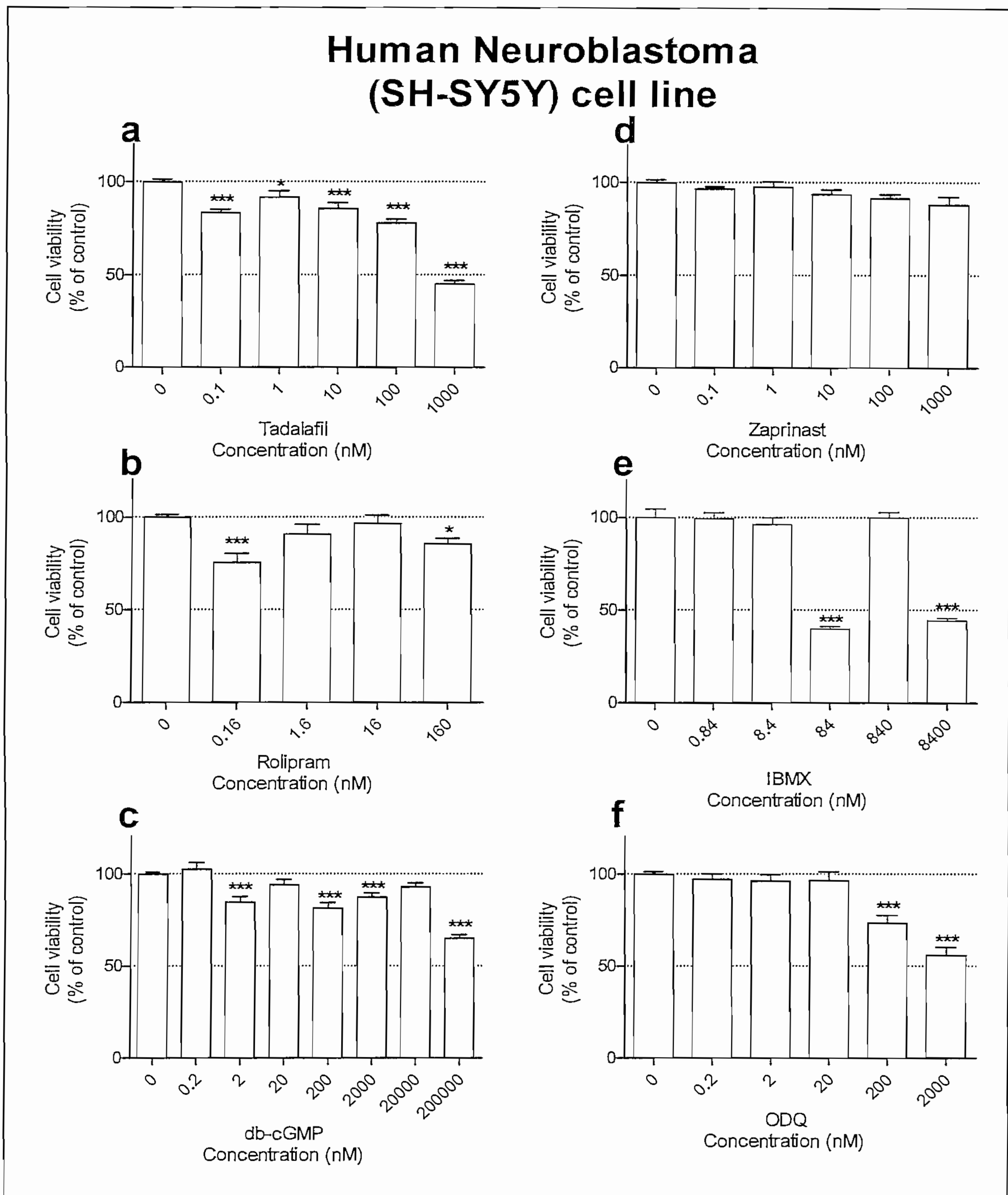


Figure B-3 The effect of 24 hour incubation with the PDE5 inhibitors, tadalafil (0-1000 nM) and zaprinast (0-1000 nM), the PDE4 inhibitor, rolipram (0-160 nM), the non-selective PDE inhibitor, IBMX (0-8400 nM), the cGMP analogue, db-cGMP (0-200000 nM) and the sGC inhibitor, ODQ (0-2000 nM) using the MTT assay. 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 analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test and with statistical significance indicated as $p < 0.01$ (*) and $p < 0.0001$ (***).

Figure B-3 depicts the results obtained from drug pre-treatment (that acts on the NO/cGMP pathway) using the MTT assay. As can be seen in **Figure B-3(a-e)** none of the drug treatments increased cell viability as would be expected from literature. In **Figure B-3f** it can be seen that 2000 nM ODQ exhibited a significant decrease in cell viability of 73% ± 4% relative to the control.

The results obtained from this experiment seem to be indicating that the MTT assay is not the most effective assay in determining the optimal concentration of the drugs acting on the NO/cGMP pathway.

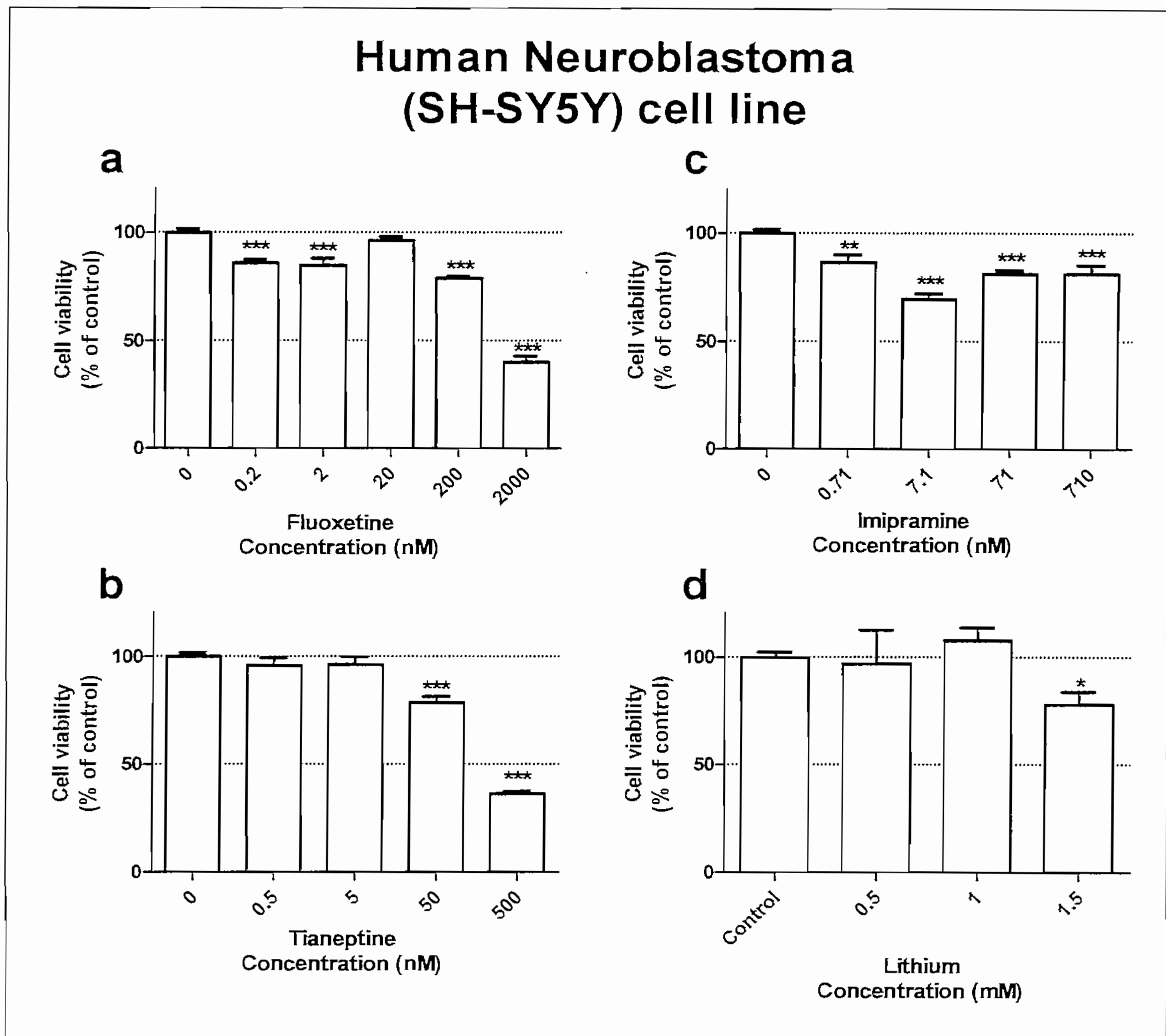


Figure B-4 The effect of 24 hour incubation with the SSRI, fluoxetine (0-2000 nM), the atypical antidepressant, tianeptine (0-500 nM), the TCA, imipramine (0-710 nM) and the mood stabilizer, lithium (0-1.5 mM) using the MTT assay. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments, and mitochondrial activity is expressed as the percentage of control. Data were analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test and with statistical significance indicated as $p < 0.01$ (*) and $p < 0.0001$ (***).

