THE MODULATING EFFECT OF SILDENAFIL ON CELL VIABILITY AND ON THE FUNCTION OF SELECTED PHARMACOLOGICAL RECEPTORS IN CELL CULTURES

B.E. EAGAR (B.Pharm)

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

Supervisor: Prof. C.B. Brink
Co-Supervisor: Prof. B.H. Harvey

2004
Potchefstroom
"For everything comes from God alone. Everything lives by His power, and everything is for His glory." – Romans 11:36

Living Bible
THE NEUROPROTECTIVE PROPERTIES OF SILDENAFIL AND ITS MODULATING EFFECTS ON MUSCARINIC ACETYLCHOLINE RECEPTOR FUNCTION

Since sildenafil's (Viagra®), a phosphodiesterase type 5 (PDE5) inhibitor, approval for the treatment of male erectile dysfunction (MED) in the United States early 1998, 274 adverse event reports were filed by the Food and Drug Administration (FDA) between 4 Jan. 1998 and 21 Feb. 2001 with sildenafil as the primary suspect of various neurological disturbances, including amnesia and aggressive behaviour (Milman and Arnold, 2002). These and other research findings have prompted investigations into the possible central effects of sildenafil.

The G protein-coupled muscarinic acetylcholine receptors (mAChRs) and serotonergic receptors (5HT-Rs), have been linked to antidepressant action (Brink et al. 2004). GPCRs signal through the phosphatidylinositol signal transduction pathway known to activate protein kinases (PKs). Since the nitric oxide (NO)-guanylyl cyclase signal transduction pathway is also known to involve the activation of PKs (via cyclic guanosine monophosphate (cGMP)), the scope is opened for sildenafil to possibly modulate the action of antidepressants by elevating cGMP levels.

It is generally assumed that excitotoxic delayed cell death is pathologically linked to an increase in the release of excitatory neurotransmitters e.g. glutamate. Glutamate antagonists, especially those that block the define NMDA-receptors, are neuroprotective, showing the importance of the NMDA-NO-cGMP pathway in neuroprotection (Brandt et al., 2003). Sildenafil may play a role in neuroprotection by elevating cGMP levels.

**Aims:** The aims of the study were to investigate any neuroprotective properties of sildenafil, as well as modulating effects of sildenafil pre-treatment on mAChR function.
Methods: Human neuroblastoma SH-SY5Y or human epithelial HeLa cells were seeded in 24-well plates and pre-treated for 24 hours in serum-free medium with no drug (control), PDE5 inhibitors sildenafil (100nM and 450 nM), dipiridamole (20 μM) or zaprinast (20 μM), non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX - 1mM), cGMP analogue N²,2'-O-dibutyrylguanosine 3',5'-cyclic monophosphate sodium salt (500 μM), guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ - 3 μM) or sildenafil + ODQ (450 nM and 3 μM respectively). Thereafter cells were used to determine mAChR function by constructing dose-response curves of methacholine or to determine cell viability utilising the Trypan blue, propidium iodide and MTT tests for cell viability.

Results: Sildenafil pre-treatments induced a 2.5-fold increase in the $E_{\text{max}}$ value of methacholine in neuronal cells but did not show a significant increase in epithelial cells. The Trypan blue test suggests that neither the PDE5 inhibitors nor a cGMP analogue show any neuroprotection. Rather, sildenafil 450 nM, dipiridamole and IBMX displayed a neurodegenerative effect. The MTT test was not suitable, since pre-treatment with the abovementioned drugs inhibited the formation of formazan. The propidium iodide assay could also not be used, due to severe cell loss.

Conclusion: Sildenafil up-regulates mAChR function in SH-SY5Y cells and displays a neurodegenerative, and not a protective property, in neuronal cells. This is not likely to be associated with its PDE5 inhibitory action, but may possibly be linked to an increase in cGMP levels via the NO-cGMP pathway.

Keywords: Sildenafil, anxiety disorders, neuroprotection, cell viability, phosphodiesterase-5, cGMP, nitric oxide, muscarinic acetylcholine receptor, cholinergic
DIE NEUROBESKERMENDE EIENSKAPPE VAN SILDENAFIL EN SY MODULERENDE EFFEkte OP MUSKARINIESE ASETIELCHOLIEN RESEPTOR FUNKSIE

Vandat sildenafil in die vroeë 1998 in die Verenigde State van Amerika vir die behandeling van manlike erektiele disfunksie goedgekeur is, is 274 verslae by die Voedsel en Geneesmiddel Administrasie ("FDA") tussen 4 Januarie en 21 Februarie 2001 aangemeld met sildenafil onder verdinking as primêre oorsaak vir verskeie neurologiese afwykings, insluitende geheueverlies en aggressiewe gedrag (Milman en Arnold, 2002). Verskeie navorsingsbevindinge, insluitend die laasgenoemde, het verdere ondersoeke na moontlike sentrale effekte van sildenafil aangespoor.

G-protein gekoppelde reseptore (GPGRe), byvoorbeeld muskariniese asetielcholien-reseptore (mAChRe) en serotenergiese reseptore (5HT-Re) is daarvoor bekend dat hulle betrokke is by die werkingsmeganisme van sekere anti-depressante (Brink et al., 2004). Hierdie GPGRe syn deur die fosfaatinositol-seintransduksieweg wat bekend is om protein kinases (PKs) te aktiveer. Aangesien die stikstof-guanilielsiklase-transduksieweg ook die aktivering van PKs insluit (via sikliese guanosientrifosfaat (sGMP)), is die moontlikheid geskep dat sildenafil die werking van antidepressante kan moduleer deur sGMP-vlakke te verhoog.

Dit word algemeen aanvaar dat eksitotoksies-vertraagde seldood patologies verbind kan word aan die verhoging van eksitatoriese neurotransmitters, bv. glutamaat. Glutamaat antagoniste, veral die wat NMDA-reseptore blokkeer, is neurobeskermend, wat die belangrike rol wat die NMDA-NO-sGMP weg in neurobeskerming speel aantoene (Brandt et al., 2003). Sildenafil kan 'n moontlike rol in neurobeskerming speel deur sGMP-vlakke te verhoog.
Doelwitte: Die doelwitte van die studie was om moontlike neurobeskermende eienskappe van sildenafil te ondersoek, sowel as enige moontlike modulerende eienskappe van sildenafil-voorbehandeling op mAChR-funksie.

Metodes: Menslike neuroblastoomselle (SH-SY5Y) of menslike epiteelselle (HeLa) is in 24-pu plate gesaai en vir 24 uur in serumvrye medium behandel met geen geneesmiddel (kontrole), sildenafil (100 nM en 450 nM), dipiridamole (20 μM), zaprinast (20 μM), 3-isobutyl-1-metielxantien (IBMX; 1 mM), N²,2'-O-dibutirielguanosine 3',5'-cyclic monofosfaat natrium sout (500 μM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ - 3 μM) of sildenafil + ODQ (450 nM en 3 μM). Dosis-responskurwes van metacholien is na die behandeling saamgestel om mAChR funksie te bepaal. Sel-lewensvatbaarheid is deur middel van die Trypan blou, propidium jodied en MTT toetse vir sel-lewensvatbaarheid bepaal.

Resultate: Sildenafil-behandeling het 'n verhoging in Eₘₐₓₖ van ongeveer 2.5 keer die waarde in neuronale SH-SY5Y selle teweeg gebring, maar geen statistiesvolle verandering in epiteel selle getoon nie. Die Trypan blou-toets het aangetoon dat fosfodiesterase type-5-inhibeerders, sowel as 'n sGMP-analoog geen neurobeskermende effek vertoon nie. Die MTT-toets was egter ongeskik vir die behandeling, aangesien die bovengenoemde geneesmiddels die vorming van formazaan geïnhibeer het. Die propidiumjodie-toets was ook ongeskik, as gevolg van 'n oormaat selverlies.

Gevolgtrekking: Sildenafil reguleer mAChR funksie in SH-SY5Y selle op en toon 'n neurodegeneratiewe effek, en nie 'n neuroprotektiewe effek nie, in neuronale selle. Hierdie kan waarskynlik nie met inhibisie van PDE5 geassosieer word nie.

Sleutelwoorde: Sildenafil, angsversteurings, neurobeskerming, sel-lewensvatbaarheid, fosfodiesterase-5, sGMP, stikstofoksied, muskariniëse asetielcholien-reseptor, cholinergiese
Above all I would like to thank the Lord Almighty for giving me the opportunity, ability, strength, courage and wisdom to accomplish this milestone in my life.

To my Mom and Dad, thank you for giving me this opportunity, there have been good times and there have been some pretty bad ones too, but we did it!

To Caroline, remember, life is your oyster and you my shining pearl.

Pastor Collins and Abraham van der Merwe and for being there when I couldn’t.

The MRC for funding this project and my study leaders for their guidance during this project.

To Jacques Petzer and Liezl Fourie for grammatical revision.

With special thanks to all the Lab-personnel for their assistance:
Maureen Steyn,
Sharlene Nieuwoudt
Francois Viljoen

And last but not least, everyone who had a kind and gentle word in these tough times (all my friends, Dennis, Renchê, Jacques, Tanya, Marina, Ramona, Marie, Sumê, Francois, Johan, Minja, Liezl and Dorê), without your support it would have been a grueling and almost impossible task.

The one thing in life I will carry with me is all the beautiful memories we made together, and the hard lessons we had to learn!
# Table of Contents

Abstract........................................................................................................... i
Opsiomming................................................................................................. iii
Acknowledgements....................................................................................... v
Table of Contents......................................................................................... vi
List of Figures................................................................................................. xi
List of Tables................................................................................................ xiii

Chapter 1 Introduction.................................................................................. 1

1.1 Problem Statement.................................................................................. 1
1.2 Study Objectives..................................................................................... 2
1.3 Study Layout............................................................................................ 3

Chapter 2 Literature Review......................................................................... 5

2.1 Clinical and Basic Pharmacology of Sildenafil................................. 5
  2.1.1 Indications for Use............................................................... 5
  2.1.2 Mechanism of Action......................................................... 6
  2.1.3 Adverse Effects & Drug Interactions................................. 7

2.2 Sildenafil and the NO-cGMP Signal Transduction Pathway.......... 8
  2.2.1 The NO-cGMP Pathway....................................................... 8
    2.2.1.1 Excitatory Amino-Acid Receptor............................... 10
      2.2.1.1.1 N-methyl-D-aspartate (NMDA)......................... 10
      2.2.1.1.2 α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)........................................ 11
      2.2.1.1.3 Kainate.............................................................. 11
      2.2.1.1.4 Orphan Glutamate Receptors......................... 12
  2.2.2 Nitric Oxide (NO)................................................................. 12
    2.2.2.1 NO Synthesis (NOS).................................................. 13
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.2.2 Nitric Oxide as Modulator of Neuronal Function</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2.3 Effects of NO on Neuronal Function</td>
<td>13</td>
</tr>
<tr>
<td>2.2.3 Soluble Guanylyl Cyclase (sGC)</td>
<td>14</td>
</tr>
<tr>
<td>2.2.4 Cyclic Guanosine Monophosphate (cGMP)</td>
<td>15</td>
</tr>
<tr>
<td>2.2.4.1 cGMP Signalling</td>
<td>15</td>
</tr>
<tr>
<td>2.2.4.2 cGMP Kinase I and Vasorelaxation</td>
<td>16</td>
</tr>
<tr>
<td>2.2.5 Phosphodiesterase (PDE)</td>
<td>16</td>
</tr>
<tr>
<td>2.2.5.1 Phosphodiesterase Activity</td>
<td>17</td>
</tr>
<tr>
<td>2.2.6 Large Conductance Ca(^{2+}) Activated Channels (BK(_{Ca}))</td>
<td>18</td>
</tr>
<tr>
<td>2.2.6.1 Two Alternative Hypothesis on How Protein Kinases Interact with BK(_{Ca})</td>
<td>20</td>
</tr>
<tr>
<td>2.2.6.1.1 Direct Interaction</td>
<td>20</td>
</tr>
<tr>
<td>2.2.6.1.2 Indirect Interaction</td>
<td>20</td>
</tr>
<tr>
<td>2.3 Introduction to Anxiety-Related Disorders</td>
<td>21</td>
</tr>
<tr>
<td>2.3.1 Depression</td>
<td>22</td>
</tr>
<tr>
<td>2.3.1.1 Clinical Presentation of Depression</td>
<td>22</td>
</tr>
<tr>
<td>2.3.1.2 The Pathophysiology of Major Depressive Episode</td>
<td>24</td>
</tr>
<tr>
<td>2.3.1.3 Classical Theories and Aetiology of Depression</td>
<td>24</td>
</tr>
<tr>
<td>2.3.1.3.1 Monoamine Hypothesis</td>
<td>25</td>
</tr>
<tr>
<td>2.3.1.3.2 Monoamine Receptor Down-Regulation</td>
<td>25</td>
</tr>
<tr>
<td>2.3.1.3.3 Muscarinic Supersensitivity Hypothesis</td>
<td>26</td>
</tr>
<tr>
<td>2.3.1.3.4 GABA Hypothesis</td>
<td>29</td>
</tr>
<tr>
<td>2.3.1.3.5 Glutamate Hypothesis</td>
<td>29</td>
</tr>
<tr>
<td>2.3.1.3.6 Neuroplasticity Hypothesis</td>
<td>30</td>
</tr>
<tr>
<td>2.3.1.4 Neuroanatomy of Depression</td>
<td>30</td>
</tr>
<tr>
<td>2.3.1.4.1 Hippocampus</td>
<td>32</td>
</tr>
<tr>
<td>2.3.1.4.2 Amygdala</td>
<td>32</td>
</tr>
<tr>
<td>2.3.1.4.3 Frontal Cortex</td>
<td>33</td>
</tr>
<tr>
<td>2.3.2 Other Anxiety and Anxiety-Related Disorders</td>
<td>33</td>
</tr>
<tr>
<td>2.3.2.1 Generalized Anxiety Disorder (GAD)</td>
<td>34</td>
</tr>
<tr>
<td>2.3.2.2 Panic Disorders</td>
<td>33</td>
</tr>
<tr>
<td>2.3.2.3 Phobic Disorders</td>
<td>35</td>
</tr>
<tr>
<td>2.3.2.4 Obsessive-Compulsive Disorder</td>
<td>36</td>
</tr>
<tr>
<td>2.3.2.5 Post Traumatic Stress Disorder</td>
<td>37</td>
</tr>
<tr>
<td>2.3.3 G-Protein Coupled Receptors (GPCRs)</td>
<td>37</td>
</tr>
</tbody>
</table>
Table of Contents

2.3.3.1 G-Protein Coupling .................................................. 38
2.3.3.2 G-Protein Coupled Receptor Signalling ....................... 39
2.3.4 Muscarinic Acetyl Choline Receptors (mACHR) .................... 39
  2.3.4.1 mACHR Families .................................................... 40
  2.3.4.2 mACHRs Function and Location ................................ 41
  2.3.4.3 mACHR and Protein Kinase C (PKC) .......................... 42
2.3.5 Current Drug Therapy of Depression ............................... 43
  2.3.5.1 Selective Serotonin Reuptake Inhibitors ..................... 44
  2.3.5.2 Tricyclic Antidepressants ...................................... 44
  2.3.5.3 Monoamine Oxidase Inhibitors ................................ 44
  2.3.5.4 Novel/atypical Antidepressants ............................... 45
2.3.6 Drugs used in Anxiety ............................................ 45
  2.3.6.1 Treatment of Generalised Anxiety Disorder ............... 45
  2.3.6.2 Treatment of Panic Disorder .................................. 46
  2.3.6.3 Treatment of Phobic Disorder ................................. 46
  2.3.6.4 Treatment of Obsessive Compulsive Disorder .............. 46
  2.3.6.5 Treatment of Post Traumatic Stress Disorder .............. 46
2.3.7 Sildenafil as Possible Antidepressant or Antidepressant Modulator? .......................................................... 47
2.4 Sildenafil and Cell Viability ........................................ 47
  2.4.1 Depression and Neurodegeneration .............................. 48
  2.4.2 Mechanisms of Neurodegeneration and Protection ............ 48
  2.4.3 Reactive Oxygen Species (ROS), Free Radicals and Oxidative Stress ........................................ 49
  2.4.4 NO-cGMP and Neuroprotection ................................. 50
Chapter 3 Experimental Procedures ................................. 51
  3.1 Introduction ....................................................... 51
  3.2 Materials and Instruments ........................................ 52
    3.2.1 Cell Lines Used .................................................. 52
      3.2.1.1 SH-SY-5Y cell line ........................................ 52
      3.2.1.2 HeLA cell line ............................................ 53
    3.2.2 Radiochemicals .................................................. 53
    3.2.3 Other Chemicals .................................................. 53
# Table of Contents