Figure B-4 depicts the results obtained from antidepressant drug pre-treatment using the MTT assay. As can be seen in **Figure B-4(a-d)** none of the drug treatments increased cell viability as would be expected from literature however in **Figure B-4d** there is an increase in cell viability, though no statistically significant neuroprotective effects were observed.

The results obtained indicated that none of these drugs possess neuroprotective properties; however literature indicates this to be the contrary. These results as depicted in Figure B-3 and Figure B-4 might indicate that these drugs do not affect the mitochondrial activity under the selected conditions.

B.2 Study objective experiments

All the experiments contributing to achievement of the study objectives were performed during Phase 2 using the neuronal (SH-SY5Y) cell line. These experiments were aimed at investigating the effects of the selected drugs (mentioned in § A.2.3) on the MTT and Trypan blue cell viability assays as well as the Comet assay measuring DNA damage. The results obtained from the Comet assay is presented in Chapter 3.

B.2.1 MTT assay (Results)

B.2.1.1 Sildenafil vs. other PDE inhibitors and their role on neuroprotection

Figure B-5 depicts the results obtained from sildenafil pre-treatment with other phosphodiesterase (PDE) inhibitors during a 24 hour serum free incubation using the MTT assay. The stressor selected was discussed in data above (§ B.1.2) while the concentrations of all the other were as described in Addendum A (see § A.2.3).

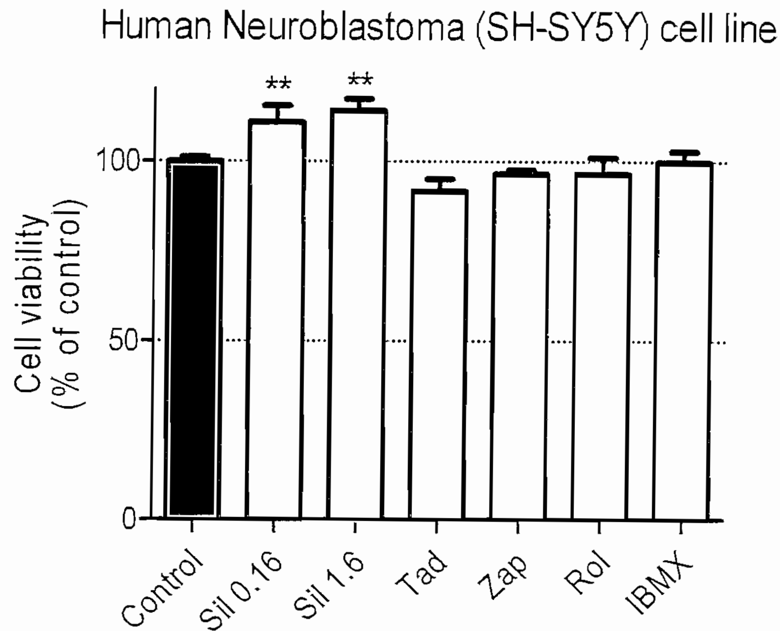


Figure B-5 Measuring the effect of the following drug pre-treatments: the PDE5 inhibitors sildenafil, tadalafil and zaprinast, the PDE4 inhibitor rolipram and the non-selective PDE inhibitor IBMX on cell viability (mitochondrial activity) in neuroblastoma cells during 24 hour serum-free incubation. Data 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 analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test, with statistical significance indicated as $p < 0.01$ (**). Abbreviations: Sil 0.16 & Sil 1.6 = sildenafil 0.16 nM & 1.6 nM 1.6 nM, Tad = tadalafil 97 nM, Zap = Zaprinast 1 nM, Rol = rolipram 16 nM and IBMX = IBMX 840 nM.

Figure B-5 depicts the results obtained from various PDE inhibitors on cell viability using the MTT assay. The PDE5 inhibitor sildenafil significantly protected against the stressor-induced decrease in cell viability, with 0.16 nM resulting in a cell viability of 111% ± 5% and 1.6 nM 114% ± 3% relative to the control. However, no other PDE inhibitor protected against the stressor-induced decrease in cell viability as measured by the MTT assay.

These results suggest that sildenafil may possess a unique protective property at relatively low concentrations. The K_D value of sildenafil for PDE5 has been reported as 3.7 ± 0.29 (Blount et al. 2004), so that the concentrations used here are 2.3 to 23x lower than that expected for PDE5 inhibition.

B.2.1.2 Sildenafil vs. antidepressant drugs and their role on neuroprotection

Most antidepressant drugs are also known to play a role in enhancing the cellular plasticity after chronic treatment (see § 2.1.3). In addition, as discussed in Chapter 2, a recent study (important for the current study), showed that the co-administration of sildenafil and atropine also possesses anti-depressant like activity in rats (Brink et al. 2008). These drugs and combinations were therefore evaluated for neuroprotective properties in SH-SY5Y cells. In figure B-6 depicts the effects on neuroplasticity during simultaneous 24 hour serum deprivation of the combination of sildenafil and atropine and of selected prototypes of classical antidepressants, as compared to sildenafil alone in the MTT assay.

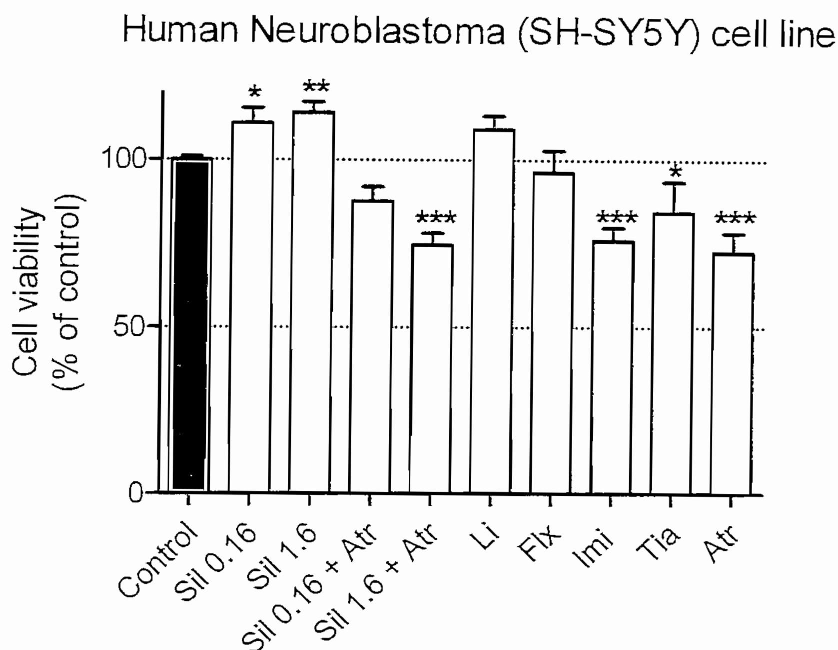


Figure B-6 The effect of 24 hour incubation with the PDE5 inhibitor, sildenafil, the mood stabilizer lithium, the SSRI fluoxetine, the TCA imipramine, the atypical antidepressant tianeptine, the anticholinergic atropine as well as a combination of sildenafil and atropine using the MTT assay during serum-free incubation. 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 analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test, with statistical significance indicated as $p < 0.01$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***). Abbreviations: Sil 0.16 & Sil 1.6 = sildenafil 0.16 nM & 1.6 nM, Sil + Atr = sildenafil + atropine, Li = lithium 1 mM, Flx = fluoxetine, Imi = imipramine, Tia = tianeptine and Atr = atropine 10 nM.

The effects of sildenafil alone were discussed before (see Figure B-5).

However as depicted in Figure B-6, the combination of sildenafil and atropine did not exhibit neuroprotective effects. This was not expected, since this combination has been shown to exert antidepressant-like properties in rats. Atropine therefore seems to abolish the neuroprotective properties of sildenafil as measured by the MTT assay. It should, however, be kept in mind that the MTT assay measured only one parameter of cell viability. It is important to note that the classical antidepressants, fluoxetine, imipramine or tianeptine (that could have served as positive controls), also did not display neuroprotective properties in the MTT and even augmented the stressor-induced reduction in cell viability in some cases. This emphasizes that the

MTT may not be an appropriate *in vitro* test for predicting neuroprotective properties of antidepressants. However, the fact that sildenafil alone still displayed neuroprotective properties in this assay, may point towards a unique property of this drug.

B.2.2 Trypan blue assay (Results)

B.2.2.1 Sildenafil vs. other PDE inhibitors on neuroprotection

The results depicted in Figure B-7 were obtained after the selected drug pre-treatments and 24 hour serum deprivation on a neuronal cell line using the Trypan blue assay. This assay was selected to measure the effects of sildenafil and other drugs acting on the NO/cGMP pathway, as well as known antidepressants since the MTT assay did not seem to justify the current antidepressant drug effects as discussed in data above (§ B.2.1.1).

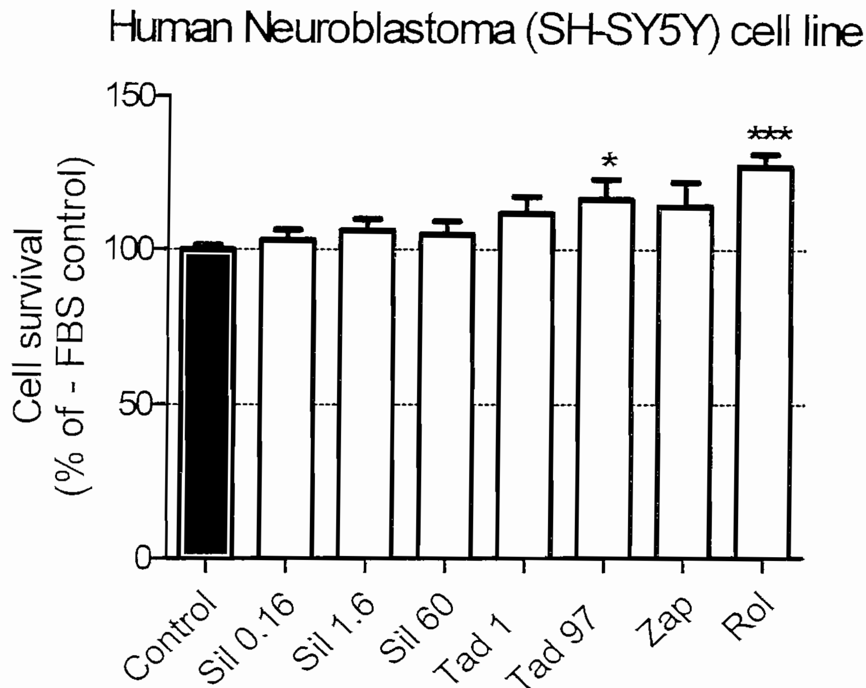


Figure B-7 The comparative effect of PDE5 inhibitors: sildenafil, tadalafil and zaprinast and the PDE4 inhibitor rolipram on membrane permeability after 24 hour serum-free drug pre-treatment incubation using the Trypan blue assay. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments, and cell survival is expressed as the percentage of serum free control. Data were analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test, with statistical significance indicated as $p < 0.01$ (*) and $p < 0.0001$ (***). Abbreviations: Sil = sildenafil 0.16, 1.6 and 60 nM, Tad = tadalafil 1 and 97 nM, Zap = zaprinast 1 nM and Rol = rolipram 16 nM.

In Figure B-7 it can be seen that the PDE5 inhibitor tadalafil (Tad 97) significantly increased the cell survival to 116% ± 6% relative to the control and no other PDE5 inhibitor exhibited a similar effect. The PDE4 inhibitor, rolipram had a significant increase in cell survival to 127% ± 4% relative to the control when using the Trypan blue assay.

These results suggest that the PDE5 inhibitors have a trend in neuroprotection however the results are inconsistent when compared to results obtained in Figure B-5.

B.2.2.2 Sildenafil vs. antidepressants and their role in neuroprotection

As discussed in Addendum B (§ B.4) known antidepressants play a role in cellular plasticity and the effect of sildenafil and the combination of sildenafil and atropine were compared to that of known antidepressants using the Trypan blue assay. The results obtained are presented in figure B-6.

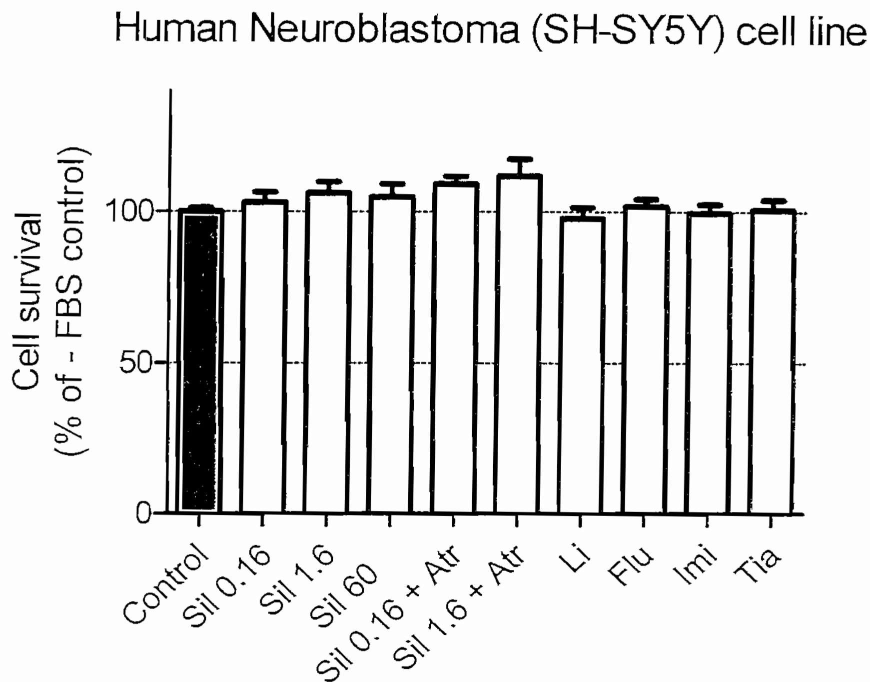
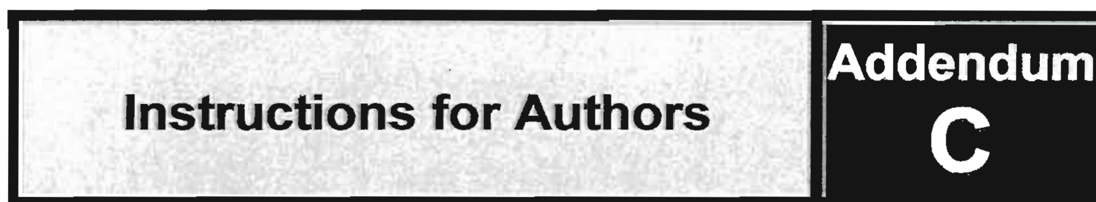


Figure B-8 The effect of 24 hour incubation with the PDE5 inhibitor, sildenafil, the mood stabilizer lithium, the SSRI fluoxetine, the TCA imipramine, the atypical antidepressant tianeptine, the anticholinergic atropine as well as a combination of sildenafil and atropine using the Trypan blue assay during serum-free incubation. Data are averages ± S.E.M of triplicate observations from three independent and comparable experiments, cell survival is expressed as the percentage of serum free control. Data were analyzed statistically by means of a one-way ANOVA and the Dunnett's Multiple-Comparison post-test and no statistical significance were found. Abbreviations: Sil 0.16 & Sil 1.6 = sildenafil 0.16 nM & 1.6 nM, Sil + Atr = sildenafil + atropine, Li = lithium 1 mM, Flx = fluoxetine, Imi = imipramine, Tia = tianeptine and Atr = atropine 10 nM.

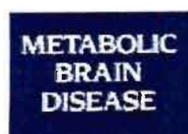
In Figure B-8 it can be seen that no drug or combinations of drugs exhibited a significant increase on cell survival when using the Trypan blue assay. These results indicate that the Trypan blue assay may not be the most effective assay to determine cellular plasticity.

B.3 Summary

The results obtained from the experiments indicate that sildenafil exhibited neuroprotective properties at low concentrations (0.16 and 1.6 nM respectively) and that only the mood stabilizer lithium (1 mM) has exhibited comparable neuroprotective effects in the MTT assay. In the Trypan blue assay only rolipram which is not used therapeutically (at 16 nM, equal to its Ki value for PDE4 inhibition) and tadalafil (97 nM) at a therapeutically relevant concentration exhibited neuroprotective effects. These results suggest that the neuroprotective properties exhibited by different PDE5 inhibitors may involve different sub-cellular mechanisms.



The inclusion of the "*Instructions for Authors*" is to indicate the guidelines given by the specific journal to which the article in *Chapter 3* will be submitted to. The *Instructions for Authors* pertaining to the Metabolic Brain Disease Journal can be found at: (http://www.springer.com/biomed/neuroscience/journal).