3.2.4 Consumables .......................................................... 54  
3.2.5 Instruments Used ................................................... 54  
3.2.6 Statistical Analyses .................................................. 54  

3.3 Assays: ........................................................................ 55  
3.3.1 Seeding and pre-treatment ........................................ 55  
3.3.2 Cell Viability ............................................................ 60  
  3.3.2.1 Assay 1 – Trypan Blue ....................................... 60  
  3.3.2.2 Assay 2 – Propidium Iodide ............................... 61  
  3.3.2.3 Assay 3 – MTT ................................................. 61  
3.3.3 Functional Assays ..................................................... 62  
  3.3.3.1 Assay 4 – IP<sub>x</sub> .......................................... 62  
  3.3.3.2 Assay 5 – cAMP .............................................. 64  

Chapter 4 Results ................................................................. 67  
4.1 Serum Deprivation and Cell Viability ............................... 68  
4.2 Anti-oxidant/oxidant and Cell Viability .............................. 69  
  4.2.1 Anti-oxidant Pre-treatment on Cell Viability ................. 70  
  4.2.2 Sodium Salicylate and Cell Viability ........................... 71  
  4.2.3 Different Drug Pre-treatments on SH-SY-5Y Cells (Cell Survival after Oxidative Stress) ...................................................... 72  
  4.2.3.1 Trypan Blue Test ............................................ 72  
  4.2.3.2 Propidium Iodide ............................................. 73  
  4.2.3.3 MTT .............................................................. 75  
4.3 IP<sub>x</sub> – production .................................................... 76  
  4.3.1 mAChR function .................................................... 76  
  4.3.2 Comparison SH-SY5Y vs. HeLa ................................ 78  
  4.3.3 cAMP production ................................................ 79  

Chapter 5 Summary, Discussion, Conclusion & Prospective Studies ......................................................... 81  
5.1 Summary ....................................................................... 81  
5.2 Conclusion ...................................................................... 82  
5.3 Recommendations ....................................................... 86
<table>
<thead>
<tr>
<th>Reference</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>88</td>
</tr>
<tr>
<td>Appendix A</td>
<td>109</td>
</tr>
</tbody>
</table>
List of Figures

Figure 2-1: Schematic representation of the nitric oxide-cGMP signal-transduction pathway and where sildenafil affects this system by inhibiting PDE5 enzyme.......................................................................................... 6

Figure 2-2 Schematic representation of the NO/cGMP biochemical pathway........ 9

Figure 2-3 Schematic representation of the effect of protein kinases on large conductance, Ca\(^{2+}\)-activated K\(^{+}\) (BK\(_{Ca}\)) channels.................................................. 19

Figure 2-4 The relation between different mood disorders........................................ 22

Figure 2-5 The relation between the limbic system and other brain structures involved in memory, planning, cognition, stress and fear.............................. 31

Figure 2-6 The major brain structures associated with the limbic system and their orientation........................................................................................................ 32

Figure 2-7 Diversity of G-protein-coupled receptors (GPCRs)........................................ 38

Figure 3-1 Schematic layout of experimental procedures........................................ 55

Figure 4-2 Schematic layout of experimental results to be discussed..................... 67

Figure 4-2 Comparisons of neuronal (SH-SY5Y) cells pre-treated for 24 hours with and without serum, as determined by the Trypan blue test for cell viability (intact cell counts only)................................................................. 68

Figure 4-3 The effect of anti-oxidant (ascorbic acid) pre-treatments on cell viability after serum deprivation, as measured by the Trypan blue test (intact cell counts only)................................................................. 69
List of Figures

Figure 4-4  The effect of an oxidative stress inhibitor (10 mM sodium salicylate) pre-treatment on cell viability during 24-hour serum deprivation, as measured by the Trypan blue test (intact cell counts only). ................................. 71

Figure 4-5  Cell viability as determined by the Trypan blue test on pre-treated SH-SY5Y cells ................................................................. 72

Figure 4-6  Cell viability as determined by the propidium iodide test on 24-hour pre-treated SH-SY5Y cells ......................................................... 74

Figure 4-7  Cell viability as determined by the MTT test on pre-treated SH-SY5Y cells .................................................................................... 75

Figure 4-8  (A) Dose-response curves of MeCh after 24-hour pre-treatment of SH-SY5Y cells with 0 M or 450 nM sildenafil in serum-free medium. (B) Whole-cell uptake of [3H]-myo-inositol (radiolable for measuring [3H]-IP, in (A)) after 24-hour pre-treatment of SH-SY5Y cells with 0 M or 450 nM sildenafil in serum-free medium .............................................. 76

Figure 4-9  Comparison of sildenafil pre-treatment on (A) neuronal (SH-SY5Y) cells. (B) Non-neuronal (HeLa) cells .............................................. 78

Figure 4-10 Changes in cAMP production after 24 hour sildenafil pre-treatment .... 79
Summar of solutions used in this essay
Table 3-4
Regeneration steps, procedures and compositions
Table 3-3
Summar of drugs and concentrations used in pre-treatments
Table 3-2
Study
Table 3-1
Summar of assays, cell lines and seeding density used in this
Table 4-1
Supplies of acetylcholine receptors
Table 1-2
well-characterized phosphodiesterase
Table 1-1
Characterization and tissue distribution of

List of Tables
1.1 Problem Statement

Sildenafil, a selective phosphodiesterase type 5 (PDE5) inhibitor is primarily indicated for the treatment of male erectile dysfunction (MED). Since sildenafil's approval for the treatment of MED in the United States early 1998, 274 adverse event reports were filed by the Food and Drug Administration (FDA) between 4 Jan. 1998 and 21 Feb. 2001 with sildenafil as the primary suspect of various neurological disturbances, including amnesia and aggressive behaviour (Milman and Arnold, 2002). These and other research findings prompted investigations into the possible central effects of sildenafil.

These, commonly precribed antidepressants and other centrally acting drugs are frequently associated with sexual dysfunction (Numberg et al., 2002; 2003; Seidman et al., 2001). This has promoted the use of sildenafil in patients suffering from depression and male erectile dysfunction. This approach alleviates depression, presumably secondary to improved sexual function (Raffaele et al., 2002; Vecchio, 2002), since there is insufficient data to suggest a direct effect of sildenafil on depression itself.

G protein-coupled receptors (GPCRs), such as muscarinic acetylcholine receptors (mAChRs) and serotonergic receptors (5HT-Rs) are known to be involved in antidepressant action (Brink et al., 2004). These GPCRs signal through the phosphatidylinositol signal transduction pathway and are known to activate protein kinases. The NO-guanylyl cyclase signal transduction pathway (see below) has also been suggested to be involved in antidepressant action. This pathway also is associated with the activation of protein kinases (via guanylyl triphosphate (GTP)). This opens the scope for sildenafil to possibly modulate the action of antidepressants.
It has also been suggested that all antidepressant therapies involve the suppression of N-methyl-D-aspartate (NMDA) receptor activity (Skolnick, 1999; Steward and Reid, 2002). Furthermore, recent studies have implicated nitric oxide (NO) in anxiety and affective disorders (Harvey, 1996; Suzuki et al., 2001). Inhibitors of the down-stream activation of NOS (Harkin et al., 1999) and cGMP (Eroglu and Caglayan, 1997; Heiberg et al., 2002) formation, including NOS inhibitors as well as GC-cGMP inhibitors, all demonstrated distinct antidepressant-like effects in animal models of depression. These same modulators of the NO-cGMP pathway have proven to be effective anxiolytic agents, comparable to traditional anxiolytic drugs (Eroglu and Caglayan, 1997; Volke et al., 1997; 1998), suggesting that the NO-cGMP pathway plays an important role in the treatment of anxiety disorders. Various antidepressants have also been shown to inhibit NOS in the hippocampus (Wegener et al., 2003), confirming the role of this pathway in the pathology and pharmacology of depression and anxiety.

Persistent stimulation of NMDA receptors by excessive levels of excitatory amino acids such as aspartate or glutamate, as well as their analogues, causes neuronal damage that is triggered by the influx of Ca\textsuperscript{2+} into the cell after NMDA receptor activation, subsequently causing membrane depolarization (Rothman and Olney, 1997). This Ca\textsuperscript{2+} overload is thought to evoke neuronal death both during aging (Verkhratsky and Toescu, 1997) and in the course of different neuropathological conditions, such as hypoxia or stroke (Choi, 1995) and dementia (Dodd et al., 1994).

NMDA/glutamate dependant neurodegeneration has also been implicated in several other neurological disorders ranging from acute insults such as hypoglycemia, trauma, and epilepsy, to neurodegenerative states such as Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease (Sauer and Fagg, 1992; Lipton and Rosenberg, 1994; Choi, 1998). The question now arises to what extend is the activation of the NO-cGMP pathway involved in the neuroprotective / degenerative process.

In this regard Monhanakumar and Steinbush (1998) described a dual role for NO in neuroprotection, where it acts as an antioxidant or in degeneration where NO enhances hydroxyl radical formation (via the formation of superoxide anions) and peroxynitrates that leads to neurotoxicity. The role of sildenafil on neuroprotection, however, is still uncertain. It has been shown that cGMP plays the role of a neuroprotective mediator and that the NO system is upregulated by cortical spreading depression, leading to an
increase of cGMP via soluble guanylyl cyclase (sGC) (Wiggins et al., 2003). NO has also been proposed to be an endogenous inhibitor of apoptosis in many cell types, where the underlying mechanism for this survival promoting effect appears to involve the activation of guanylyl cyclase and the generation of cGMP (Bredt and Snyder, 1992). These findings suggest that cGMP may play a major role in neuroprotection, possibly countering the neurotoxic actions of its precursor, NO.

To the contrary, NO in its role as a neuronal messenger is also implicated in neuropathology (Dawson et al., 1992), which is believed to involve the NMDA pathway. Thus excitotoxicity is caused via a NO mediated, calcium-calmodulin dependent mechanism (Dawson et al., 1991). Interestingly, neurodegeneration after a kainate-induced status epilepticus has some features of excitotoxic delayed cell death (Pollard et al., 1994; van Lookeren Campagne et al., 1995; Bengzon et al., 1997; Fukijkawa et al., 2000). It is generally thought that this kind of cell death is pathologically linked to an increase in the release of excitatory neurotransmitter glutamate and that glutamate antagonists, especially those that block the NMDA-receptors, are neuroprotective, again suggesting the importance of the NMDA-NO-cGMP pathway in neurodegeneration (Brandt et al., 2003).

1.2 Study Objectives

In the light of the suspected / putative antidepressant activity of sildenafil, the current study explored the effect of sildenafil on cell function and selected biological markers of depression and antidepressant action.

The main objective of the study is to investigate the modulating effect of sildenafil on cell viability and on the function of selected pharmacological receptors in cell cultures.

Two specific objectives/parts can be formulated, namely to investigate in vitro:

a) the effect of sildenafil pre-treatment on cell viability and the mechanism thereof

b) any modulating effects of sildenafil pre-treatment on muscarinic acetylcholine receptor (mAChR) and β-adrenoceptor (β-AR) function in neuronal and non-neuronal cultured cells.
1.3 Study Layout

All studies were performed in the Laboratory for Applied Molecular Biology at the North-West University (PUK), Potchefstroom, South Africa. In order to achieve the abovementioned objectives the following project layout was followed:

a) Human neuroblastoma (SH-SY5Y) cells were pre-treated with no drug, sildenafil (100 nM & 450 nM), sodium salycilate, sildenafil + sodium salycilate, cGMP analogue, PDE inhibitors dipiridamole, zaprinast or IBMX, ODQ or sildenafil + ODQ for 24 hours. Thereafter the cells were rinsed and incubated in drug-free medium for 18 hours, followed by the trypan blue assay in order to determine the cell viability after pre-treatment with the appropriate drugs.

b) Human neuroblastoma (SH-SY5Y) and human epithelial (HeLa) cells were pre-treated with or without sildenafil for 24 hours, whereafter mAChR and β-AR function, respectively, was determined by measuring agonist-induced second messenger formation.

The modulation of agonist-induced mAChR function was determined by performing whole cell [3H]-IP₅ (inositol-multiposphates) accumulation assay, constructing dose-response curves with the mAChR full agonist metacholine. Similarly, the modulation of agonist-induced β-AR function was determined by performing whole cell [3H]-cAMP accumulation assay, constructing dose-response curves with the β-AR full agonist l-isoproterenol. [3H]-MI and [3H]-cAMP cellular uptake studies were also performed in order to investigate the effect of the pre-treatment on cellular [3H]-MI and [3H]-cAMP uptake (a process intrinsic to the functional assays).
In view of the proposed important role of the NO-cGMP signal transduction pathway in depression and the involvement of sildenafil in this pathway, as well as its putative role in neuroprotection, this chapter will discuss relevant aspects of depression. This chapter will also review the pharmacology of sildenafil, different hypotheses of the bio-molecular mechanisms underlying depression (as well as other anxiety-related disorders) and the mechanisms and relevance of neurodegeneration/protection. The three related main topics for this literature review therefore include:

- Sildenafil and the NO-cGMP signal transduction pathway.
- Depression and other anxiety-related disorders.
- Neurodegeneration/-protection.

### 2.1 Clinical and Basic Pharmacology of Sildenafil

#### 2.1.1 Indications for Use

Sildenafil is a selective phosphodiesterase type 5 (PDE5) inhibitor and is used as effective first-line therapy for the treatment of male erectile dysfunction (MED). This includes MED associated with prostatectomy, radiation therapy, diabetes mellitus and certain antidepressant drug therapies (e.g. selective serotonin reuptake inhibitors (SSRIs)) (Boyce and Umland, 2001).

Although still controversial, sildenafil has been used for the treatment of pulmonary hypertension in both newborn babies and adults. In a trial that was conducted by
Michelakis et al., 2002, a single oral dose of sildenafil was shown to be as selective and effective as the pulmonary vasodilators in the treatment of patients suffering with pulmonary arterial hypertension.

2.1.2 Mechanism of Action

In MED, sildenafil acts by enhancing the relaxation of both vascular and trabecular smooth muscle cells which leads to the filling of the corpus cavernosum and consequently causing penile erection. This smooth muscle relaxing effect is mediated via the NO-cGMP signal transduction pathway, which is depicted in Figure 2-1.

![Figure 2-1 Schematic representation of the nitric oxide-cGMP signal-transduction pathway and where sildenafil affects this system by inhibiting the PDE5 enzyme. Abbreviations: GC = guanyly cyclase; cGMP = cyclic guanosine monophosphate; GMP = guanosine monophosphate; GTP = guanosine triphosphate; NANC nonadrenergic-noncholinergic neurons; NO = nitric oxide; PDE5 = phosphodiesterase type 5 - adapted form Kloner and Zusman, (1999)
Following the formation of gaseous nitric oxide (NO) by NOS (see section 2.2.2 NO), NO diffuses into neighbouring smooth muscles and activates guanylate cyclase (GC), an enzyme that converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). This increase in intracellular cGMP, together with the resulting activation of PKC, then provides the signal for smooth muscle relaxation (Ballard et al., 1998; Boolell et al., 1996). This signalling cascade involves intracellular transport of Ca\(^{2+}\) and thus Ca\(^{2+}\) regulation (Lincoln et al., 1995) – see section 2.2.6 Large Conductance Ca\(^{2+}\) Activated Channels (BK\(_{Ca}\)).

Phosphodiesterases (PDEs) are responsible for the hydrolyses of cGMP to inactive GMP. Sildenafil elevates the cGMP signal by inhibiting the degradation of cGMP, thereby increasing cGMP levels and strengthening signal transduction. This explains the clinical effect of sildenafil in MED (Ballard et al., 1998; Jeremy et al., 1997).

### 2.1.3 Adverse Effects & Drug Interactions

The pharmacodynamic and adverse effect profiles observed in clinical trials with sildenafil are consistent with the in vitro profile of the tissue distribution of PDE5 and its best described mechanism of action as a selective inhibitor of PDE5. Sildenafil has many adverse effects, possibly due to its potent vasodilatory effect, as well as its weaker interaction with phosphodiesterase type 6 (PDE6), the prominent PDE found in the retina causing blurred vision. Other adverse effects include headache, flushing, nasal congestion and gastrointestinal effects such as dyspepsia (Morales et al., 1998).

Vallance et al., (1989) found that sildenafil enhanced smooth muscle relaxation induced by glyceryl trinitrate in isolated aortic rings contracted by exposure to phenylephrine. Glyceryl trinitrate is a drug that acts by releasing NO once it is biotransformed. (Vallance et al., 1989).

Sildenafil has the ability to elevate smooth muscle cGMP levels by blocking cGMP breakdown in the cGMP-signalling pathway, whereas NO has the ability to increase cGMP synthesis. Therefore a clear synergistic action between PDE inhibitors like sildenafil and NO donating drugs such as glyceryl trinitrate exists.
This suggests that sildenafil can potentiate the effects of NO donor agents on vascular tissues, which may cause a decrease in systemic vascular resistance, resulting in hypotension (Valiance et al., 1989; Umans et al., 1995).

2.2 Sildenafil and the NO-cGMP Signal Transduction Pathway

Sildenafil acts by inhibiting the breakdown of cGMP by phosphodiesterase enzymes in the NO-cGMP pathway, therefore potentiating cGMP’s effects (see Figure 2-1) and ultimately leading to smooth muscle relaxation/vasodilatation.

2.2.1 The NO-cGMP pathway

Several drugs exert their effect through the NO-cGMP signal transduction pathway, interfering at different steps in the signalling cascade. This pathway includes both NO and cGMP, which are implicated in both neuroprotection and degeneration (also see section 2.4.2 Mechanism of Neurodegeneration and Protection). The NO-cGMP pathway is a complex pathway that is able to influence/manipulate a number of systems within the human cell and ultimately influence body functions by producing NO and cGMP as second messenger molecules.

Figure 2-2 gives a schematic representation of the NO-cGMP pathway:
Figure 2-2 Schematic representation of the NO/cGMP biochemical pathway. After the activation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptors on the postsynaptic terminal by glutamate, calcium (Ca\(^{2+}\)) enters the cell and activates nitric oxide synthase (NOS). However, the activation of the NMDA channel will only result in a low current due to inhibiting Mg\(^{2+}\) ions that also enter through the channel, causing a block in the current. NOS, after stimulation by Ca\(^{2+}\), produces nitric oxide (NO) by utilising l-arginine, oxygen (O\(_2\)) and NADPH. l-Citrulline and NADP is formed as by-products. The NO then diffuses to the presynaptic terminal and activates soluble guanylyl cyclase (sGC), which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). This increase in the intracellular cGMP, resulting in the activation of protein kinase C (PKC) that provides the signal for smooth muscle relaxation. (Ballard et al., 1998; Boolell et al., 1996). This process is terminated by phosphodiesterase (PDE) enzymes that convert the cGMP to inactive GTP. Sildenafil inhibits the phosphodiesterase type 5 (PDE5) enzymes thereby increasing cGMP levels.

In order to gain a better understanding of the NO-cGMP signal transduction pathway, a few key components of this pathway will be discussed, including excitatory amino acid receptors (EAAR), nitric oxide (NO), soluble guanylyl cyclase (sGC), cyclic guanosine monophosphate (cGMP), phosphodiesterases (PDE) and large-conductance Ca\(^{2+}\)-activated (BK\(_{Ca}\)) channels (responsible for smooth muscle tuning).
2.2.1.1 Excitatory Amino-Acid Receptor:

Four different kinds of glutamate receptors can be distinguished namely N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate and orphan receptors. In addition to the first three groups of ionotropic receptors, glutamate also activates G-protein-coupled receptors (GPCRs), called metabotropic glutamate receptors (Foreman and Torben, 2003).

2.2.1.1.1 N-methyl-D-aspartate (NMDA)

NMDA receptors play a unique and profound role in synaptic transmission, neurophysiology and pathological processes, which include the regulation of the NO-cGMP pathway. The hippocampus which is primarily concerned with short term memory formation (Leonard, 1997), and which has been implicated in depression (also see section 2.3.1.3.5 Glutamate Hypothesis) shows a high density of these receptors (Cooper et al., 1996).

Receptors that are characterised by slow kinetics and high Ca²⁺ permeability are activated by the endogenous agonist NMDA. In addition to glutamate (or NMDA), these receptors require glycine as co-agonist (Foreman and Torben, 2003).

At -70mV (membrane resting potential) the activation of the channel will result in only a low current due to inhibiting Mg²⁺ ions entering the channel. However, as the membrane potential becomes less negative the affinity of Mg²⁺ decreases (Schubert and Nelson, 2001), thereby negating its inhibitory effect (see Figure 2-2).

The NMDA receptors are co-localized with the AMPA receptors in many synapses. Due to the slow kinetics of the NMDA receptor, receptor activation after a single release of presynaptic glutamate release is minimal resulting in Mg²⁺ block of the NMDA receptor. However, after extensive stimulation of the synapse when repetitive activation of the AMPA receptors evokes sufficient depolarisation of the postsynaptic membrane to relieve the NMDA receptors of the Mg²⁺ block, the NMDA receptor will become fully excitable.
However, prolonged stimulation of NMDA receptors can damage or kill target cells via a process referred to as excitotoxicity. This process is probably linked to an increased influx of Ca$^{2+}$ ions through the NMDA ion channel, resulting in an increase in intracellular proteases and lipases, impaired mitochondrial function activation and free radical generation. The net result of this is cell death (Leonard, 1997).

This use-depandant influx of Ca$^{2+}$ has been interpreted to be one of the mechanisms underlying many different neuronal processes, including learning and memory (Foreman and Torben, 2003).

2.2.1.1.2 α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)

Another class of ionotropic glutamate receptors, known as α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (AMPA), exhibits fast kinetics and in most neurons, a low Ca$^{2+}$ permeability when activated by glutamate.

The agonist α-amino-3-hydroxy-5-methyl-4-isoxazole propionate as well as glutamate activates a fast desensitizing current in the majority of these receptors (Foreman and Torben, 2003). The distribution of AMPA receptors parallels that of the NMDA receptors and is widespread in the central nervous system (CNS). AMPA receptor function can be enhanced by zinc and sulfhydryl agents and is suppressed or antagonised by barbiturates and several spider and wasp toxins (Cooper et al., 1996).

AMPA receptors are responsible for the majority of fast excitatory neurotransmission in the mammalian brain because of its characteristic of extremely rapid rate of desensitization after stimulation by glutamate (Foreman and Torben, 2003).

2.2.1.1.3 Kainate

Kainate activates a non-desensitizing current at AMPA receptors, but activates a fast desensitizing current on another receptor, namely the kainate receptor (Foreman and Torben, 2003). These receptors can be found in/on:

- glia cells of the cerebellum (Bumashev et al., 1992),
2.2.1.4 Orphan Glutamate Receptors

Receptors that share sequence similarities with the glutamate receptors, but cannot be activated by glutamate or any of the common glutamate receptor agonists, have been identified. Since the endogenous agonist(s) is unknown, these receptors are referred to as orphan glutamate receptors. They consist of two subunits, namely the δ₁ and δ₂ subunits (Foreman and Torben, 2003).

2.2.2 Nitric Oxide (NO)

Since the discovery that endothelium-derived relaxing factor (EDRF) is in fact the same compound as nitric oxide (NO), many publications have recognised its key role in cell-to-cell communication, including endothelium and neuronal signalling, as well as immune response following pathogen infection (Rees et al., 1989; Lancaster, 1992; Moncada and Higgs, 1995).

NO has diverse functions in the periphery and CNS and can act as both second messenger and intercellular messenger (neurotransmitter). In the periphery it is implicated in numerous functions, such as the regulation of vascular tone (essential for the regulation of blood pressure), as well as the control of platelet aggregation and the regulation of cardiac contractility (Moncada and Higgs, 1995).

NO acts as neurotransmitter by activating soluble (cytoplasmic) guanylyl cyclase (sGC), which catalyses the conversion of GTP to the signalling molecule cyclic GMP (cGMP) (Murad et al., 1978). cGMP then activates protein kinases, which is involved in the intracellular transport of Ca²⁺. The specificity of the cellular response to cGMP is dictated by cGMP-binding motifs in target proteins (Lincoln et al., 1994). In addition to its normal physiological roles, NO is also indicated in the pathophysiology of several neurodegenerative diseases in the CNS such as Alzheimer's disease, Huntington's disease, as well as psychiatric illness such as depression and anxiety (Harvey, 1996).
2.2.2.1 NO Synthase (NOS)

NO is synthesised from L-arginine (Schmidt et al., 1998) in a reaction that is catalysed by the enzyme nitric oxide synthase (NOS) (Bredt and Snyder, 1990). Once it is activated, NOS binds to calmodulin and together with \( O_2 \) and NADPH, (Schmidt et al., 1998) results in the subsequent oxidation of L-arginine, resulting in the formation of L-citrulline and NO.

The interaction between nNOS and eNOS with calmodulin depends on the availability of free \( Ca^{2+} \) molecules. Calmodulin and \( Ca^{2+} \) is an absolute requirement for this process in both neuronal nNOS and endothelial eNOS activity (Garthwaite, 1991).

2.2.2.2 Nitric Oxide as Modulator of Neuronal Function

Experimental data suggest that this free radical (NO) is probably implicated in the regulation of firing and excitability, long-term potentiation (LTP) and long-term depression (LTD) of central neurons (Prast and Philippu, 2000), as well as in the formation of memory (Fazeli et al., 1992). Prast and Philippu, (2000) document that previous in vivo and in vitro brain studies have shown that endogenous NO modulates the release of several neurotransmitters, e.g. acetylcholine, histamine, catecholamines, excitatory and inhibitory amino acids, serotonin, and adenosine.

2.2.2.3 Effects of NO on Neuronal Excitability and Firing

cGMP synthesis plays a major role in the majority of NO effects on excitability. It has been suggested that the activation of the sGC, an increase of cGMP formation and the action of cGMP-dependent protein kinases acts as the main signal transduction pathway of NO (Smolenski et al., 1998).

NO reduces the function of \( \gamma \)-aminobutyric acid (GABA)A receptors in the cerebellum (Zarri et al., 1994; Robello et al., 1996) and that of AMPA receptors in forebrain, cerebellum and in the horizontal cells of the retina (Dev and Morris, 1994; McMahon and Ponomareva, 1996) via a cGMP dependant mechanism. In addition, NO seems to modulate neuronal function in a cGMP-independent way which comprises direct reaction with proteins leading to nitrosylation and reaction of NO with superoxide, resulting in the
formation of peroxynitrite and subsequent protein nitration and oxidation. The formation of peroxynitrate may be involved in the NMDA receptor-mediated cascade of cell death (Schultz et al., 1997).

NO may exert a significant function in LTP or LTD, even in brain areas where no acute effects of NO on excitability are manifested (Prast and Philippu, 2000). During high signal transmission, short-lasting inhibition of GABA<sub>α</sub> receptors will lead to a transient enhancement of excitability at synapses. On the other hand, the inhibition of NMDA receptor response by NO is persistent and may provide a feedback mechanism which attenuates NMDA receptor-mediated effects in case of excessive receptor stimulation (Aizenman et al., 1989; Levy et al., 1990), without blocking LTP.

NO modulates neurotransmitter release and excitability in several brain regions such as the striatum, the hippocampus, and the hypothalamus and locus coeruleus. NO influence on excitability and firing and on hippocampal LTP is predominantly mediated via cGMP and its target proteins. In the striatum and hippocampus, NO modulates action potential-dependent neurotransmitter release in a cGMP-, Ca<sup>2+</sup>- and tetrodotoxin (TTX)-sensitive mechanism. The NO-induced changes in transmitter release in the striatum are probably the consequence of a primary increase in glutamate release by NO. Low concentrations of NO seem to decrease glutamate release in the hippocampus, while it facilitates LTP which, in turn, leads to long-term increase of glutamate release (Prast and Philippu, 2000).

### 2.2.3 Soluble Guanylyl Cyclase (sGC)

cGMP is an important signalling molecule generated by guanylyl cyclases (GCs) which is involved in a variety of physiological processes, including smooth muscle relaxation, inhibition of retinal signal transduction (by direct interaction with Na<sup>+</sup> channels), and platelet aggregation (Moncada and Higgs, 1995).

Guanylyl cyclase (GC) is mainly found as membrane (pGC) and soluble guanylyl cyclase, (sGC). pGC and sGC enzymes share similar structural characteristics and are homologous based on amino acid sequences, but differ in their mechanisms of physiological regulation. This is illustrated by the ability of natriuretic peptides to
stimulate several isoforms of the pGCs, while most of the functions for NO and other nitro-vasodilators are mediated through the stimulation of sGCs (Andreopoulos & Papapetropoulos, 2000).

2.2.4 Cyclic Guanosine Monophosphate (cGMP)

As an important second messenger, cGMP mediates a considerable part of its effects by cGMP-dependent protein kinase, a dimeric protein that is assembled from two homologous subunits (Hofmann et al., 1992; Pfeifer et al., 1998). Two types of mammalian cGMP kinase isoforms exist, namely cGMP kinase I and cGMP kinase II (Wernet et al., 1989; De Jonge, 1981; Jarchau et al., 1994). cGMP kinase I is expressed at high levels in all types of smooth muscle, platelets, and cerebellar Purkinje cells and in the hippocampus (Keilbach et al., 1992; Kleppisch et al., 1999). cGMP kinase II is expressed in the brush border of the intestinal mucosa (Markert et al., 1995), juxtaglomerular cells of the kidney (Gambaryan et al., 1996), chondrocytes (Pfeifer et al., 1996) and in specific brain regions (El-Husseini et al., 1998) but not in cardiovascular cells (Ruth, 1999).

The presence of different receptors for cGMP complicates the identification of the specific cGMP receptor protein that elicits the cellular effect in response to increases in cGMP levels. cAMP kinase and cGMP kinase can act in concert as functional co-regulators of cellular activity in many tissues (Heaslip et al., 1987) and it was for this reason that the biological role of the cGMP kinases had been obscured for a long time (Ruth, 1999).

2.2.4.1 cGMP Signalling

The cGMP signalling pathway produces many cellular responses after stimulation by a variety of chemicals, including hormones, neurotransmitters, drugs and toxins. The biochemical mechanisms underlying these responses include the synthesis of the nucleotide following the activation of either the soluble or the particulate guanylate cyclase and its degradation by numerous PDEs (Beltman et al., 1995). The recent availability of selective and potent PDE inhibitors, such as sildenafil, highlights the role of cGMP in cellular biology and thereby opening broad clinical applications.
2.2.4.2 cGMP Kinase I and Vasorelaxation

Francis et al., (1988) found that cyclic nucleotide analogues that potently activate purified cGMP kinase I, induced relaxation of vascular smooth muscle, suggesting that the activation of cGMP kinase mediates the relaxation of smooth muscle in response to an increase of intracellular cGMP. Studies done in mice carrying null mutations of the genes coding for the eNOS and for the receptor guanylyl cyclase A (GCA) clearly showed that the NO-cGMP signalling cascade mediates basal vasodilatation in vivo (Huang et al., 1995; Lopez et al., 1995).