Manuscript Submission

Manuscripts, in triplicate and in English, and books for review should be submitted to the Editor-in-Chief:

Dr. David W. McCandless
Department of Cell Biology and Anatomy
Finch University of Health Sciences/
The Chicago Medical School
3333 Green Bay Road
North Chicago, Illinois 60064

Or, please email your manuscript to the Managing Editor, Vilmary Friederichs, at vilmary.friederichs@rosalindfranklin.edu.

Publication Policies

Submission is a representation that the manuscript has not been published previously and is not currently under consideration for publication elsewhere. A statement transferring copyright from the authors (or their employers, if they hold the copyright) to Springer Science + Business Media, LLC will be required before the manuscript can be accepted for publication. The Editor–in–Chief will supply the necessary forms for this transfer. Such a written transfer of copyright, which previously was assumed to be implicit in the act of submitting a manuscript, is necessary under the U.S. Copyright Law in order for the publisher to carry through the dissemination of research results and reviews as widely and effectively as possible.

Manuscript Style

Type double spaced, and submit the original and two copies (including copies of all illustrations and tables) plus a copy on a disk.

Order the elements comprising the manuscript as follows:

- Title page
- Abstract
- Key words
- Introduction
- Materials and methods
- Results
- Discussion
- Conclusions
- Appendix
- Acknowledgements
- References
- Tables
- Figure caption list
- And figures

Short communications should comprise a maximum of 6 (journal) pages including references and are limited to 2 tables or figures, and should carry no text headings or subheadings. Abbreviations should be those recommended in the Council of Biology Editors Style Manual. Generic names should be used when referring to drugs.

Title Page

A title page is to be provided and should include

- The title of the article
- Author's name (no degree)
- Author's affiliation
- And suggested running head

The affiliation should comprise

- the department
- institution (usually university or company)
- city
- and state (or nation)

and should be typed as a footnote to the author's name. The suggested running head should be less than 80 characters (including spaces) and should comprise the article title or an abbreviated version thereof.

For office purposes, the title page should include the complete mailing address and telephone number of the one author designated to review proofs.

Abstract

An abstract, preferably not more than 200 words (120 for short communication), is to be provided as the second page.

Key Words

A list of 4–6 key words is to be provided directly below the abstract. Key words should express the precise content of the manuscript, as they are used for indexing purposes.

Acknowledgements

All acknowledgements (including those for grants and financial support) should be typed in one paragraph on a separate page that directly precedes the reference section.

References

List references alphabetically at the end of the paper and refer to them by name and year in parenthesis. Where there are three or more authors, only the first author's name is given in the text, followed by et al.

References should include (in this order):

- last names and initials of all authors
- year published
- title of article
- name of publication
- volume number
- and inclusive pages

Abbreviations for journal names should conform to those in Index Medicus. The style and punctuation of the references should conform to that used in the journal–illustrated by the following examples:

Journal Article

- Sipertein, M.D., Unger, R.H., and Madison, L.L. (1968). Studies of muscle capillary basement membranes in normal subjects, diabetic and pre-diabetic patients. J. Clin. Invest. 47:1973–1999.

Book

- Himwich, H.E. (1951). Brain Metabolism and Cerebral Disorders, Williams and Wilkins, Baltimore.

Contribution to a Book

- O'Neil, J.J. and Holtzman, D. (1985). Heavy metal toxicity and energy metabolism in the developing brain: Lead as the Model. In (D.W. McCandless, ed.), Cerebral Energy Metabolism and Metabolic Encephalopathy, Plenum Press, New York, pp. 391–424.

Footnotes

Footnotes should be avoided. When their use is absolutely necessary, footnotes should be numbered consecutively using Arabic numerals and should be typed at the bottom of the page to which they refer. Place a line above the footnotes, so that they are set off from the text. Use the appropriate superscript numeral for citation in the text

Illustration Style

Illustrations

Illustrations (photographs, drawings, diagrams, and charts) are to be numbered in one consecutive series of Arabic numerals. The captions for illustrations should be typed on a separate sheet of paper.

Photographs should be large, glossy prints, showing high contrast. Drawings should be prepared with India ink. Either original drawings or good quality photographic prints are acceptable.

Identify figures on the back with the author's name and number of the illustration

Tables

Tables should be numbered (preferably with Roman numerals) and referred to by number in the text. Each table should be typed on a separate sheet of paper.

Submission of Accepted Manuscripts

After a manuscript has been accepted for publication and after all revisions have been incorporated, manuscripts should be submitted to the Editor's office as a hardcopy accompanied by electronic files on disk. Label the disk with identifying information – software, journal name, and first author's last name. The disk must be the one from which the accompanying manuscript (finalized version) was printed out. The Editor's Office cannot accept a disk without its accompanying, matching hardcopy manuscript.

Page Charges

The journal makes no page charges. Reprints are available to authors, and order forms with the current price schedule are sent by the Editor in Chief with proofs.

Congress contribution

Addendum D

In this addendum the study abstract that was presented at the congress of the South African Basic and Clinical Pharmacology Society (2009, Potchefstroom, South Africa) Young Scientist Category, is shown.

The effects of phosphodiesterase type 5 inhibitors on cellular plasticity in a neuronal and non-neuronal cell line

Riaan Reay and Christiaan B. Brink

Division of Pharmacology, School of Pharmacy, North-West University, Potchefstroom 2520, South Africa. Tiaan.Brink@nwu.ac.za

Background:

Depression is the most debilitating psychiatric disorder of our time, whilst clinically used drug treatments are plagued with troublesome side-effects, delayed onset of action and treatment resistance. In addition, a comprehensive understanding of the biological basis of depression and its treatment is remains elusive, prompting extensive ongoing research. While the neuroplasticity hypothesis of depression has gained recent support from various lines of experimental and clinical evidence, the nitric-oxide (NO)/cGMP pathway is believed to play an important role in dysregulated neuroplasticity in depression and in its reversal and enhanced synaptogenesis by antidepressants. Recent studied in our laboratory demonstrated antidepressant-like effects of the phosphodiesterase type 5 (PDE5) inhibitors sildenafil and tadalafil when combined with the antimuscarinic drug atropine in rats, while unpublished data also suggest that sildenafil may upregulate genes encoding for the expression of anti-apoptotic proteins *in vitro*. These data warranted the current study to investigate the effects of PDE5 inhibitors and other modulators of the NO/cGMP pathway on neuroplasticity.

Methods:

Human neuroblastoma (SH-SY5Y) and Chinese hamster ovary (CHO-K1) cell lines were maintained in culture medium at 37°C, 10% CO_2 and saturated humidity. Cells were subjected to stress (modelling neuronal stress associated with major depression) by inducing excitotoxicity (glutamate) or oxidative stress (serum deprivation or H_2O_2), and the condition (concentration and duration of exposure) that induces a 50% reduction in mitochondrial activity, as measured by the standard MTT cell viability assay, was determined for each of these stressors. Thereafter, implementing these optimal stress conditions, the effects of the PDE5 inhibitors sildenafil, tadalafil and zaprinast, rolipram (PDE4 inhibitor), 3-isobutyl-1-methylxanthine (nonselective PDE inhibitor), imipramine (tricyclic antidepressant), fluoxetine (selective serotonin reuptake inhibitor) and tianeptine (neuroprotective antidepressant), ODQ (selective inhibitor of soluble guanylyl cyclase), db-cGMP (cGMP analogue), sildenafil + atropine, as well as sildenafil + ODQ were determined on neuroplasticity, measuring mitochondrial activity (MTT).

Results:

Optimal stress conditions (50% reduction in mitochondrial activity) in both neuroblastoma SH-SY5Y and CHO-K1 cells were demonstrated to be either 24-hour serum-deprivation, 15 minutes incubation with 1.75 μM H_2O_2, or a 24-hour incubation with 12.5 mM glutamate (for SH-SY5Y) and 17.5 mM glutamate (for CHO-K1), respectively. From a concentration series, only 0.16 nM sildenafil significantly improved mitochondrial activity (MTT) after 24-hour serum deprivation, while all other concentrations, drugs and in all other stress conditions there was no neuroprotective effect.

Conclusions:

Sildenafil protects against neurodegenerative effects of serum deprivation in neuronal cells at relatively low concentrations (about 10-fold below its K_D value for PDE5), while this effect was not observed in non-neuronal cells. Neuroprotection may play a role in the antidepressant-like activity by sildenafil, as observed in rats in earlier studies.

Abbreviations

Addendum E

Numerals

5'-cAMP	5'-cyclic adenosine monophosphate
5'-cGMP	5'-cyclic guanylyl monophosphate
5-HT	Serotonin
$5\text{-}HT_{1A}$	Serotonin-1A-receptor
$5\text{-}HT_{2}$	Serotonin-2-receptor
$5\text{-}HT_{2C}$	Serotonin-2C-receptor

A

Abs	Absorbance
AC	Adenyl cyclase
ACTH	Adrenocorticotrophin hormone
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate
ATF	Activating Transcription Factor

B

BDNF	Brain derived neurotrophic factor
Bcl-2	B-cell lymphoma/leukemia-2 gene family
Bax	Bcl-2 associated protein x

C

Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
CHO-K1	Chinese Hamster Ovary cells
CNS	Central Nervous System
CRE	cAMP Response Element
CREB	cAMP Response Element Binding protein
CREM	cAMP Response Element Modulator
CRH	Corticotrophin Releasing Hormone
Cu/Zn-SOD	Copper/Zinc- superoxide dismutase

D

DA	Dopamine
DALYs	Disability-adjusted life years
db-cGMP	Dibutryl-cyclic guanosine monophosphate
$_{dd}H_2O$	Double distilled water
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and statistical manual for mental disorders - IV

E

ED	Erectile dysfunction
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial NOS

F

FBS	Foetal bovine serum

G

GC	Guanylate cyclase
GMP	Guanosine monophosphate
GSH	Glutathione peroxidase
GTP	Guanosine triphosphate

H

HPA	Hypothalamic-pituitary-adrenocortical

I

IBMX	3-Isobutyl-1-methylxanthine
ICER	Inducible cAMP element repressor
IDO	Indolamine
Iκβ	Inhibitory kappa beta
Iκβα	Inhibitory kappa beta alpha
iNOS	inducible NOS

L

L-Arg	L-Arginine
l-NE	Norepinephrine
LTP	Long-term potentiation

M

mAChR	Muscarinic acetylcholine receptor

MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MAOIs	Monoamine oxidase inhibitor
MDD	Major depressive disorder
mGluR	Metabotropic glutamate receptor
mGluRs	Metabotropic glutamate receptors
Mn-SOD	Manganese- superoxide dismutase
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

N

NARIs	Norepinephrine re-uptake inhibitors
NF-κβ	Nuclear factor kappa beta
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal NOS

O

O_2^-	Superoxide
ODQ	1H-[1,2,4] oxadiazolol [4,3-a] quinoxallin-1-one
•OH	Hydroxyl free radical
$ONOO^-$	Peroxynitrate

P

PBS	Phosphate buffer solution
PDE	Phosphodiesterase
PDE4	Phosphodiesterase 4
PDE5	Phosphodiesterase 5

PDEs	Phosphodiesterases
PET	Positron Emission Tomography
PKA	Protein kinase A
PKG	Protein kinase G

R

ROS	Reactive oxygen species
RP-PET-cGMPS	Rp-8-bromob-phenyl-1, N^2-etheno-guanosine 3'5' cyclic monophosphorothioate
rt-PCR	Real-time polymerase chain reaction

S

sGC	Soluble guanylate cyclase
SH-SY5Y	Human neuroblastoma cells
SOD	Superoxide dismutase
SSRIs	Selective serotonin reuptake inhibitors

T

TCA	Tricyclic antidepressant
Trk	Tyrosine kinase protein family
TrkB	Tyrosine kinase protein B

Y

YLD	Years of life lived with disability

Addendum F

Addendum F

AHMED, T. & FREY, J.U. 2005. Plasticity-specific phosphorylation of Ca-MKII, MAP-kinases and CREB during late-LTP in rat hippocampal slices *in vitro*. *Neuropharmacology,* 49:477-492.

ALTAR, C.A. 1999. Neurotrophins and depression. *Trends in Pharmacological Sciences,* 20:59-62.

AMERICAN PSYCHIATRIC ASSOCIATION. 2000. Diagnostic and statistical manual of mental disorders. 4th ed. Washington, DC: American Psychiatric Publishing Inc.

ANDREEVA, S.G., DIKKES, P., EPSTEIN, P.M. & ROSENBERG, P.A. 2001. Expression of cGMP-specific phosphodiesterase 9A mRNA in the rat brain. *The Journal of Neuroscience,* 21:9068-9076.

ANDREOPOULOS, S. & PAPAPETROPOULOS, A. 2000. Molecular aspects of soluble guanylyl cyclase regulation. *General Pharmacology,* 34:147-157.

AYUSO-MATEOS, J.L., VAZQUEEZ-BARQUERO, J.L., DOWRICK, C., LEHTINEN, V., DALGARD, O.S., CASEY, P., WILKINSON, C., LASA, L., PAGE, H., DUNN, G. & WILKINSON, G. 2001. Depressive disorders in Europe: Prevalence figures from the ODIN study. *The British Journal of Psychiatry,* 179:308-316.

BALU, D.T., HOSHAW, B.A., MALBERG, J.E., ROSENZWEIG-LIPSON, S., SCHECHTER, L.E. & LUCKI, I. 2008. Differential regulation of central BDNF protein levels by antidepressant and non-antidepressant drug treatments. *Brain Research,* 1211:37-43.

BARLOW, D.H. & DURAND, V.M. 2002. Mood disorders. (In Taflinger, M. & Strandberg, J., eds. Abnormal psychology. 3rd ed. CA: Wadsworth. p. 190-235.).

BATTISTI, C., FORMICHI, P., RADI, E. & FEDERICO, A. 2008. Oxidative-stress-induced apoptosis in PBLs of two patients with Parkinson disease secondary to alpha-synuclein mutation. *Journal of the Neurological Sciences,* 267:120-124.

BELMAKER, R.H. & AGAM, G. 2008. Major depressive disorder. *New England Journal of Medicine,* 358:55-68.

BENDER, A.T. & BEAVO, J.A. 2006. Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use. *Pharmacological Reviews,* 58:488-520.

BERMAN, R.M., CAPPIELLO, A., ANAND, A., OREN, D.A., HENINGER, G.R., CHARNEY, D.S. & KRYSTAL, J.H. 2000. Antidepressant effects of ketamine in depressed patients. *Biological Psychiatry,* 47:351-354.

BLENDY, J.A. 2006. The role of CREB in depression and antidepressant treatment. *Biological Psychiatry,* 59:1144-1150.

BLIER, P. 2003. The pharmacology of putative early-onset antidepressant strategies. *European Neuropsychopharmacology,* 13:57-66.

BLOKLAND, A., SCHREIBER, R. & PRICKAERTS, J. 2006. Improving memory: A role for phosphodiesterases. *Current Pharmaceutical Design,* 12:2511-2523.

BOESS, F.G., HENDRIX, M., VAN DER STAAY, F., ERB, C., SCHREIBER, R., VAN STAVEREN, W., DE VENTE, J., PRICKAERTS, J., BLOKLAND, A. & KOENIG, G. 2004. Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. *Neuropharmacology,* 47:1081-1092.

BOSWELL-SMITH, V., SPINA, D. & PAGE, C.P. 2006. Phosphodiesterase inhibitors. *British Journal of Pharmacology,* 147:S252-S257.

BREMNER, J.D., VYTHILINGAM, M., VERMETTEN, E., NAZEER, A., ADIL, J., KHAN, S., STAIB, L.H. & CHARNEY, D.S. 2002. Reduced volume of orbitofrontal cortex in major depression. *Biological Psychiatry,* 51:273-279.

BRINK, C.B., CLAPTON, J.D., EAGAR, B.E. & HARVEY, B.H. 2008. Appearance of antidepressant-like effect by sildenafil in rats after central muscarinic receptor blockade: Evidence from behavioural and neuro-receptor studies. *Journal of Neural Transmission,* 115:117-125.

BRINK, C.B., HARVEY, B.H. & BRAND, L. 2006. Tianeptine: A novel atypical antidepressant that may provide new insights into the bio-molecular basis of depression. *Recent Patents on CNS Drug Discovery,* 1:29-41.

BRINK, C.B., VILJOEN, S.L., DE KOCK, S.E., STEIN, D.J. & HARVEY, B.H. 2004. Effects of *myo*-inositol versus fluoxetine and imipramine pre-treatments on serotonin $5HT_{2A}$ and muscarinic acetylcholine receptors in human neuroblastoma cells. *Metabolic Brain Disease,* 19:51-70.

CARLSON, P.J., SINGH, J.B., ZARATE, J.,CARLOS A., DREVETS, W.C. & MANJI, H.K. 2006. Neural circuitry and neuroplasticity in mood disorders: Insights for novel therapeutic targets. *Neurotherapeutics,* 3:22-41.

CARROLL, B.J. & RUBIN, R.T. 2008. Mifepristone in psychotic depression? *Biological Psychiatry,* 63:e1-e1.