Apart from cGMP, elevation of CAMP has also long been known to cause relaxation of vascular smooth muscle (Hardman, 1984). However, compounds that elevate CAMP are only weakly correlated with smooth muscle relaxation (Schultz et al., 1977). Furthermore, the activation of CAMP kinase by CAMP analogues and the potencies by which these analogues relax smooth muscle are weakly correlated (Francis et al., 1988). This led to the suggestion that cGMP kinase is cross activated by CAMP, resulting in relaxation. To confirm this hypothesis, coronary arteries from pig were stimulated with isoproterenol, resulting in an increase of both CAMP kinase and cGMP kinase activity (Jiang et al., 1992).

Activated cGMP kinase I lowers cytosolic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{i}\)) in various cell types including smooth muscle cells (Felbel et al., 1988; Cornwell and Lincoln, 1989; Geiger et al., 1992; Ruth et al., 1993). This effect is compatible with the smooth muscle relaxing activity of the enzyme.

2.2.5 Phosphodiesterase (PDE)

PDE families play a major role in neuronal messenger regulation and are distributed in different parts of the human body. These families can also differ from tissue to tissue, each family with its own distribution and effect (Wallis et al., 1999).

By using anion exchange chromatography they can be isolated and divided into families based on their primary structures and their catalytic and regulatory properties, e.g.,

- the relative selectivity in hydrolyzing cGMP or CAMP,
the ability of cGMP to stimulate or inhibit cAMP hydrolytic activity,
> the existence of allosteric cGMP-binding sites on the PDE,
> and the effects of calcium on enzyme activity.

2.2.5.1 Phosphodiesterase Activity

In a study that was done by Wallis et al., (1999) – it was shown that the major PDE activity in the human cardiac ventricle was calcium/calmodulin-dependent PDE1; in contrast, there was no detectable level of PDE5. It was also found that the human saphenous vein contained PDEs 1, 4, and 5, and the human mesenteric artery contained PDEs 1, 2, 3, 4, and 5. Sildenafil, had no effect on the isolated trabeculae carneae, unlike milrinone, a selective PDE3 inhibitor; this is consistent with the lack of PDE5 expression in cardiac myocytes.

PDE1 and PDE5 have higher affinity for cGMP, whereas PDE2, PDE3, and PDE4 have a higher affinity for cAMP. Evidence of high concentrations of PDE5 has been found in the corpus cavernosum (Boolell et al., 1996), but is also known to exist in the vasculature.

Vasodilator drugs, such as theophylline and papaverine, both non-specific PDE inhibitors produce vasodilatation by increasing cAMP and cGMP levels while other vasodilating drugs such as amrinone and milrinone, act by inhibition of PDE3 (Wallis et al., 1999) and possibly PDE1 and PDE2. (Fischer et al., 1992)

Sildenafil has a substantially lower affinity for the other PDE isozymes, manifested by the much higher concentrations of sildenafil needed to inhibit 50% of the enzyme activity (IC50) as shown in Table 2-1.

Although sildenafil is PDE5 selective, it is also able to exert an effect on PDE6 that is mainly found in the retina.
Table 2-1 Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro (Wallis et al., 1999).

<table>
<thead>
<tr>
<th>Nomenclature, Characterization and tissue distribution of well-characterized Phosphodiesterase</th>
<th>Inhibition of Human PDEs by sildenafil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family</td>
<td>Substrate</td>
</tr>
<tr>
<td>PDE1</td>
<td>cGMP&gt; cAMP</td>
</tr>
<tr>
<td>PDE2</td>
<td>cGMP &amp; cAMP</td>
</tr>
<tr>
<td>PDE3</td>
<td>cAMP</td>
</tr>
<tr>
<td>PDE4</td>
<td>cAMP</td>
</tr>
<tr>
<td>PDE5</td>
<td>cGMP</td>
</tr>
<tr>
<td>PDE6</td>
<td>cGMP</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PDE7-10 families have been identified but not been well characterized

Abbreviations: cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; IC<sub>50</sub> = concentration needed that reduces enzyme activity with 50%; PDE = phosphodiesterase

2.2.6 Large-Conductance Ca<sup>2+</sup> Activated Channels (BK<sub>Ca</sub>)

Although sildenafil inhibits the breakdown of cGMP, it alone is not enough to cause an effect (vasodilatation). As mentioned in section 2.2.4.2 cGMP Kinase I and Vasorelaxation, cGMP production causes a decrease in Ca<sup>2+</sup> concentrations. The decreased Ca<sup>2+</sup> has a direct effect on BK<sub>Ca</sub> channel membrane potential and eventually smooth muscle contraction/relaxation.
Several protein kinases such as cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C (PKC) can also affect tissue function by modulating the apparent Ca\(^{2+}\) sensitivity of the BK\(_{Ca}\) channel to physiological changes (Schubert and Nelson, 2001).

![Diagram of BK\(_{Ca}\) channel regulation by protein kinases](image)

**Figure 2-3** Schematic representation of the effect of protein kinases on large conductance, Ca\(^{2+}\) -activated K\(^+\) (BK\(_{Ca}\)) channels – adapted from Schubert and Nelson (2001).

The activity of the BK\(_{Ca}\) channel is determined by the membrane potential and intracellular Ca\(^{2+}\) concentration. Both membrane depolarization and elevations in intracellular Ca\(^{2+}\) concentrations activate the channel. The latter process is often based on local, high elevations of intracellular Ca\(^{2+}\) ('Ca\(^{2+}\) sparks') caused by Ca\(^{2+}\) release through ryanodine receptors in the sarcoplasmic reticulum. Protein kinases also modulate the activity of BK\(_{Ca}\) channels.
It is well known that PKC is responsible for the inhibition of BK_{Ca} channel activity while PKA or PKG activate these channels. This is manifested by an apparent sensitization of the channel to Ca^{2+}. Jagger (2000) found that the relaxation of several types of smooth muscle tissues, due to an elevation of cAMP and cGMP by pharmacological agents was properly due to the activation of BK_{Ca} channels.

2.2.6.1 Two Alternative Hypotheses on How Protein Kinases Interact with BK_{Ca}

2.2.6.1.1 Direct Interaction

Recent studies done by Toro et al., (1998) have shown that cloned smooth muscle BK_{Ca} channels have strong phosphorylation sites for PKG and PKC on the α-subunit, a strong phosphorylation site for PKG on the β-subunit and possible phosphorylation sites for PKA on both channel subunits. Thus, for these channels at least, the effects of PKG are accompanied by phosphorylation of the channel itself. However, in all experimental situations BK_{Ca} channels can potentially associate with regulatory proteins and thus the effects of kinases can always be indirect.

2.2.6.1.2 Indirect Interaction

In contrast to the direct effect of protein kinases on BK_{Ca} channels, the activating effect of PKG on BK_{Ca} channels from bovine trachea can be mimicked by phosphatase 2Ac and reversed by phosphatase inhibitors (Zhou, 2000). These data show that protein kinases activate the channel indirectly, following kinase-induced activation of a phosphatase. It cannot be excluded that the effects observed are indirect and mediated by a phosphatase. It is unclear whether the indirect phosphatase mediated effect occurs by phosphorylation of the phosphatase or by protein kinases. It is also possible that they phosphorylate a phosphatase-binding protein that is responsible for the membrane localization of the phosphatase near the channel (Zhou, 1996).
2.3 Introduction to Anxiety-Related Disorders

Depression is a common disorder, with up to 30% of primary care patients suffering from depressive symptoms. Epidemiological evidence suggests that there is a fourfold increase in death rates in individuals with major depressive disorder (MDD) who are over the age of 55 years (DSM-IV-TR., 2000). Several factors may contribute to the final manifestation of depression and involves genetic factors (e.g. neurotransmitter dysfunction), developmental problems (e.g. personality defects, childhood events) or psychosocial stresses (e.g. divorce or unemployment). In general, sadness and grief can be seen as normal responses to loss, and these do not necessarily imply depression. Normal grief is often accompanied by an intact self-esteem, while depression is characterised by a sense of guilt or worthlessness (Tierney et al., 1999). Other related and common psychological disorders include anxiety and anxiety related disorders. Stress, fear and anxiety tend to be interactive as can be seen in Figure 2-4 (below).

The main components of anxiety are psychologic, (tension, fears, difficulty in concentration, apprehension) and somatic (tachycardia, hyperventilation, sweating and tremors). Anxiety can become self-generating, since the symptoms reinforce the reaction, causing it to spiral. This is often the case when anxiety is an epiphenomenon of other medical or psychiatric disorders (Tierney et al., 1999).
Figure 2-4 The relation between different mood disorders (Boon, 2002). Fig. 2-4 shows the relationship between the activation of the sympathetic and parasympathetic systems in terms of performance and the role of cortisol and DHEA production on stress and emotion. An elevation in cortisol levels, together with sympathetic stimulation by adrenaline may lead to frustration, anger, worry and anxiety while an increase in DHEA levels in the same system leads to exhilaration, joy and happiness. However, parasympathetic stimulation together with a decrease in DHEA may lead to burnout, withdrawal, boredom and apathy. Decreased cortisol levels in the latter system may give raise to serenity, balance, compassion and contentment.

When looking at the high incidence of psychiatric disorders, such as depression and anxiety, it is clear that these psychiatric disorders play a prominent role in society. The relevance and general interest of these disorders has lead to numerous studies over many years, so that we currently have several hypotheses and proposed underlying biomolecular mechanisms, while there is also to a host of remaining and new questions. In general the biomolecular bases of anxiety and anxiety related disorders are still poorly understood.

Although effective drug treatments of these disorders are available, tolerance, a high incidence of treatment-resistance and delayed onset of action remain challenges. In order to address these challenges a better understanding of the underlying mechanisms of these disorders is necessary, motivating intensive ongoing research.
2.3.1 Depression

2.3.1.1 Clinical Image of Depression

Depression carries the second greatest burden of all illnesses in industrialized countries and has a lifetime prevalence of around 20% (Murry and Lopez, 1997). It is accompanied by a state of lowered mood and accompanying disturbances that may include altered sleep patterns, lowered general energy levels, changes in appetite, reduced mental concentrations and lowered libido. Four mood episodes that may be present can be distinguished, namely major depressive episodes, manic episodes, mixed episodes and hypomanic episode. As a biologically heterogeneous illness, depression involves multiple neurotransmitter and receptor systems. Also, depression can be divided into different subtypes, namely endogenous or nonendogenous, primary or secondary, unipolar or bipolar and psychotic or nonpsychotic. Consequently patients respond differently to various kinds of anti-depressant pharmacotherapy.

On a clinical basis it is useful to distinguish between bipolar and unipolar mood disorders:

- Bipolar depression is characterized by mood swings between manic and depressive states. Bipolar mood disorder commonly begins with depression and is characterized by at least one "excited" period sometime during the illness. It is subdivided into Bipolar 1 and Bipolar 2 disorders where Bipolar 1 disorder has the distinct characteristic of an alternation between full-blown manic and major depressive episodes while Bipolar 2 disorder can be distinguished by an alternation between depressive episodes and hypomanias (mild, nonpsychotic periods of excitement) of short duration.

- Unipolar depression is characterized by continuous depressive mood. Another term used for unipolar mood disorder is major depressive disorder and occurs as syndromal depression with several episodes over a lifetime. Melancholia is a form of unipolar depression and is reserved for the most full-blown expression of major depressive disorder. Typical manifestations of this disorder includes a marked psychomotor slowing or agitation, pathologic guilt, middle or early
morning insomnia, diurnal variation in mood and activity with nadir in the morning, and the loss of capacity to experience pleasure (Bondy et al., 1992).

2.3.1.2 The Pathophysiology of Major Depressive Episode

The pathophysiology of Major Depressive Episodes (MDE) may involve a dysregulation of a number of neurotransmitter systems, including l-norepinephrine (l-NE), serotonin (5-HT), dopamine (DA), acetylcholine (ACh) and gamma-aminobutyric acid (GABA) systems. Evidence also exists that several neuropeptide alternations, including corticotropin-releasing hormone may play a role in the pathophysiology of depression. According to the DSM-IV-TR (2000) hormonal disturbances have been observed in some depressed individuals, including an elevation in glucocorticoid secretion (e.g., elevated urinary free cortisol levels or dexamethasone nonsuppression of plasma cortisol) and blunted growth hormone, thyroid-stimulating hormone as well as prolactin responses to various challenge tests. Alterations in cerebral blood flow in limbic and paralimbic regions as well as a decrease in blood flow in the lateral prefrontal cortex has been observed by using functional brain imaging studies. Late life depression is associated with an alternation in brain structure, including periventricular vascular changes. It is also worth mentioning that none of these changes are present in all MDE suffering individuals, nor is any particular disturbance specific to depression (DSM-IV-TR, 2000). Important to note that depression, especially recurrent depression, is associated in hippocampal shrinkage and is of great importance for neurodegenerative theories of depression.

2.3.1.3 Classical Theories and Aetiology of Depression

Given the complexity of the neural circuitry and all the systems involved it is not surprising that no single hypothesis appears to be sufficient to explain the mechanisms of antidepressant action. It is also naive to assume that depression is caused by a synaptic deficiency in only a single group of indolamine and/or catecholamine neurotransmitters, seeing that this assumption fails to explain why cerebrospinal fluid, urinary and serum transmitter metabolites do not reveal any consistent pattern of abnormality in depressed patients (DSM-IV-TR, 2000).
2.3.1.3.1 Monoamine Hypothesis

The idea of the involvement of biogenic monoamine's in the aetiology of depression initially emerged from three main lines of evidence. Firstly, when looking at a drug such as reserpine that causes depletion of brain monoamine stores e.g. l-norepinephrine (l-NE), dopamine (DA) and serotonin (5HT) (Cooper et al., 1996), it is able to induce depressive-like symptoms. Secondly, some depressed patients have reduced levels of monoaminergic metabolites in some body fluids, particularly cerebrospinal fluid. Lastly, drugs that relieve depression seem to immediately attenuate the mechanisms by which 5-HT and l-NE are metabolically inactivated (Blier, 2003).

Although evidence exists for the role of both 5HT and l-NA neurotransmission in the aetiology of depression and the mechanisms of action of antidepressants, the focus has shifted to the 5HT system over the past decade. This shift in focus could be contributed to the great success of the selective serotonin re-uptake inhibitors (SSRIs) as antidepressants. Unlike the tri-cyclic antidepressants (TCAs), the SSRIs form a class of drugs with unrelated chemical structures. Indeed the only property that they share is the inhibition of 5HT reuptake and membership of this class specifically excludes significant inhibition of l-NE reuptake. It has therefore become difficult to sustain a hypothesis for the mechanism of action of antidepressants that places l-NE, rather than 5HT, in the pivotal role (Blier, 2003).

However, one cannot say that l-NE does not play a significant role in the mechanism of action of other types of antidepressants. Blier (2003) mentions that the l-NE system may be crucial in the mechanism whereby putative faster-onset antidepressants exert their action. Nevertheless, the monoamine hypothesis, as initially conceived, fails to adequately explain the discrepancy between the acute effects of antidepressants and the delay in the onset of their therapeutic action.

2.3.1.3.2 Monoamine Receptor Down-Regulation Hypothesis

A modification of the monoamine hypothesis suggests that, although monoaminergic neurotransmission may be reduced in patients with depression, it is the consequent supersensitivity of post-synaptic monoamine receptors in adaptation to reduced amounts of neurotransmitter that is responsible for depression. It is suggested that enhanced
synaptic neurotransmission (brought about by decreased transmitter reuptake or inhibition of monoamine oxidase, for example) causes a down-regulation of postsynaptic monoamine receptors and therefore a relief from depressive symptoms.

Several lines of experimental data support this hypothesis of antidepressant-induced down-regulation of postsynaptic receptors. This has been shown in particular for β-adrenoceptors (β-ARs) and 5HT receptors (5HT-Rs), in animal experiments and also generally occurs along a similar time-course to the development of antidepressant response in patients. The down-regulation of post-synaptic β-ARs are indeed an ubiquitous finding among classical antidepressants and is still often regarded as indicative of antidepressant potential for new agents (Blier., 2003).