CHAN, P.H. 1996. Role of antioxidants in ischemic brain damage. *Stroke,* 27:1124-1129.

CHEN, A.C.-., SHIRAYAMA, Y., SHIN, K., NEVE, R.L. & DUMAN, R.S. 2001. Expression of the cAMP response element binding protein (CREB) in hippocampus produces an antidepressant effect. *Biological Psychiatry,* 49:753-762.

CONSTANTINE-PATON, M. & CLINE, H.T. 1998. LTP and activity-dependent synaptogenesis: The more alike they are, the more different they become. *Current Opinion in Neurobiology,* 8:139-148.

COPPEN, A. 1967. The biochemistry of affective disorders. *The British Journal of Psychiatry,* 113:1237-1264.

CORBIN, J.D. & FRANCIS, S.H. 1999. Cyclic GMP phosphodiesterase-5: Target of sildenafil. *The Journal of Biological Chemistry,* 274:13729-13732.

CORBIN, J.D., FRANCIS, S.H. & OSTERLOH, I.H. 2000. Effects of sildenafil on cAMP and cGMP levels in isolated human cavernous and cardiac tissue. *Urology,* 56:545-545.

CULL-CANDY, S., KELLY, L. & FARRANT, M. 2006. Regulation of Ca^{2+}-permeable AMPA receptors: Synaptic plasticity and beyond. *Current Opinion in Neurobiology,* 16:288-297.

DANBOLT, N.C. 2001. Glutamate uptake. *Progression in Neurobiology,* 65:1-105.

DAWS, L.C. & OVERSTREET, D.H. 1999. Ontogeny of muscarinic cholinergic supersensitivity in the flinders sensitive line rat. *Pharmacology Biochemistry and Behavior,* 62:367-380.

DEBATTISTA, C. & BELANOFF, J. 2006. The use of mifepristone in the treatment of neuropsychiatric disorders. *Trends in Endocrinology & Metabolism,* 17:117-121.

DENNINGER, J.W. & MARLETTA, M.A. 1999. Guanylate cyclase and the ·NO/cGMP signaling pathway. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1411:334-350.

DEVAN, B.D., SIERRA-MERCADO, J., DEMETRIO, JIMENEZ, M., BOWKER, J.L., DUFFY, K.B., SPANGLER, E.L. & INGRAM, D.K. 2004. Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats. *Pharmacology Biochemistry and Behavior,* 79:691-699.

DILSAVER, S.C. 1986. Cholinergic mechanisms in depression. *Brain Research Review,* 11:285-316.

DOWLATSHAHI, D., MACQUEEN, G.M., WANG, J.F. & YOUNG, L.T. 1998. Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. *The Lancet,* 352:1754-1755.

DREVETS, W.C., FRANK, E., PRICE, J.C., KUPFER, D.J., HOLT, D., GREER, P.J., HUANG, Y., GAUTIER, C. & MATHIS, C. 1999. PET imaging of serotonin 1A receptor binding in depression. *Biological Psychiatry,* 46:1375-1387.

DRZYZGA, Ł.R., MARCINOWSKA, A. & OBUCHOWICZ, E. 2009. Anti-apoptotic and neurotrophic effects of antidepressants: A review of clinical and experimental studies. *Brain Research Bulletin,* 79:248-257.

DU BOIS, T.M. & HUANG, X. 2007. Early brain development disruption from NMDA receptor hypofunction: Relevance to schizophrenia. *Brain Research Review,* 53:260-270.

DUMAN, R.S. 2002. Pathophysiology of depression: The concept of synaptic plasticity. *European Psychiatry,* 17:306-310.

DUMAN, R.S., MALBERG, J. & THOME, J. 1999. Neural plasticity to stress and antidepressant treatment. *Biological Psychiatry,* 46:1181-1191.

DUMAN, R.S. & MONTEGGIA, L.M. 2006. A neurotrophic model for stress-related mood disorders. *Biological Psychiatry,* 59:1116-1127.

ESPLUGUES, J.V. 2002. NO as a signalling molecule in the nervous system. *British Journal of Pharmacology,* 1079-1095.

FAVA, M. & PAPAKOSTAS, G.I. 2008. Monoaminergic-based pharmacotherapy for depression. *Biology of Depression,* 1:87-209.

FAVA, M. 2003. Diagnosis and definition of treatment-resistant depression. *Biological Psychiatry,* 53:649-659.

FUCHS, E., CZÉH, B., KOLE, M.H.P., MICHAELIS, T. & LUCASSEN, P.J. 2004. Alterations of neuroplasticity in depression: The hippocampus and beyond. *European Neuropsychopharmacology,* 14:S481-S490.

FURCHGOTT, R.F. 1996. The discovery of endothelium-derived relaxing factor and its importance in the identification of nitric oxide. *Journal of the American Medical Association,* 276:1186-1188.

FUREY, M.L. & DREVETS, W.C. 2006. Antidepressant efficacy of the antimuscarinic drug scopolamine: A randomized, placebo-controlled clinical trial. *Archives of General Psychiatry,* 63:1121-1129.

FURGESON, J. 2001. SSRI antidepressant medications adverse effects and tolerability. *The Primary Care Companion,* 3:22-27.

GACKOWSKI, D., ROZALSKI, R., SIOMEK, A., DZIAMAN, T., NICPON, K., KLIMARCZYK, M., ARASZKIEWICZ, A. & OLINSKI, R. 2008. Oxidative stress and oxidative DNA damage is characteristic for mixed Alzheimer disease/vascular dementia. *Journal of the Neurological Sciences,* 266:57-62.

GARTHWAITE, J. & BOULTON, C.L. 1995. Nitric oxide signaling in the central nervous system. *Annual Review of Physiology,* 57:683-706.

GARTHWAITE, J., CHARLES, S.L. & CHESS-WILLIAMS, R. 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature,* 385-388.

GILGUN-SHERKI, Y., MELAMED, E. & OFFEN, D. 2001. Oxidative stress induced-neurodegenerative diseases: The need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology,* 40:959-975.

GONZALEZ, M., RUGGIERO, F.P., CHANG, Q., SHI, Y.J., RICH, M.M. & KRANER, S. 1999. Disruption of TrkB-mediated signaling induces disassembly of postsynaptic receptor clusters at neuromuscular junctions. *Neuron,* 24:567-583.

GREENE, J.G. & GREENAMYRE, J.T. 1996. Bioenergetics and glutamate excitotoxicity. *Progression in Neurobiology,* 48:613-634.

HALLIWELL, B. & GUTTERIDGE, J.M.C. 1992. Biologically relevant metal ion-dependent hydroxyl radical generation an update. *FEBS Letters,* 307:108-112.

HARVEY, B.H. 1997. The neurobiology and pharmacology of depression. A comparative overview of serotonin selective antidepressants. *South African Medical Journal,* 87:540-550.

HARVEY, B.H., CARSTENS, M. & TALJAARD, T. 1994. Evidence that lithium induces a glutamatergic: Nitric oxide-mediated response in rat brain. *Neurochemistry,* 19:469-474.

HARVEY, B.H., CARSTENS, M. & TALJAARD, T. 1990. Lithium modulation of cortical cyclic nucleotides: Evidence for the yin-yang hypothesis. *European Journal of Pharmacology,* 175:129-136.

HARVEY, B.H. 2006. Adaptive plasticity during stress and depression and the role of glutamate-nitric-oxide pathways. *South African Psychiatry Review,* 9:132-139.

HEBB, A.L. & ROBERTSON, H.A. 2007. Role of phosphodiesterases in neurological and psychiatric disease. *Current Opinion in Pharmacology,* 7:86-92.

HERMES-LIMA, M., STOREY, J.M. & STOREY, K.B. 1998. Antioxidant defences and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 120:437-448.

JANOWSKY, D.S., OVERSTREET, D.H. & NURNBERGER, J.I. 1994. Is cholinergic sensitivity a genetic marker for the affective disorders? *American Journal of Medical Genetics,* 54:335-344.

JANOWSKY, D., DAVIS, J., EL-YOUSEF, M.K. & SEKERKE, H.J. 1972. A cholinergic-adrenergic Hypothesis of Mania and Depression. *The Lancet,* 300:632-635.

KAPCZINSKI, F., VIETA, E., ANDREAZZA, A.C., FREY, B.N., GOMES, F.A., TRAMONTINA, J., KAUER-SANT'ANNA, M., GRASSI-OLIVEIRA, R. & POST, R.M. 2008. Allostatic load in bipolar disorder: Implications for pathophysiology and treatment. *Neuroscience & Bio-behavioral Reviews,* 32:675-692.

KASPER, S., MOISES, H. & BECKMANN, H. 1982. The anticholinergic biperiden in depressive disorders. *Journal of Clinical Psychopharmacology,* 2:295.