Recent evidence, particularly from electrophysiological studies of monoaminergic neurones suggest that the interactions between the two candidate monoamine neurotransmitters, I-NE and 5HT, are more complex than previously envisaged. Evidence exists that suggests the down-regulation of β-ARs is dependent on the 5HT system, which argues against an exclusive central role for 5HT and I-NE in the mechanism of action in antidepressants. SSRIs, which do not down-regulate β-ARs (Goodnough and Baker, 1994), is also not easily accommodated in the β-adrenoceptor down-regulation hypothesis. Finally, the observation that the β-AR antagonist propranolol that crosses the blood-brain barrier is not an antidepressant is difficult to reconcile with the β-AR hypothesis; blocking a receptor or inducing its desensitization should produce the same impact on neuronal function (Blier, 2003).

2.3.1.3.3 Muscarinic Supersensitivity Hypothesis

The stimulation of central cholinergic transmission with cholinomimetics or cholinesterase inhibitors can cause severe depressed mood, dysphoria, behavioural withdrawal, a reduction in hedonic capacity and psychomotor retardation. This drug-induced syndrome fits the profile of major depressive disorder, which gave rise to the cholinergic/muscarinic hypothesis of depression (Dilsaver, 1986).
Evidence in support of the cholinergic involvement in affective disorders is provided by the following: the efficacy of antimuscarinic drugs (e.g. imipramine) in the treatment of depression (Goldman and Erickson, 1983), the euphorigenic properties of antimuscarinic agents that is frequently abused (Smith, 1980), evidence of cholinergic-monoamine antagonism in regulation of reward, punishment and hedonic capacity and the antimuscarinic agent withdrawal effects (anxiety, sleep disturbances and rebound exacerbation of motor dysfunction) which is also similar to those of well-known antidepressants (Dilsaver and Greden, 1984).

In addition, central cholinomimetics display antimanic properties as opposed to antidepressant effects of anticholinergics (Fritze and Beckmann, 1998). Previous studies have also shown that the cholinergic system of depressed subjects is supersensitive compared to those without depression. However, it is still unclear whether this is a cause or a consequence of depression (Daws and Overstreet, 1999). A normal subject's response to pharmacologically induced central cholinergic overdrive is not necessarily relevant to the question of whether central cholinergic overdrive plays a role in the genesis of affective disorders like depression. Furthermore, subjects that develop a depressed mood in response to cholinergic agents may possess pre-existing aberrations at central cholinergic loci, resembling those of affective disorder patients. These aberrations may, however, not be specific to an affective disorder. Such disturbances may permissibly promote development of affective disorders; alternatively they could be a factor or one of a group of factors necessary, but not sufficient, to produce affective illness (Dilsaver, 1986).

Hypercortisolaemia and the unresponsiveness of the limbic-hypothalamic-pituitary axis (LHPA) to feedback inhibition are confirmed features of depressive illness (Rudorfer et al., 1982). The cleavage of pro-opiomelanocortin forms peptides which are co-released along the LHPA. These peptides interact with one another and can undergo selective cleavage, resulting in the formation of ACTH and β-endorphin, which are then co-released and co-regulated peptides (Kalin et al., 1982). Muscarinic agonists are able to stimulate corticotrophin releasing factor (CRF) both in vivo and in vitro. Atropine crystals implantation into the anterior hypothalamus inhibits stress-induced ACTH secretion, suggesting that acetylcholine activates the LHPA axis under certain conditions (e.g. baseline stress in response to diurnal changes). It is also known that depressed
patients show a greater physostigmine-induced increase in \(\beta\)-endorphin release by compared to those of normal patients. It has been demonstrated that patients with affective disorder exhibit concurrent behavioural and neuroendocrine hyperresponsiveness to physostigmine as compared to normal subjects (control) (Dilsaver, 1986).

Several cholinergic systems that are distributed in different parts of the brain seem to be involved in certain aspects of depression. The cholinergic system in the basal forebrain has dense projections to the core limbic structures such as the hippocampus and the amygdala. As illustrated in Fig 2.4, these systems may be implicated in depression since they are important for learning, cognition, planning, fear and motivation, all of which are affected by depression. A large cholinergic component can also be found in the reticular activating system (RAS) which is involved in sleep and behavioural arousal. Dilsaver (1986) suggested that this system’s interaction with the monoaminergic system may contribute to depression and mania.

Sleep abnormalities are also very common in patients that are suffering from depressive disorder. These abnormalities can be produced by cholinoreceptor blockade (McCarley, 1982). Sitram and Gilian (1980) reports that anticholinesterases produce subjective disturbances in sleep continuity and are also able to produce vivid terrifying dreams, showing this pathway may be involved in depressive disorders.

Cholinergic interneurons are also located in the nucleus accumbens, an important brain area since it mediates aspects of motivation and reinforcement. These interneurons are well-suited for controlling the transmission of limbic information to the accumbens target nuclei, because they are able to interact with mesencephalic dopamine as well as medial thalamus and limbic cortex glutaminergic inputs.

Cholinomimetics were found to exacerbate behavioural depression in animals that was subjected to the Porsolt swimming test, while antimuscarinic agents and several antidepressants inhibited this response. This again shows that the cholinergic pathway plays an important role in the aetiology of depression (Chau et al., 2001).
2.3.1.3.4 GABA Hypothesis

As already mentioned a major group of cholinergic interneurons, located in the neostriatum can directly alter the excitability of GABA output neurons, (Chau, et al., 2001), this gave raise to a more recent hypothesis that proposes that GABA is reduced in mood disorders, in particular also in depression. The importance of GABA may lie in its ability to act as a glutamate inhibitor. Shaikh and Yatham (1998) suggested that brain GABA activity is involved in local interneurons and circuits which may lead to the facilitatory effect on serotonin or noradrenergic function.

This hypothesis is supported by the following evidence:

a) GABA agonist administration caused an increase in 5HT and l-NE release and cortical serotonin 5-HT2 receptor (5HT2-R) density in various animal models.

b) GABA agonists or potentiators (e.g. alprasolam, a benzodiazepine) are effective in the treatment of affective disorders.

Since the discovery that plasma GABA levels are low in bipolar and unipolar depressives, Petty (1995) proposed that excessive glutamate/low GABA tone may be a genetic marker of vulnerability for the development or recurrence of mood disorders in a subgroup of patients (Petty, 1995). This can be supported by the fact that GABA potentiators, such as alprasolam (Reynolds, 1989), are effective in the treatment of depression and can also enhance existing antidepressant therapy by reversing GABA hypofunction/glutamate hyperfunction (Harvey, 1996).

2.3.1.3.5 Glutamate Hypothesis

Significant evidence implicates the N-methyl-D-aspartate (NMDA-) glutamate pathway in affective disorders (Harvey, 1996). Structurally dissimilar antidepressants, including fluoxetine and imipramine, and electro-convulsive therapy (ECT) have been found to suppress glutamate activity at NMDA receptors by suppressing allosteric coupling between glycine and glutamate recognition sites in the NMDA ion channel (Skolnick, 1999).

Skolnick also found that NMDA receptor antagonists possess antidepressant-like actions in various animal models. Regulation of NMDA glutaminergic mechanisms has
been implicated in the behavioural and adaptive neuronal response to antidepressants, suggesting that this pathway may play a key role in the neuropathology of affective disorders (Skolnick, 1999).

2.3.1.3.6 Neuroplasticity Hypothesis

A more recent hypothesis implicates the alteration of the neuroplasticity in different brain areas that are involved mood control and dysfunctions thereof (e.g. depression). Experimental studies in animals submitted to emotionally intense experiences, as well as post-mortem analysis of certain brain areas taken from depressed patients support this hypothesis. A point worth mentioning is that post mortem analysis taken from depressed patients showed a decrease in adult neurogenesis in the hippocampus, atrophy of pyramidal neurons in the hippocampus, reduction in the density of glia and size of neurons in the prefrontal cortex. All of these pathophysiological changes may contribute to the morphological changes of the prefrontal cortex, the amygdala and the hippocampus, as has been observed in brain imaging studies that was done by Olië et al., 2004 on depressed patients.

2.3.1.4 Neuroanatomy of Depression

Although there is little reliable evidence to suggest that major depression is accompanied by structural abnormalities in the central nervous system (CNS), Berkov and Fletcher (1992) implicated the limbic system in the aetiology of primary mood disorders, such as depression.

The evidence for pathophysiological changes in depression, as mentioned above, would also implicate the limbic system in depression (Olië et al., 2004). The limbic system (together with other brain structures in a complex system) is responsible for several brain functions such as planning, cognition, stress, fear and memory (see illustration in Figure. 2-5)
Figure 2-5 illustrates that planning, cognition, stress memory and fear are all part/consequences of the limbic system. The arrowsheads show the direction in which the structures are innervated to one another and how they interact to form part of the limbic system in order to exert the first mentioned actions.

Figure 2-5 The relation between the limbic system and other brain structures involved in memory, planning, cognition, stress and fear (Azitma, 2004).

In order to better understand the orientation of the limbic system and the brain structures involved, fig 2-6 shows the 3D orientation, together with a short description of every structure and its involvement in the limbic system.
Corpus callosum is the densest region of the brain, connecting the two hemispheres. Not a part of the limbic system.

Corpus callosum contains pleasure centers, particularly sexual ones.

The thalamus is an important gathering place for sensory information before distribution to higher areas.

Hippocampus is involved mainly with memory.

The major brain structures associated with the limbic system

Figure 2-6 The major brain structures associated with the limbic system and their orientation (Walsh, 2004).

It is generally accepted that the limbic system plays a very important role in the homeostasis of brain functioning. To better understand the involvement of the limbic system in mood disorders, such as depression, the possible role of the hippocampus, amygdala and the frontal cortex in depression will be discussed in more detail.

2.3.1.4.1 Hippocampus

As one of the most extensively studied structures in patients suffering from mood disorders, researchers found the hippocampus to be involved in episodic, contextual and spatial learning and memory, with deficits in these functions in depressed patients, implicating this structure in mood disorders such as depression.

By using magnetic resonance imaging (MRI), researchers have found significant reductions in hippocampal volume in depressed patients. However, others found no changes in hippocampal volume (Bremner et al., 2000; Mervaala et al., 2000; Sheline et al., 1996). One possible explanation for these volumetric discrepancies may be contributed to methodological differences. It is also worth mentioning that in one study
hippocampal volume loss was only observed in patients suffering from chronic depression, but not in those with remitted depression (Olië et al., 2004).

2.3.1.4.2 Amygdala

Since the amygdala merges with the surrounding cortex (cortical amygdala), it is difficult to determine specific boundaries, causing wide-ranging results between studies. This will explain the varying results found with regards to amygdala volumes in major depression.

Functional neuroimaging studies have demonstrated that the amygdala is activated during negative affective states such as sadness and anxiety. A very interesting point is that depressed patients present with hyperarousal in the left amygdala, even when processing stimuli in unconscious awareness. This hyperarousal is normalized by antidepressant treatment. In addition, further support for the role of the amygdala is found in functional studies that have shown that the amygdala is involved in the generation of responses to emotional stimuli such as fearful faces. Bilateral damage to the amygdala impairs the processing of fearful facial expressions (Olië et al., 2004).

2.3.1.4.3 Frontal Cortex

The prefrontal cortex is a particularly important target of monoamine projections. Abnormalities of monoamine receptors, transporters and second messenger systems are reported to occur in major depression. Frontal cortex volume reductions in major depressed patients have also been reported with an overall reduction of 7% in frontal lobe volume and up to 48% in the subgenual prefrontal cortex. It is also worth mentioning that the orbitomedial prefrontal cortex has a high concentration of glutocorticoid receptors, potentially rendering it vulnerable to stress-mediated damage (Olië et al., 2004).

From all of the above it is clear that dysfunction in the limbic system and its structures play an important role in the pathophysiology of mood disorders.
2.3.2 Other Anxiety and Anxiety-Related Disorders

The word anxiety is derived from the Latin, angere, which means to choke or strangle. Anxiety disorders affect people of all ages and involve feelings of unrealistic fear and worry. It can also interfere with sleep, concentration, appetite, libido and overall quality of life.

Recent studies suggest that some patients may be biochemically more susceptible to the development of anxiety symptoms in the presence of particular diseases, including adrenal dysfunction, Cushing's disease, hyperglycaemia, pancreatic tumours, phaeochromocytoma and several thyroid diseases, including hypo/hyperthyroidism and thyroiditis.

Although anxiety is capable of paralyzing the individual into inaction or withdrawal, it is often not attributable to a real threat (McCarthy et al., 2001). Anxiety disorders have been classified according to the severity and duration of their symptoms and specific behavioural characteristics. Categories include:

1. Generalized anxiety disorder (GAD),
2. Panic disorder
3. Phobias,
4. Obsessive-compulsive disorder (OCD),
5. Post-traumatic stress disorder (PTSD).

The two most common forms of the anxiety and related disorders are GAD and panic disorder. These disorders are the consequence of a combination of psychological, physical, and genetic factors, and the treatment is generally very effective.

When looking at the treatment of anxiety disorders, benzodiazepines are the most broadly effective medication for most anxiety-related disorders; including panic disorder, agoraphobia and GAD. However, traditional antidepressants, such as the SSRIs, are increasingly being used as the initial treatment for anxiety-related disorders. SSRI treatment has the advantage of being the less addictive and having relative minor side effects compared to that of the benzodiazepines (McCarthy et al., 2001)
2.3.2.1 Generalized Anxiety Disorder (GAD)

People with GAD may be likely to experience bouts of depression between episodes of anxiety. GAD symptoms may cause significant distress and impair normal functioning which is not due to a medical condition or to another mood disorder or psychosis. GAD can be characterised by a more-or-less constant state of worry and anxiety, where subjects find it extremely difficult to control worry (DSM-IV-TR., 2000). This state occurs on most days for more than six months, despite the lack of an obvious or specific stressor. Symptoms include gastrointestinal complaints, difficulty in concentrating, irritability, muscle tension and disturbed sleep. People with GAD tend to be unsure of themselves and overly perfectionistic and conforming (DSM-IV-TR., 2000).

Some researchers have also reported that patients with GAD have higher plasma catecholamine levels than normal controls. These patients may downregulate catecholamine receptors as a result of these higher plasma concentrations and thus experience reduced receptor sensitivity in the adrenergic nervous system.

2.3.2.2 Panic Disorders

The essential feature of panic disorder is the presence of recurrent, unexpected panic attacks followed by at least one month of persistent concern about having another panic attack and worrying about the possible consequences or implications of the attacks or any significant behavioural changes related to the attacks (DSM-IV-TR., 2000).

This disorder is characterized by periodic attacks of anxiety or terror (panic attacks) that usually last 15 to 30 minutes. The frequency and severity of acute states of anxiety determine the diagnosis. It is also worth mentioning that panic attacks can occur in nearly every anxiety-related disorder. In other anxiety-related disorders, however, there is always a cue or specific trigger for the attack (McCarthy et al., 2001).

2.3.2.3 Phobic Disorders

Phobias are common anxiety-related disorders and are manifested by overwhelming and irrational fears. In most cases, phobic situations can be avoided, but in other cases,
such as agoraphobia, the anxiety associated with the feared object or situation can be incapacitating.

- **Agoraphobia** is characterized by a paralyzing terror of being in places or situations from which the patient feels there is neither escape nor accessible help in case of an attack. Therefore people with agoraphobia confine themselves to places in which they feel safe, usually at home.

- **Social Phobia** is also known as social anxiety disorder. It is the fear of being publicly scrutinized and humiliated and is manifested by extreme shyness and discomfort in social settings. This phobia includes performance anxiety, also known as stage freight. It often causes individuals to avoid social situations and is not due to a physical or mental problem.

- **Specific Phobias** were formally known as simple phobias. Specific Phobias are irrational fear of specific situations or objects. Although most cases are mild some may require treatment. Specific Phobias includes the fear of:
  - heights (**acrophobia**),
  - water, injections, public transportation, confined spaces (**claustrophobia**),
  - dentists (**odontiatophobia**),
  - flying (**pterigophobia**),
  - animals (usually spiders, snakes, or mice),

When confronted with the object or situation, the phobic person may experience panic, excessive sweating, breathing difficulty and sometimes palpitations (McCarthy et al., 2001).