KASPER, S., BALDWIN, D.S., LARSSON LÖNN, S. & BOULENGER, J. 2009. Superiority of escitalopram to paroxetine in the treatment of depression. *European Neuropsychopharmacology,* 19:229-237.

KATO, T. 2008. Molecular neurobiology of bipolar disorder: A disease of 'mood-stabilizing neurons'? *Trends Neuroscience,* 31:495-503.

KATZUNG, B.G., TREVOR, A.J. & MASTERS, S.B. 2002. Antidepressants. (In Foltin, J., Lebowitz, H. & Boyle, P.B., eds. Katzung & Trevor's pharmacology: Examination &Board review. sixth ed. New-York: McGraw-Hill Medical Publishing Division. p. 269-278.).

KOLE, M.H.P., SWAN, L. & FUCHS, E. 2002. The antidepressant tianeptine persistently modulates glutamate receptor currents of the hippocampal CA3 commissural associational synapse in chronically stressed rats. *European Journal of Neuroscience,* 16:807-816.

KOLLER, G., BONDY, B., PREUSS, U.W., ZILL, P. & SOYKA, M. 2006. The C(-1019)G 5-HT1A promoter polymorphism and personality traits: No evidence for significant association in alcoholic patients. *Behavioral and Brain Functions,* 2:7.

KRONENBERG, G., TEBARTZ VAN ELST, L., REGEN, F., DEUSCHLE, M., HEUSER, I. & COLLA, M. 2009. Reduced amygdala volume in newly admitted psychiatric in-patients with unipolar major depression. *Journal of Psychiatric Research,* 43:1112-1117.

LAI, T., PAYNE, M.E., BYRUM, C.E., STEFFENS, D.C. & KRISHNAN, K.R.R. 2000. Reduction of orbital frontal cortex volume in geriatric depression. *Biological Psychiatry,* 48:971-975.

LEADER, J.B. & KLEIN, D.N. 1996. Social adjustment in dysthymia, double depression and episodic major depression. *Journal of Affective Disorders,* 37:91-101.

LEE, H.J., KIM, J.W., YIM, S.V., KIM, M.J., KIM, S.A., KIM, Y.J., KIM, C.J. & & CHUNG, J.H. 2001. Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. *Molecular Psychiatry,* 6:725-728.

LEHTO, S.M., TOLMUNEN, T., KUIKKA, J., VALKONEN-KORHONEN, M., JOENSUU, M., SAARINEN, P.I., VANNINEN, R., AHOLA, P., TIIHONEN, J. & LEHTONEN, J. 2008. Midbrain serotonin and striatum dopamine transporter binding in double depression: A one-year follow-up study. *Neuroscience Letters,* 441:291-295.

LEONARD, B.E. 1997. Noradrenaline in basic models of depression. *European Neuropsychopharmacology,* 7:S11-S16.

LEONARD, B.E. 2001. The immune system, depression and the action of antidepressants. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 25:767-780.

LONZE, B.E. & GINTY, D.D. 2002. Function and regulation of CREB family transcription factors in the nervous system. *Neuron,* 35:605-623.

LUCASSEN, P.J., FUCHS, E. & CZÉH, B. 2004. Antidepressant treatment with tianeptine reduces apoptosis in the hippocampal dentate gyrus and temporal cortex. *Biological Psychiatry,* 55:789-796.

MANJI, H.K. & DUMAN, R.S. 2001. Impairments of neuroplasticity and cellular resilience in severe mood disorders: Implications for the development of novel therapeutics. *Psychopharmacology Bulletin,* 35:5-49.

MANJI, H.K., QUIROZ, J.A., SPORN, J., PAYNE, J.L., DENICOFF, K., A. GRAY, N., ZARATE, C.A. & CHARNEY, D.S. 2003. Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biological Psychiatry,* 53:707-742.

MATHERS, C., STEIN, C. and SHIBUJA, K., 2003. *The Global Burden of Disease: A response to the need for comprehensive, consistent and comparable global information on diseases and injuries.* www.who.int: World Health Organization.

MCEWEN, B.S. & OLIE, J.P. 2005. Neurobiology of mood, anxiety, and emotions as revealed by studies of unique antidepressant: Tianeptine. *Molecular Psychiatry,* 10:525-537.

MICHEL, T.M., FRANGOU, S., THIEMEYER, D., CAMARA, S., JECEL, J., NARA, K., BRUNKLAUS, A., ZOECHLING, R. & RIEDERER, P. 2007. Evidence for oxidative stress in the frontal cortex in patients with recurrent depressive disorder—a postmortem study. *Psychiatry Research,* 151:145-150.

MILLER, A.H. & MANJI, H.K. 2006. On redefining the role of the immune system in psychiatric disease. *Biological Psychiatry,* 60:796-798.

MITANI, H., SHIRAYAMA, Y., YAMADA, T., MAEDA, K., ASHBY, J.,CHARLES R. & KAWAHARA, R. 2006. Correlation between plasma levels of glutamate, alanine and

serine with severity of depression. *Progression in Neuro-Psychopharmacology,* 30:1155-1158.

MOERK, K.C. & KLEIN, D.N. 2000. The development of major depressive episodes during the course of dysthymic and episodic major depressive disorders: A retrospective examination of life events. *Journal of Affective Disorders,* 58:117-123.

MONTELEONE, P., CATAPANO, F., TORTORELLA, A., DI MARTINO, S. & MAJ, M. 1995. Plasma melatonin and cortisol circadian patterns in patients with obsessive-compulsive disorder before and after fluoxetine treatment. *Psychoneuroendocrinology,* 20:763-770.

MORYL, E., DANYSZ, W. & QUACK, G. 1993. Potential antidepressive of amantadine, memantine and bifemelane. *Pharmacology and Toxicology,* 72:394-397.

MURESANU, D.F. 2007. Neuroprotection and neuroplasticity – A holistic approach and future perspectives. *Journal of Neurological Sciences,* 257:38-43.

MYINT, A.M. & KIM, Y.K. 2003. Cytokine–serotonin interaction through IDO: A neurodegeneration hypothesis of depression. *Medical Hypotheses,* 61:519-525.

MYINT, A., KIM, Y.K., VERKERK, R., SCHARPÉ, S., STEINBUSCH, H. & LEONARD, B. 2007. Kynurenine pathway in major depression: Evidence of impaired neuroprotection. *Journal of Affective Disorders,* 98:143-151.

NAIR, A. & VAIDYA, V.A. 2006. Cyclic AMP response element binding protein and brain-derived neurotrophic factor: Molecules that modulate our mood? *Journal of Bioscience,* 31:423-434.

NAKAMIZO, T., KAWAMATA, J., YOSHIDA, K., KANWAI, Y., KANKI, R., SAWADA, H., KIHARA, T., YAMASHITA, H., SHIBASAKI, H., AKAIKE, A. & SHIMOHAMA, S. 2003. Phosphodiesterase inhibitors are neuroprotective to cultured spinal motor neurons. *Journal of Neuroscience Research,* 71:485-495.

NESTLER, E.J., BARROT, M., DILEONE, R.J., EISCH, A.J., GOLD, S.J. & MONTEGGIA, L.M. 2002. Neurobiology of depression. *Neuron,* 34:13-25.

NGUYEN, P.V. & WOO, N.H. 2003. Regulation of hippocampal synaptic plasticity by cyclic AMP-dependent protein kinases. *Progression in Neurobiology,* 71:401-437.

NIBUYA, M., NESTLER, E.J. & DUMAN, R.S. 1996. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *Journal of Neuroscience,* 16:2365-2372.

PARKS, C.L., ROBINSON, P.S., SIBILLE, E., SHENK, T. & TOTH, M. 1998. Increased anxiety of mice lacking the serotonine-1A receptor. *Proceedings of the National Academy of Sciences,* 1,95:10734-10739.

PILZ, R.B. & CASTEEL, D.E. 2003. Regulation of gene expression by cyclic GMP. *Circulation Research,* 93:1034-1046.

PITTENGER, C. & DUMAN, R.S. 2008. Stress, depression, and neuroplasticity: A convergence of mechanisms. *Neuropsychopharmacology,* 33:88-109.

PRAST, H. & PHILIPPU, A. 2001. Nitric oxide as modulator of neuronal function. Progression in Neurobiology, 64:51-68.

PRICKAERTS, J., VAN STAVEREN, W.C.G., SIK, A., MARKERINK-VAN ITTERSUM, M., NIEWÖHNER, U., VAN DER STAAY, F.J., BLOKLAND, A. & DE VENTE, J. 2002. Effects of two selective phosphodiesterase type 5 inhibitors, sildenafil and vardenafil, on object recognition memory and hippocampal cyclic GMP levels in the rat. *Neuroscience,* 113:351-361.