### 2.3.2.4 Obsessive-Compulsive Disorder

Obsessive-compulsive disorder (OCD) has been described as hiccups of the mind, including an over inflated sense of responsibility, in which the patient's thoughts centre around possible dangers and an urgent need to do something about it (McCarthy et al., 2001). Essential features of OCD includes recurrent obsessions that are severe enough to be time consuming (taking more than one hour per day) or cause marked distress or significant impairment (DSM-IV-TR., 2000).
• Obsessions are persistent or recurrent mental images, ideas or thoughts, ranging from mundane worries about thoughts, impulses or images that are experienced as intrusive and inappropriate, that can cause marked anxiety or distress.

• Compulsive behaviours are repetitive, rigid, and self-prescribed routines that are intended to prevent the manifestation of an associated obsession. Some people are compelled to wash their hands every few minutes or to spend inordinate amounts of time cleaning their surroundings in order to subdue the fear of contagion.

OCD often accompanies depression or other anxiety disorders. There is some evidence that the symptoms improve over time and that nearly half will eventually recover completely or have only minor symptoms. Serotonin is a major player in OCD.

OCD should not be confused with obsessive-compulsive personality, which defines certain character traits (e.g. being a perfectionist, excessively consciousness, morally rigid or preoccupied with rules and order (McCarthy et al., 2001).

2.3.2.5 Post-Traumatic Stress Disorder

PTSD is triggered by violent or traumatic events that are usually outside the norm of human experience. The person’s response to the event/stressor usually involves intense fear, helplessness or horror, especially in children.

The symptoms are the same whether the triggering event is a violent action (being kidnapped, military combat or a personal assault), or natural or man-made disasters (DSM-IV-TR., 2000).

2.3.3 G-Protein Coupled Receptors (GPCRs)

G-protein coupled receptors (GPCRs), such as mAChR and 5-HT receptors, have been implicated in mood disorders. It is therefore important to gain a better understanding of sildenafil’s role in GPCR signalling and how it influences several neurotransmitter systems implicated in depression (e.g. 5-HT, I-NE and acetylcholine).
GPCRs is a superfamily of receptors that are characterised structurally by their seven transmembrane helices that are connected by three intracellular and three extracellular loops, as well as an extracellular NH$_2$ terminal and an intracellular COOH terminal. These receptors are known to couple to signalling heterotrimeric GTP-binding proteins (G proteins - consisting of an α-, β-, and γ-subunit) that, upon activation, stimulate effectors such as second messenger systems.

### 2.3.3.1 G-Protein Coupling

![Diversity of G-protein-coupled receptors (GPCRs)](image)

**Figure 2-7 Diversity of G-protein-coupled receptors (GPCRs) - adopted from Marinissen and Gutkind (2001).** Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, leutinizing hormone; LPA, lysophosphatidic acid; PAF, plateletactivating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone. (Marinissen and Gutkind, 2001).

A wide variety of ligands, including biogenic amines, amino acids, ions, lipids, peptides and proteins bind to GPCRs to activate cytoplasmic and nuclear targets via
heterotrimeric G-protein-dependent and -independent pathways. Such signalling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis.

2.3.3.2 G-Protein Coupled Receptor Signalling

Upon the stimulation of GPCRs, it couples to and activates G-proteins, which leads to a dissociation into a GTP-bound Go and a Gβγ subunit. Both of these subunits are active and can interact with different effector molecules, including adenylyl and guanylyl cyclases (production of second messengers CAMP and cGMP), phosphodiesterases (catabolism of cAMP and cGMP), phospholipase A2 (PLA2), phospholipase C (PLC - production of second messengers phosphatidyl inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG)), phosphoinositide 3-kinases (PI3Ks) and ion channels (Fig. 2.7) (Marinissen and Gutkind, 2001).

The GTP bound to the Go-subunit is rapidly hydrolyzed to GDP by intrinsic GTPase-activity and the G-protein cycle is terminated by the re-association of GDP-Go and the Gβγ subunit. Importantly, however, the intrinsic GTPase-activity of the Go subunit is barely sufficient to hydrolyze GTP as rapidly as required, but this property is greatly enhanced by additional proteins which can either be a target protein (e.g. phospholipase Cβ1) or a specific modulator known as a regulator of G-protein signalling (RGS).

There are several mechanisms by which the GPCR can be regulated; they include the regulation by PIP3 and Ca2+/CaM, the regulation by 14-3-3, the regulation by PDZ domain and the regulation by the GGL domain.

2.3.4 Muscarinic Acetyl Choline Receptors (mAChR)

There is no truly inclusive hypothesis to describe all the different mechanisms by which antidepressant drugs are proposed to act. The cholinergic hypothesis (see §2.3.1.3.3) is still one hypothesis that may explain a contributory mechanism of action for certain antidepressants. Brink et al. (2004) showed that antidepressants such as fluoxetine and imipramine are able to modulate mAChR function, probably by a
mechanism that involve the phosphatidyl inositol metabolic pathway. These data provided further support for the cholinergic hypothesis of depression and implicates GCPRs such as mAChR in the aetiology of depression.

More evidence in support of the cholinergic involvement in affective disorders is provided by the efficacy of antimuscarinic drugs (e.g. imipramine) in the treatment of depression (Goldman and Erickson, 1983), the euphorigenic properties of antimuscarinic agents that is frequently abused (Smith, 1980), and the antimuscarinic agent withdrawal effects (anxiety, sleep disturbances and rebound exacerbation of motor dysfunction) which is also similar as those of well known antidepressants (Dilsaver and Greden, 1984).

2.3.4.1 mAChR Families

mAChRs belong to the GPCR super family of receptors. These receptors mediate most of the actions of the neurotransmitter ACh in the CNS and peripheral nervous system. Gene cloning has revealed that five types of mammalian mAChRs exist, but only four have been distinguished as pharmacologically functional (Rang et al., 1999).

The odd number of the group namely M₁, M₅, and M₅ receptor subtypes are efficiently coupled to the pertussis toxin-insensitive Gαq/11 and Gα13 subtypes of G proteins, leading to, for example, activation of phospholipase C (PLC) and phospholipase D (PLD), thus exerting their effect through the phosphatidyl inositol metabolic pathway. On the other hand, M₂ and M₄ receptors preferentially couple to pertussis toxin-sensitive Gᵢ and Gₒ proteins, leading to the inhibition of adenylyl cyclase (Caulfield, 1993; Rümenapp et al., 2001) and eventually a reduction in cAMP (Rang et al., 1999).
Table 2-2 Subtypes of acetylcholine receptors – adapted from “Forth Edition Pharmacology” (Rang et al., 1999)

<table>
<thead>
<tr>
<th>Type</th>
<th>M₁ ‘neural’</th>
<th>M₂ ‘cardiac’</th>
<th>M₃ ‘glandular’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main actions</td>
<td>Neural</td>
<td>Cardiac</td>
<td>Exocrine glands</td>
</tr>
<tr>
<td></td>
<td>CNS (cortex, hippocampus)</td>
<td>Atria</td>
<td>Smooth muscle</td>
</tr>
<tr>
<td></td>
<td>Ganglia (enteric, autonomic)</td>
<td>Conducting tissue</td>
<td>Vascular endothelium</td>
</tr>
<tr>
<td></td>
<td>Gastric</td>
<td>Neural</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parietal cells</td>
<td>Presynaptic terminals</td>
<td></td>
</tr>
<tr>
<td>Effects Cellular</td>
<td>↑ IP₃ ; DAG ;</td>
<td>↓ cAMP</td>
<td>↑ IP₃</td>
</tr>
<tr>
<td></td>
<td>Depolarization</td>
<td>Inhibition (↑Gₖ, ↓ IₗCa)</td>
<td>Stimulation</td>
</tr>
<tr>
<td></td>
<td>Excitation (Slow epsp)</td>
<td>Slow ipsp</td>
<td>(↑ [Ca]₃)</td>
</tr>
<tr>
<td></td>
<td>(↓ Gₖ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional</td>
<td>CNS excitation</td>
<td>Cardiac Inhibition</td>
<td>Secretion</td>
</tr>
<tr>
<td></td>
<td>Gastric acid secretion</td>
<td>Presynaptic Inhibition</td>
<td>Smooth muscle</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal motility</td>
<td>Neural Inhibition</td>
<td>contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vasodilatation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(via NO)</td>
</tr>
<tr>
<td>Agonists</td>
<td>ACh</td>
<td>ACh</td>
<td>ACh</td>
</tr>
<tr>
<td></td>
<td>Oxotremorine</td>
<td>CCh</td>
<td>CCh</td>
</tr>
<tr>
<td></td>
<td>McN3A343</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antagonists</td>
<td>Atropine</td>
<td>Atropine</td>
<td>Atropine</td>
</tr>
<tr>
<td></td>
<td>Pirenzepine</td>
<td>Gallamine</td>
<td>Hexahydrosiladifenol</td>
</tr>
<tr>
<td></td>
<td>Dicyclomine</td>
<td>AF-DX 116</td>
<td></td>
</tr>
</tbody>
</table>

2.3.4.2 mAChRs - Function and Location

The main mAChRs that mediate ACh-induced MAP kinase activation in the CNS is M₁ mAChR. MAP kinase activation plays an important role in memory processing (Hamilton
M2 mAChRs are essential for mAChR-dependent bradycardia and contribute in a lesser extent to agonist-induced contraction of the stomach fundus, urinary bladder, and trachea (Stengel et al., 2000). M2 mAChRs can also be found in the heart (cardiac) and presynaptic terminals of peripheral and central neurons.

M3-receptors are responsible for the mediation of smooth muscle relaxation (mainly vascular) resulting from the release of NO from neighbouring endothelial cells (Rang et al., 1999). It is also worth mentioning that M3 mAChRs are also involved in the regulation of food intake and appetite (Yamada et al., 2001). M3 mAChRs play a key role in salivary secretion, pupillary constriction, and bladder detrusor contraction (Matsui et al., 2000).

Brain M4 mAChRs, like the M1 mAChRs, are also involved in the modulation of central dopaminergic responses. Gomez et al., 1999 found that M4 mAChR-deficient mice show an increase in basal locomotor activity and greatly enhanced locomotor responses after activation of D1 dopamine receptors. It also appears that M4 mAChRs play a negligible role in the regulation of peripheral smooth muscle tone (Stengel et al., 2000). mAChR-induced dopamine release in the striatum is facilitated by M5 mAChR agonists and modulates both morphine reward and withdrawal processes (Basile et al., 2002). M6 mAChRs are required for cholinergic dilation of cerebral blood arteries and arterioles (Yamada et al., 2001).

### 2.3.4.3 mAChR and Protein Kinase C (PKC)

Wall et al., (1992) have shown that the in vivo administration of mAChR antagonists, which blocks the action of the released ACh or induce the inactive state of mAChR can evoke mAChR up-regulation. Just like a large number of GPCRs, agonist-induced desensitization of mAChRs usually involves receptor phosphorylation (Haga et al., 1990; Kwatra and Hosey, 1986). Several protein kinases are able to phosphorylate mAChRs. This includes various GPCR kinases (GRKs), diacylglycerol-regulated PKC and casein kinase 1a (CK1a).
It has been shown that $M_1$ and $M_3$ mAChRs can be phosphorylated by PKC in an agonist-independent and $G\beta\gamma$-independent manner, both in vitro and in vivo (Haga et al., 1996; Richardson and Hosey, 1990; Uchiyama et al., 1990). In contrast to PKC, GRKs phosphorylate only agonist occupied receptors, leading to the binding of arrestins, which sterically suppress further G protein interaction, thus terminating the signal.

GRKs includes the following:

- GRK1 (or rhodopsin kinase) and GRK7 (a cone opsin kinase), both of which are exclusively expressed in retina;
- GRK2 (or β-adrenergic receptor kinase-1, bARK1) and GRK3 (or bARK2), both ubiquitously expressed;
- GRK4 (expressed predominantly in the testes and brain); and the ubiquitously expressed GRK5 and GRK6 (Ferguson, 2001).

Koenig and Edwardson, (1996) have shown that prolonged activation of mAChRs does not only facilitate uncoupling of the receptor from the G-protein, but can also induce mAChR internalization in a large number of cell types.

### 2.3.5 Current Drug Therapy of Depression:

Although no uniform classification system for antidepressants is currently available, antidepressants can be divided into four basis groups namely: 1) selective serotonin reuptake inhibitors (SSRIs), 2) tricyclic antidepressants, (TCAs) 3) monoamine oxidase inhibitors (MAOls) and 4) novel/atypical antidepressants.

The existence and supporting data for the various hypothesis of the pathophysiology of depression, suggest that it would be possible to achieve therapeutic effects by antidepressants via the manipulation of a variety of systems, including serotonergic, dopaminergic, noradrenergic, cholinergic and other distinct neurochemical components (Figber, 1995). The following drug groups that are currently used in the treatment of depression will be discussed: SSRIs, TCAs, MAOls and atypical antidepressants.
2.3.5.1 Selective Serotonin Reuptake Inhibitors

SSRIs include examples of drugs such as fluoxetine, sertraline, paroxetine, citalopram and fluvoxamine. As current first line treatment in the management of depression (Kamil et al., 1996), SSRIs are a heterogeneous group of compounds that has a selective effect on the reuptake of 5HT in presynaptic neurons. All of these compounds are structurally distinct entities with fewer antihistaminic, α-adrenergic, and anticholinergic effects as compared to the TCAs (Harvey, 1997; Kamil, 1996).

2.3.5.2 Tricylic Antidepressants

TCAs include examples of drugs such as amitriptyline, doxepin, and imipramine. All TCAs inhibit the presynaptic reuptake of monoamine neurotransmitters e.g. l-NE and 5HT, thereby enhancing the availability of these monoamines. However, not all TCAs affect the monoamines equally, for example, imipramine and amitriptyline affect 5HT and l-NE reuptake almost equally while others preferentially affect l-NE. Regardless of their varying action on l-NE and 5HT reuptake, most TCAs are equivalent in their antidepressant action (Kamil, 1996).

It is also worth mentioning that these compounds also have affinity for several other receptors, including histamine type 1 receptors (H1-Rs), α-ARs and mAChRs (Kamil., 1996). The TCAs are potent antagonists at these receptors and this antagonism is primarily responsible for the side effects and toxicity of the TCAs (Leonard, 1997).

2.3.5.3 Monoamine Oxidase Inhibitors

The primary action of monoamine oxidase inhibitors (MAOIs) is to block the action of monoamine oxidase (MAO), a mitochondrial enzyme responsible for the degradation of biogenic monoamines (i.e. l-NE, DA, 5HT and melatonin), thereby increasing the postsynaptic availability of these neurotransmitters (Leonard, 1997).

Two subtypes of MAO exist, namely MAO-A and MAO-B. The two different subtypes are responsible for the catabolism of different monoamines; MAO-A catabolises l-NE, 5-HT and DA while MAO-B is responsible for the catabolism of phenylethylamine, DA and tyramine (Kamil, 1996). MAOIs can be divided into selective/nonselective and either
reversible or irreversible. A selective MAOI can inhibit either MAO-A or MOA-B while a nonselective MAOI has the ability to inhibit both. Irreversible MAOIs inhibit the enzyme irreversibly, so that new enzymes must be synthesized to overcome the effect. Examples of reversible MAO-A inhibitors include moclobemide and brofarimine, while selegiline acts as a irreversible MAO-B inhibitor (Rang et al., 1999). Irreversible MAOIs include isocarboxazid, pargyline, phenelzine and tranylcypromine (Kamil, 1996).

2.3.5.4 Novel/atypical Antidepressants

Atypical antidepressant drugs do not share structural or functional similarities with the TCAs, SSRIs or MAOIs. Trazodone and nefazodone are examples of novel antidepressants. They are weak 5HT reuptake antagonists but are potent 5-HT2-R antagonists (Harvey, 1997). It has also been shown that the active metabolite of trazodone, m-chlorophenylpiperazine, has affinity at serotonin 5-HT1-Rs and 5-HT2-Rs and may also indirectly facilitate noradrenergic transmission (Baldessarini, 1996).

2.3.6 Drugs Used in Anxiety

2.3.6.1 Treatment of Generalized Anxiety Disorder

As mentioned above, people with GAD may be likely to experience bouts of depression between episodes of anxiety. Drugs used in the treatment of GAD, includes benzodiazepines, such as alprazolam, clonazepam, diazepam, lorazepam and chlor Diazepam. Another unique anti-anxiety agent belonging to the azapirones is buspirone. Buspirone is not very effective in the treatment of panic attacks, but clinical studies have demonstrated that is just as effective in the treatment of GAD as the benzodiazepines. Venlafaxine, a designer antidepressant that inhibits the reuptake of both I-NE and 5HT (NSRI), has also shown the ability to reduce anxiety in patients with GAD (as with all the SSRIs), but it still has the side effect of sexual impairment.

Imipramine, a TCA, has also been shown to be effective in the treatment of anxiety disorders (McCarthy et al., 2001).
2.3.6.2 Treatment of Panic disorder

Panic disorder can be treated with several drugs, including the SSRls, benzodiazepines, TADs, MAOls and anticonvulsants. The most frequently used SSRls for this disorder include fluvoxamine and sertraline, but with typical side-effects such as nausea and sexual dysfunction. Alprazolam and clonazepam are the benzodiazepines used in panic disorder. Imipramine, which can also be used in the treatment of GAD, is the most commonly used TCA for panic disorder, although clomipramine, is also effective in patients suffering from panic disorder. MAOls used include phenelzine or tranylcypromine (McCarthy et al., 2001).

2.3.6.3 Treatment of Phobic disorders

Peripheral symptoms are treated with β-AR blockers, such as propanalol and atenolol, to control the peripheral symptoms of anxiety, including a reduction in heart rate and, in the case of propranolol, skeletal muscle tremors. Other treatments for phobias include the benzodiazepines (alprazolam and clonazeepam) and SSRls (fluoxetine and sertraline) (McCarthy et al., 2001).

2.3.6.4 Treatment of Obsessive Compulsive Disorders

With OCD, SSRls is recommended as first line treatment, reducing the symptoms by 25-30%. SSRls used includes fluoxetine, sertraline, citalopram and fluvoxamine. Clomipramine has been shown to be effective in the treatment of OCD. However, other TCAs do not appear to show benefit in OCD patients (McCarthy et al., 2001).

2.3.6.5 Treatment of Post Traumatic Stress Disorder

Currently the only SSRls approved by the FDA for the treatment of PTSD are sertraline and paroxetine. Clonidine has also been used in the treatment of PTSD (McCarthy et al., 2001).
2.3.7 Sildenafil as Possible Antidepressant or Antidepressant Modulator?

Inhibitors of the down-stream activation of NOS and cGMP formation, including NOS (Harkin et al., 1999) and GC-cGMP (Heiberg et al., 2002) inhibitors have all demonstrated distinct antidepressant-like effects in animal models of depression (Harvey, 1996). Sildenafil exerts its effects through the NO-cGMP pathway, which has been implicated in depression. However, there is insufficient data to suggest a direct effect of sildenafil on depression. From studies in rats Hirata et al., (1990) concluded that cGMP pre-treatment leads to the inhibition of agonist-stimulated G-protein activation, as well as the interactions between the G-protein and phospholipase C. These effects were probably mediated through the activation of cGMP-dependent protein kinase and protein kinase C respectively, implicating cGMP’s role in G-protein receptor signalling. Treatment of rats with the mood stabilizing drug, LiCl was found to increase cGMP levels with a reciprocal reduction of cAMP (Harvey et al., 1990; 1994), suggesting a possible role for NO-cGMP in the action of the drug in affective illness.

mACHRs and serotonergic receptors (5HT-Rs) are known to be involved in antidepressant action. These GPCRs signal through the phosphatidylinositol signal transduction pathway and since the NO-guanylyl cyclase signal transduction pathway is also known to involve the activation of protein kinases via GTP, a scope for sildenafil to possibly modulate the action of antidepressants by elevating GTP levels is opened.

2.4 Sildenafil and Cell Viability

Sildenafil is responsible for the inhibition of PDE5 and cGMP breakdown, thus leading to an increase of cGMP concentration. cGMP has been implicated in neuroprotection, secondary to NO production (Kim et al., 1999). It is therefore possible for sildenafil to display neuroprotective properties. To better understand cGMP’s role in neuroprotection, neurodegeneration and the possible mechanisms in this process will be discussed in more detail.
2.4.1 Depression and Neurodegeneration

Neurodegeneration disturbs all major coping strategies in the brain and can result in major depression or treatment-resistant depression. This process is reinforced by the neurotoxic effect of high cortisone levels during stress (Watanabe et al., 1992; Luine, 1994). This depression-neurodegeneration hypothesis is supported by the findings of Bremner and colleagues (Bremner et al., 2000), and Sheline and co-workers (Sheline, 1996; Sheline et al., 1999) who have found that the atrophy of hippocampus in major depression increases with longer duration of depression. It is also supported by the findings that neurogenesis occurs in brain structures that exhibit a high degree of neural plasticity, particularly the hippocampus (Eriksson et al., 1998).

Post mortem analysis in depressed patients show a decrease in adult neurogenesis in the hippocampus, atrophy of pyramidal neurons in the hippocampus, reduction in the density of glia and reduction of neuron size in prefrontal cortex, therefore implicating neurodegeneration in the aetiology of depression (Olie et al., 2004).

Interesting data suggesting the possible role of cGMP in depression is that sildenafil has been found to exacerbate anxiety (Volke et al., 2003; Kurt et al., 2004) while studies done at the North West University, Potchefstroom campus, have found that sildenafil can exacerbate stress evoked hippocampal nitric oxide synthase activation in rats (Bothma, 2004).

2.4.2 Mechanisms of Neurodegeneration and Protection

Persistent stimulation of N-methyl-D-Aspartate (NMDA) receptors by excessive levels of excitatory amino acids such as aspartate or glutamate, as well as their analogues causes neuronal damage. This is triggered by the influx of Ca2+ into the cell after NMDA receptor activation, subsequently causing membrane depolarization (Rothman and Olney, 1997). This process is described in figure 2-2.

Neuroprotection can be facilitated via multiple pathways. Caspase inhibitors have been shown to inhibit apoptotic cell death of cultured neurons in response to several stimuli, including trophic factor deprivation (Wirtz-Brugger and Giovanni, 2000).
NO is a synaptic signalling molecule in the nervous system that can act as an endogenous inhibitor of apoptosis in many cell types (Wirtz-Brugger and Giovanni, 2000). According to Kim et al., (1999), the underlying mechanism for this survival promoting effect appears to involve the activation of guanylyl cyclase (GC) and the generation of cGMP. These studies suggest that cyclic nucleotides such as cGMP, and possibly cAMP, play an important role in promoting cell survival in these models (Wirtz-Brugger and Giovanni, 2000).

The brain has two different corticosteroid receptor types that are able to mediate the genomic effects of corticosteroids, i.e. the mineralocorticoid receptor (MR), and the glucocorticoid receptor (GR). The MRs, are localized particularly in neurons of the limbic system including the hippocampus and bind corticosterone with high affinity. GR expression is much more widespread, and is also localized at high concentrations in the hippocampus, binding glucocorticoids with a lower affinity than MRs. Corticosteroids have an effect on the activity of the stress-adaptive and stress-responsive hypothalamus–pituitary–adrenal (HPA) system, which is mainly regulated by the hippocampus. It is worth reminding that depressed patients show alterations in the HPA system (Behl, 1998). Stress evoked hippocampal atrophy evidence in depression has been associated with hypercortisolism, followed by an increase in glutamate and NO release (Olié et al., 2004).

2.4.3 Reactive Oxygen Species (ROS), Free Radicals and Oxidative Stress

Since aerobic cells in general (neurons in particular) are permanently challenged by exogenous oxidative stressors, cellular antioxidant defence mechanisms provided by enzymes (e.g., catalase, superoxide dismutase) and other compounds (e.g., vitamins A & G and glutathione) in these cells have to maintain an equilibrium between reactive oxygen species (ROS) formation and detoxification in order to keep the cell functional and alive (Behl, 1998). This finely-tuned balance can be disturbed by exogenous insults and intracellular alterations which can lead to oxidative stress.

The accumulation of ROS or free radicals in the brain renders the brain vulnerable to oxidative stress. This can be attributed to the fact that neuronal membranes contain high
concentrations of polyunsaturated fatty acids that are substrates for peroxidation reactions by ROS or free radicals. There are numerous sources of oxygen free radicals, which include several neuronal tissues. ROS are regularly formed in the oxidative phosphorylation during ATP formation, but can also be generated by various enzymatic (e.g., oxidases, lipooxygenases) and nonenzymatic (e.g., catecholamine metabolism) mechanisms. Ultimately, it is the very reactive hydroxyl radical generated from hydrogen peroxide that causes the peroxidation of lipids leading to the lyses of the cell in most cases (Behl, 1998).

2.4.4 NO-cGMP Pathway and Neuroprotection

As mentioned in § 2.3.3, radicals generated from hydrogen peroxide as well as free radicals and ROS can cause the peroxidation of lipids, leading to the lyses of the cells (Behl, 1998).

While the neurotoxic potential of NO is well recognised, NO is also able to play the role of endogenous inducer or inhibitor of apoptosis in many cell types. However, it is clear that toxicity is not mediated by NO itself, but by its reaction products with O₂ and ROS.

Excess NO production over peroxide formation will mitigate peroxynitrate-induced membrane toxicity (Gordge, 1998). It is important to note that this can only occur when large amounts of NO are produced. Under normal physiological conditions, NO can reduce or inhibit oxidative stress toxicity by binding to alkoxyl and peroxy radicals to form non-radical nitroso derivatives (Gordge, 1998).

According to Prast and Philippu (2000), the underlying mechanism of NO’s neuroprotective property seems to involve the production of cGMP.

NO donors and NO synthase inhibitor’s use have revealed that NO can act as a free radical which is probably implicated in the regulation of firing and excitability, long-term potentiation (LTP) and long-term depression (LTD) (Prast and Philippu, 2000).
3.1 Introduction

The main objective of the study was to investigate the modulating effect of sildenafil on cell viability and on the function of selected pharmacological receptors in cell cultures.

Cell viability studies were done in a human neuroblastoma cell line (SH-SY5Y), applying the Trypan blue method before and after pre-treatment of the cells with different drugs (including sildenafil) in serum-free medium. Drugs used in the pre-treatments were selected according of their action on the NO-cGMP pathway and cGMP production. It is noteworthy to mention that the propidium iodide and MTT tests were also used to investigate cell viability after drug pre-treatments.

Functional studies were done on both SH-SY5Y and HeLa cell lines that endogenously express the muscarinic acetylcholine receptor (mAChR). The cells were pre-treated with different drugs in serum-free medium, followed by the whole cell \([^3H]\)-IP\(_x\) assay which was used to construct dose-response curves (DRC) of the mAChR agonist methacholine (MeCh) in the SH-SH5Y cells and to determine minimum and maximum responses in the HeLa cell line.
3.2 Materials and Instruments

3.2.1 Cell lines used

SH-SY5Y (ATCC Number: CCL-2) and HeLa (ATCC Number: CRL-2266) cell lines were obtained from the American Type Culture Collection (American Type Culture Collection, U.S.A.)

3.2.1.1 SH-SY5Y cell line

SH-SY5Y is a human neuroblastoma cell line that endogenously expresses the muscarinic acetylcholine receptor (mACHR) (see below) and was obtained from the American Type Culture Collection (American Type Culture Collection, catalogue number CRL-2266). This neuronal cell line was established in 1970 and is a trice-cloned sub-line of the neuroblasoma cell line SK-N-SH (SK-N-SH → SH-SY→ SH-SY5→ SH-SY5Y) (American Type Culture Collection, 2004).

The primary reasons for the selection of this cell line in this study:

➢ SH-SY5Y is a neuronal cell line and therefore related to cells of the central nervous system (CNS), suitable for the investigation of possible neuronal effects of sildenafil.

➢ M3-mACHRs (Slowiejko et al., 1996) are endogenously expressed in the SH-SY5Y cell line, with some evidence for M1 and M2-mACHRs (Kukkonen et al., 1992).

The SH-SY5Y cells were maintained in 1:1 DMEM:Ham's F12, 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone in cell culture flasks at 37°C in 5% CO2 and humidified environment. Under these conditions the cell duplication time is approximately 48 hours.
3.2.1.2 HeLa cell line:

HeLa is a human non-neuronal adenocarcinoma cell line also obtained from the American Type Culture Collection (catalogue number CRL-2266). In this study, the HeLa cell line was used to compare the effect of sildenafil pre-treatments on receptor function in non-neuronal cells to its effect in neuronal SH-SY5Y cells.

HeLa cells were maintained in similar conditions as that of the to SH-SY5Y cells (above), except that using DMEM was used as growth medium. Under these conditions the cell duplication time was approximately 36 hours.

3.2.2 Radiochemicals

myo-[2-3H]-inositol ([3H]-mlns) (17 Ci/mmol) was obtained from AEC Amersham, (Johannesburg, South Africa). [2-3]-adenine (19-23 Ci/mmol) was obtained from Amersham Pharmacia Biotech (U.K.).

3.2.3 Other Chemicals

myo-Inositol (mlns), metacholine (MeCh), trichloroacetic acid (TCA), N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES), lithium chloride (LiCl), N2,2′-O-Dibutyrylguanosine 3′-5′-cyclic monophosphate (db-cGMP), Propidium iodide, MTT, dipyridamole, guanosine, zaprinast, IBMX, ATP, cAMP, UK 14,034, Dowex 1x8-400, 200-400 mesh (1-chloride form) was obtained from Sigma Aldrich (Johannesburg, South Africa). Dulbecco's Modified Eagles Medium (DMEM), Ham's F12, foetal calf serum (FCS), Minimum Essential Media with Earle's Base (EMEM), trypsin-verse (0.05% trypsin 1:250 + 0.02% EDTA) and bovine serum albumin was obtained from Highveld Biologicals (Johannesburg, South Africa). Sodium-citrate was kindly provided by the Division of Biochemistry (North-West University, South Africa). Penicillin, streptomycin and fungizone were obtained from Bio-Wittaker (Walkersville, MD, U.S.A.). NaCl, KCl, Na2HCO3, formic acid and acetone were obtained from Merck
(Johannesburg, South Africa). Ultima Gold XR scintillation fluid was obtained from Packard BioScience (Meriden, CT, U.S.A.).

3.2.4 Consumables

Consumables such as 24-well plates, 150 cm² culture flasks, 50 ml sterile conical tubes and serological pipettes, etc. were obtained from Corning (New York, U.S.A.). Cover slips were obtained from LASEC (Johannesburg).

3.2.5 Instruments Used

Tri-carb 2100TR liquid scintillation analyser (Packard, A.D.P. South Africa) was used for the functional studies. Cell viability (counts) was done with a 0.1 mm dept, 0.0025 cm² haematocytometer and a Nikon TMS inverted microscope (model number: 31771). Eppendorf pipettes (10–100 uM, 100–1000 uM and multi pipette) were used for analytical measurements. For spectroscopic analyses, a 96-well spectroscopic plate reader with a 560 nm filter was used, obtained from Labsystems Multiskan RC.

3.2.6 Statistical Analyses

3.3 Assays:

Experimental Layout

As indicated in Fig 3-1, (A) neuronal and non-neuronal cells were in 24 well plates and (B) incubated with different drugs for a period of 24 hours where after different experiments were initiated in order to achieve the objectives set in § 1.2. These experiments included, (C) cell viability (trypan blue, propidium iodide & MTT) and (B) receptor function (IP$_x$ production & cAMP production) experiments.