PUZZO, D., SAPIENZA, S., ARANCIO, O. & PALMERI, A. 2008. Role of phosphodiesterase 5 in synaptic plasticity and memory. *Neuropsychiatric Disease Treatment,* 4:371-387.

RAMBOZ, S., OOSTING, R., AMARA, D.A., KUNG, H.F., BLIER, P., MENDELSOHN, M., MANN, J.J., BRUNNER, D. & HEN, R. 1998. Serotonin receptor 1A knockout: An animal model of anxiety-related disorder. *Proceedings of the National Academy of Sciencesm,* 95:14476-14481.

RAO, V.R. & FINKBEINER, S. 2007. NMDA and AMPA receptors: Old channels, new tricks. *Trends in Neuroscience,* 30:284-291.

REAGAN, L.P., HENDRY, R.M., REZNIKOV, L.R., PIROLI, G.G., WOOD, G.E., MCEWEN, B.S. & GRILLO, C.A. 2007. Tianeptine increases brain-derived neurotrophic factor expression in the rat amygdala. *European Journal of Pharmacology,* 565:68-75.

REIERSON, G.W., MASTRONARDI, C.A., LICINIO, J. & WONG, M. 2009. Repeated antidepressant therapy increases cyclic GMP signaling in rat hippocampus. *Neuroscience Letters,* In Press, Uncorrected Proof:.

REZAK, M. 2007. Current pharmacotherapeutic treatment options in Parkinson's disease. *Disease-a-Month,* 53:214-222.

RHEBERGEN, D., BEEKMAN, A.T., GRAAF, R.D., NOLEN, W.A., SPIJKER, J., HOOGENDIJK, W.J. & PENNINX, B.W. 2009. The three-year naturalistic course of major depressive disorder, dysthymic disorder and double depression. *Journal of Affective Disorders,* 115:450-459.

ROSENKRANZ, J.A., MOORE, H. & GRACE, A.A. 2003. The prefrontal cortex regulates lateral amygdala neuronal plasticity and responses to previously conditioned stimuli. *The Journal of Neuroscience,* 23:11054-11064.

RUBIN, L.L. & STADDON, J.M. 1999. THE CELL BIOLOGY OF THE BLOOD-BRAIN BARRIER. *Annual Review of Neuroscience,* 22:11-28.

RUDORFER, M. & POTTER, W. 1999. Metabolism of tricyclic antidepressants. *Cellular and Molecular Neurobiology,* 19:373-409.

SALA, G., BERETTA, S., CERESA, C., MATTAVELLI, L., ZOIA, C., TREMOLIZZO, L., FERRI, A., CARRÌ, M.T. & FERRARESE, C. 2005. Impairment of glutamate transport and increased vulnerability to oxidative stress in neuroblastoma SH-SY5Y cells expressing a Cu/Zn superoxide dismutase typical of familial amyotrophic lateral sclerosis. *Neurochemistry International,* 46:227-234.

SAN, L. & ARRANZ, B. 2008. Agomelatine: A novel mechanism of antidepressant action involving the melatonergic and the serotonergic system. *European Psychiatry,* 23:396-402.

SANACORA, G., ZARATE JR., C.A., KRYSTAL, C. & MANJI, H.K. 2008. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nature Review Drug Discoveries,* 7:426-437.

SAPOLSKY, R.M. 2000. The possibility of neurotoxicity in the hippocampus in major depression: A primer on neuron death. *Biological Psychiatry,* 48:755-765.

SARANDOL, A., SARANDOL, E., EKER, S.S., ERDINC, S., VATANSEVER, E. & KIRLI, S. 2007. Major depressive disorder is accompanied with oxidative stress: Short-term antidepressant treatment does not alter oxidative-antioxidative systems. *Human Psychopharmacology: Clinical and Experimental,* 22:67-73.

SARGENT, P., NASH, J., HOOD, S., RABINER, E., MESSA, C., COWEN, P., NUTT, D. & GRASBY, P. 2000. 5-HT1A receptor binding in panic disorder; comparison with depressive disorder and healthy volunteers using PET and [11C]WAY-100635. *Neuroimage,* 11:S189-S189.

SCHILDKRAUT, J. 1995. The catecholamine hypothesis of affective disorders: A review of supporting evidence. 1965 [classical article]. *Journal of Neuropsychiatry and Clinical Neuroscience,* 7:524-533.

SCHWEDE, F., MARONDE, E., GENIESER, H. & JASTORFF, B. 2000. Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacology and Therapeutics,* 87:199-226.

SHELINE, Y.I., BARCH, D.M., DONNELLY, J.M., OLLINGER, J.M., SNYDER, A.Z. & MINTUN, M.A. 2001. Increased amygdala response to masked emotional faces in

depressed subjects resolves with antidepressant treatment: An fMRI study. *Biological Psychiatry,* 50:651-658.

SHIRAYAMA, Y., CHEN, A.C.-., NAKAGAWA, S., RUSSELL, D.S. & DUMAN, R.S. 2002. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. *The Journal of Neuroscience,* 22:3251-3261.

SKOLNICK, P. 1999. Antidepressants for the new millennium. *European Journal of Pharmacology,* 375:31-40.

SONNENBURG, W.K., SEGER, D., KWAK, K.S., HAUNG, J. & CHARBONNEAU, H. 1995. Identification of inhibitory and calmodulin-binding domains of the PDE1A1 and PDE1A2 calmodulin-stimulated cyclic nucleotide phosphodiesterases. *Journal of Biological Chemistry,* 270:30989-31000.

SOUTH AFRICAN DEPRESSION AND ANXIETY GROUP. 2009. Suicide treatment and referral guide. http://www.sadag.co.za/index.php/Mood-Disorders/Suicide-Treatment-and-Referral-Guide.html.

STOCKMEIER, C.A., MAHAJAN, G.J., KONICK, L.C., OVERHOLSER, J.C., JURJUS, G.J., MELTZER, H.Y., UYLINGS, H.B.M., FRIEDMAN, L. & RAJKOWSKA, G. 2004. Cellular changes in the postmortem hippocampus in major depression. *Biological Psychiatry,* 56:640-650.

STOKES, P.E. & HOLTZ, A. 1997. Fluoxetine tenth anniversary update: The progress continues. *Clinical Therapeutics,* 19:1135-1250.

SWAAB, D.F., BAO, A. & LUCASSEN, P.J. 2005. The stress system in the human brain in depression and neurodegeneration. *Ageing Research Reviews,* 4:141-194.

SZABÓ, C. 1996. Physiological and pathophysiological roles of nitric oxide in the central nervous system. *Brain Research Bulletin,* 41.:131-141.

UTHAYATHAS, S.K., S.S. TAMER, S.I. PARAMESHWARAN, K. DEGIM, T. SUPPIRAMANIAM, V. & DHANASEKARAN, M. 2007. Versatile effects of sildenafil: Recent pharmacological applications. *Pharmacological Reports,* 59:150-163.

UZBAY, T.I. 2008. Tianeptine: Potential influences on neuroplasticity and novel pharmacological effects. *Progression in Neuro-Psychopharmacology,* 32:915-924.

VAN STAVEREN, W.C.G., MARKERINK-VAN ITTERSUM, M., STEINBUSCH, H.W.M., BEHRENDS, S. & DE VENTE, J. 2005. Localization and characterization of cGMP-immunoreactive structures in rat brain slices after NO-dependent and NO-independent stimulation of soluble guanylyl cyclase. *Brain Research,* 1036:77-89.

WONG, M., WHELAN, F., DELOUKAS, P., WHITTAKER, P., DELGADO, M., CANTOR, R.M., MCCANN, S.M. & LICINIO, J. 2006. Phosphodiesterase genes are associated with susceptibility to major depression and antidepressant treatment response. *Proceedings of the National Academy of Sciences,* 103:15124-15129.

YAMADA, M. & YASUHARA, H. 2004. Clinical pharmacology of MAO inhibitors: Safety and future. *Neurotoxicology,* 25:215-221.

YAMADA, S., YAMAMOTO, M., OZAWA, H., RIEDERER, P. & SAITO, T. 2003. Reduced phosphorylation of cyclic AMP-responsive element binding protein in the postmortem orbitofrontal cortex of patients with major depressive disorder. *Journal of Neural Transmission,* 110:671-680.

YOUNG, A.H. 2005. Metyrapone is an effective adjuvant treatment for major depression. *Evidence Based Mental Health,* 8:72.

ZHANG, R., WANG, Y., ZHANG, L., ZHANG, Z., TSANG, W., LU, M., ZHANG, L. & CHOPP, M. 2002. Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke,* 33:2675-2680.