The experimental layout is schematically represented in Figure 3-1

![Experimental Layout Diagram]

Figure 3-1 Schematic layout of experimental procedures. Abbreviations: IP$_x$ = inositol-multiposphates; SS = sodium salicylate; IBMX = 3-isobutyl-1-methyloanthine; ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; MTT = methylthiazol tetrazolium; db-cGMP = N$^2$2'-O-dibutyrylguanosine 3'-5'-cyclic monophosphate.
3.3.1 Seeding and pre-treatment

Cells were seeded in 24-well or 96-well plates in preparation of the pre-treatments and assays. Cell counts were obtained from cell suspensions by using a haemacytometer, followed by the dilution of the cell suspension to the appropriate volume with growth medium. Table 3-1 illustrates the cell numbers and plates used for the different cells lines and assays.

Table 3-1 Summary of assays, cell lines and seeding density used in this study

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cell line</th>
<th>Plate</th>
<th>Cell density (cells/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP(_x) (mACHR)</td>
<td>SH-SY5Y</td>
<td>24-well</td>
<td>5 x 10(^6)</td>
</tr>
<tr>
<td>IP(_x) (mACHR)</td>
<td>HeLa</td>
<td>24-well</td>
<td>5 x 10(^6)</td>
</tr>
<tr>
<td>cAMP</td>
<td>SH-SY5Y</td>
<td>24-well</td>
<td>5 x 10(^6)</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>SH-SY5Y</td>
<td>24-well</td>
<td>3 x 10(^6)</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>SH-SY5Y</td>
<td>96 well</td>
<td>2.5 x 10(^6)</td>
</tr>
<tr>
<td>MTT</td>
<td>SH-SY5Y</td>
<td>24 well and 96 well</td>
<td>500 000; 250 000; 100 000; 75 000 and 50 000</td>
</tr>
</tbody>
</table>

After seeding (1 ml/well), the cells were allowed to attach to the well bottoms for 5 hours at 37\(^\circ\) C and 5% CO\(_2\). Thereafter the medium was aspirated and the wells twice rinsed with 500 µl MEM. The appropriate pre-treatments (drug in serum-free MEM + 0.1 mM mlns\(^1\)) was added to the wells and incubated for 24 hours at 37\(^\circ\) C in 5% CO\(_2\).

After the 24 hour pre-treatment, the medium was aspirated and the wells rinsed with 100 µl or 500 µl (for 96-well or 24-well plates, respectively) sterile PBS (37\(^\circ\) C), followed by the addition of 100 µl or 500 µl EMEM + 1% bovine serum albumin (BSA) per well for 5 minutes at 37\(^\circ\) C in 5% CO\(_2\) (final rinsing step to remove all free drug from the pre-treatments). The medium was then aspirated again and radio labelled with 100 µl or 300 µl.

\(^1\) See explanation for enriching MEM with mlns below.
μl EMEM + 1% bovine serum + either 1 μCi/ml $[^3]$H]-mlns (IP$_3$ assay), 1 μCi/ml $[^3]$H]-adenine (cAMP assay) or dummy labelled with MI (cell viability assays) and incubated overnight for 18-20 hours at 37°C in 5% CO₂.

**Incubation medium and drug concentrations for pre-treatments**

- **Pre-treatment incubation medium:**
  
  **Serum-free**
  
  Most drugs used are bound to plasma proteins, so that serum protein may influence the free drug concentration. Therefore serum-free medium was used.

  **mlns enrichment**
  
  DMEM: Ham's F12 1:1 mixture contains 12.51 mg/L myo-inositol (mlns) (0.07mM), whereas Ham's F12 medium contains 18mg/ml mlns (0.1mM). These concentrations of mlns show no difference in muscarinic receptor stimulated IP$_3$ synthesis (Viljoen, 2002) and can be considered to be in the normal MI concentration range in cell cultures as well as in the normal physiological range (2 – 15 mM) in normal brain (Fisher et al., 2002; Levine, 1997; Häussinger et al., 1994). MEM used during the 24-hour pre-treatments is low in mlns and was therefore enriched with 0.1 mM mlns as described in Brink et al., (2004).

- **Sildenafil concentrations used in pre-treatments:**
  
  Peak plasma levels of sildenafil in humans are 212 ng/ml, which is equal to 446 nM = 450 nM (sildenafil citrate MM = 192,122$^3$). Therefore 100 nM was chosen as a low concentration and 450 nM as a high concentration of sildenafil.

  For the current study a concentration of 100 or 450 nM sildenafil was used in SH-SY5Y neuroblasoma cells when indicated.

---

$^2$ Pfizer Material data sheet, Revision date 10/23/03, Version 1.2.0 Viagra; CAS number : 171599-83-0
- Sodium salysilate concentration used in pre-treatments:
  
  Sodium salysilate was used at a concentration of 1 mM. At this concentration sodium salysilate acts as a protecting agent against oxidative stress induced by serum-deprivation during the 24-hour pre-treatments (Andoh et al., 2002).

  For the current study a concentration of 1 mM sodium salysilate was used in SH-SY5Y neuroblasoma cells when indicated.

- N²,2'-O-Dibutyrylguanosine 3'-5'-cyclic monophosphate (db-cGMP) concentration used in pre-treatments:

  Rooney, (1996) found that 200 μM of the cell permeable cGMP analogue, N²,2'-O-Dibutyrylguanosine 3'-5'-cyclic monophosphate (db-cGMP), is able to induce a significant increases in intracellular Ca²⁺ concentrations. Others found that db-cGMP can be used in a concentration range of 100-1000 μM, causing an increase in Ca²⁺ concentrations within this range (Sato and Kawatani, 1998).

  For the current study a concentration of 500 μM db-cGMP was used as cell permeable cGMP analogue in SH-SY5Y neuroblasoma cells when indicated.

- Dipyridamole concentration used in pre-treatments:

  Dipyridamole is known as a selective inhibitor of PDE5. Hogan et al. (1998) reported a significant inhibition of basal ganglia transmission by 2 μM dipyridamole. Denis and Riendeau (1999) used a concentration of 20 μM dipyridamole in order to inhibit PDE4 dependant regulation of cAMP.

  For the current study a concentration of 20 μM dipyridamole was used in SH-SY5Y neuroblasoma cells when indicated.

- Zaprinast concentration used in pre-treatments:

  Santschi et al., (1999) reported that 20 μM zaprinast raised cGMP concentrations sufficiently to reversibly depress synaptic potentials in Sprague Dawley rats.

³ Concentration used is not published and was obtained from correspondence with Dr. Andoh (Andoh et al., 2002).
For the current study a concentration of 20 µM zaprinast was used in SH-SY5Y neuroblasoma cells when indicated.

- **IBMX concentration used in pre-treatments:**

  IBMX is typically used at a concentration of 1 mM to inhibit PDE activity non-selectively in cAMP measurement assays (Bodenstein and Venter, 2003). Also, according to Enna et al., (2004), the addition of 10 to 100 µM IBMX can potentiate adrenergic response. 1 mM IBMX may maximally stimulate lipolyses and mask the lipolytic response to some agonists (e.g. β2-agonists).

  For the current study a concentration of 1 mM IBMX was used in SH-SY5Y neuroblasoma cells when indicated.

- **ODQ concentration used in pre-treatments:**

  In an article published by Brunner et al., (1995), ODQ was used at a concentration of 3 µM in order to inhibit nitric oxide synthase.

  For the current study a concentration of 3 µM ODQ was used in SH-SY5Y neuroblasoma cells when indicated.

**Table 3-2 Summary of drugs and concentrations used in pre-treatments:**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sildenafil</td>
<td>450 nM</td>
</tr>
<tr>
<td></td>
<td>100 nM</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>1 mM</td>
</tr>
<tr>
<td>Sildenafil + sodium salicylate</td>
<td>450 nM + 1 mM</td>
</tr>
<tr>
<td>db-cGMP</td>
<td>500 µM</td>
</tr>
<tr>
<td>Dipiridamole</td>
<td>20 µM</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>20 µM</td>
</tr>
<tr>
<td>IBMX</td>
<td>1E-3 M</td>
</tr>
<tr>
<td>ODQ</td>
<td>1 x 10^-3 µM</td>
</tr>
<tr>
<td>Sildenafil + ODQ</td>
<td>450 nM+3 µM</td>
</tr>
</tbody>
</table>
3.3.2 Cell viability

3.3.2.1 Assay 1 – Trypan blue

After the indicated pre-treatments (see § 3.3.1) the Trypan blue assay for cell viability was initiated as described below:

Assay:

➢ The incubation medium was aspirated and the cells lifted with 200 µl Trypsin/versene (T/V) 4.

➢ Thereafter 800 µl phosphate-buffered saline (PBS) was added to each well and suspended cell clusters broken by pipetting up and down. The cell suspensions of each well was transferred to an eppendorf tube and centrifuged for 10 minutes at 5 000 rpm (± 2 300 × g) at 4 °C in a bench top centrifuge.

➢ The supernatant aspirated and the pellet resuspended in 1 ml PBS. The cell suspension was centrifuged again as described above and the supernatant aspirated. This procedure removed T/V that may interfere with the Trypan blue assay.

➢ Just before cell counting: 50 µl of 0.4% Trypan blue was added to the eppendorf tubes that contained the cell pellets. These tubes were then vortexed to resuspend the cells in the 0.4% Trypan blue medium.

➢ 20 µl of the suspended Trypan blue-cell mixture was used in the haematocytometer and the “white and blue” cells were counted, where “white” cells represent intact cells (viable) and “blue” cells represent damaged cells.

4 Each well was rinsed with 200 µl T/V, which was aspirated immediately and followed by another 200µl T/V. The well plates were again incubated for 20 minutes.
3.3.2.2 Assay 2 – Propidium Iodide\textsuperscript{5}

After the indicated pre-treatments (see § 3.3.1) the propidium iodide assay for cell viability was initiated as described below:

**Assay:**

- The incubation medium of the remaining wells was aspirated and the wells were rinsed twice with 100 µl PBS solution.
- The PBS was aspirated and 100 µl propidium iodide was added to each well and incubated for 30 minutes at room temp.
- Propidium iodide was also added to 3 wells not containing any cells (background control).
- The fluorescence intensity was measured in a 96-well spectrophotometric plate reader, using a 650 nm filter.

Note: The percentage of propidium uptake was determined by subtracting the fluorescence measured in untreated cells containing propidium iodide. Normalised values to the fluorescence representing 100% neuronal death was obtained by exposing cells to 10 µM glutamate for the same period of pre-treatment or by adding lyses solution to the cells (5% TCA for 90 minutes). Thus, the percentage of propidium iodide uptake is equivalent to the % of dead cells above control levels.

3.3.2.3 Assay 3 – MTT \textsuperscript{6}

After the indicated pre-treatments (see § 3.3.1) the MTT assay for cell viability was initiated as described below:

\textsuperscript{5} Propidium iodide should be opened under argon because it is oxidized in O\textsubscript{2}

\textsuperscript{6} Light sensitive, prepare in laminar flow chamber and in dark room.
Chapter 3 - Experimental Procedures

Assay:

- Cell growth was terminated by adding 40 μl MTT reagent/well where after the well plates were incubated for 2h at 37°C in 5% CO₂ - a purple colour occurred.

- The medium was aspirated, 250 μl isopropanol added to the wells and incubated for 5 minutes at room temperature.

- 100 μl from each well was transferred to a corresponding well in a 96-well plate and absorbance was recorded with a spectrophotometer at a wavelength of 570 nm.

3.3.3 Functional Assays

3.3.3.1 Assay 4 - IPₓ

After the indicated pre-treatments (see § 3.3.1) the IPₓ assay for mAChR function was initiated as described in Brink et al., (2004), briefly as follows:

Assay:

- The labelling medium in the 24-well plates was aspirated and 500 μl/well assay medium (DMEM + 0.5 M HEPES + 0.4 M LiCl) added for 10 minutes at 37°C in 5% CO₂.

- After the 10 minutes of incubation, the assay medium was aspirated and 500 μl assay medium plus the appropriate concentration of the mAChR agonist metacholine (MeCh) was added to each well and incubated for another 60 minutes at 37°C in 5% CO₂.

- The assay medium was aspirated and the reaction was terminated by adding 1 ml ice-cold lyses solution (10 mM formic acid dissolved in double distilled (dd) water). The cells were left to lyse at 4°C for 90 minutes.
Dowex columns (Dowex 1x8 - 400, 200 - 400 mesh, 1-chloride form - 250µl Bio Rad Poly prep columns) were employed to separate the inositol phosphates (IP₆) from other cellular compounds. Each well's lyses solution (containing the cell supernatant) was transferred to a Dowex column and left to run through into the collecting bucket, with the IP₆ retained in the resin.

Each well was rinsed with another 1 ml ice-cold lyses solution and also transferred to its corresponding Dowex column and again left to run through into the collecting bucket, with the IP₆ still retained in the resin.

Thereafter the columns were rinsed with 2 x 5 ml of solution 3 (see Table 3-4) and left for the solution to run through into the collecting bucket.

Thereafter the columns were mounted over the scintillation vials and the IP₆ in each column was eluted with 3 ml of solution 4 (see Table 3-4).

7 ml of Ultima Gold XR scintillation fluid was added to each scintillation vial and the [³H]- IP₆ was counted in a scintillation counter.

Note: The Dowex columns must be regenerated before use, in order to clean the columns of any impurities that might have been left behind in previous experiments. The regeneration steps were done as follows:

Table 3-3 Regeneration steps, procedures and compositions:

<table>
<thead>
<tr>
<th>Step nr.</th>
<th>Procedure</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Add 5 ml H₂O per column and let it run through column</td>
<td>dd water</td>
</tr>
<tr>
<td>Step 2</td>
<td>Add 2,5 ml Sol 1 per column and let it run through column</td>
<td>Ammonium Formate (5 M) Formic Acid (3M)</td>
</tr>
<tr>
<td>Step 3</td>
<td>Add 10 ml H₂O per column and let it run through column</td>
<td>dd water</td>
</tr>
</tbody>
</table>
**Step 4**
Add 2 x 5 ml Sol 2 per column and let it run through column

<table>
<thead>
<tr>
<th>Step 4</th>
<th>Add 2 x 5 ml Sol 2 per column and let it run through column</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3-4 Summary of solutions used in this IP₄ assay:

<table>
<thead>
<tr>
<th>Summary of solutions used in this IP₄ assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution number</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

**[^3]H]-myo-inositol uptake studies**

The aim of this assay was to examine the possible effect that sildenafil may have on[^3]H]-myo-inositol uptake into SH-SY5Y and HeLa cells after 24 hour pre-treatment with sildenafil - pre-treatment conditions as described in § 3.3.1. This is important to interpret the results obtained from the mAChR functional studies described above.

Three wells for each drug pre-treatment were reserved for the measurement of[^3]H]-myo-inositol uptake. The labelling medium was aspirated as for the mAChR functional assay, but 1 ml ice-cold lyses solution (10 mM formic acid) was added directly. The lyses solution (containing the cell supernatant) from each well was then transferred directly to scintillation vials. Again 7 ml of Ultima Gold XR scintillation fluid was added to each scintillation vial and the radioactivity was counted in a scintillation counter.

**3.3.3.2 Assay 5 - cAMP**

After the indicated pre-treatments (see § 3.3.1) the cAMP assay was initiated as described in Brink et al., (2004):
Chapter 3 - Experimental Procedures

Assay:

- The labelling medium was aspirated and the cells were rinsed with 500 µl PBS.
- The assay medium (DMEM + 1 mM IBMX + 30 µM Forskolin) was added and incubated for 20 min at 37°C and 5% CO₂.
- Thereafter the assay medium was aspirated and the reaction terminated by adding 1 ml of ice-cold lyses solution (5% TCA + 1 mM ATP+ 1 mM cAMP) per well. The cells were left to lyse for 30 min at 4°C.

IPx Dowex columns: (Separation of [³H]-ATP)

- The Dowex columns (Dowex AG50W×4 resin) were placed over the revised number of scintillation bottles. The different well mediums were carried over into the Dowex columns and the elute collected in the scintillation vials (ATP collected).
- 2 ml of double distilled water was added to the columns, again collecting the run-through in the scintillation bottles (ATP collected).
- Thereafter 12 ml of Ready Gel scintillation cocktail was added to each scintillation vial, mixed and radioactivity counted in scintillation counter.

Alumina columns: (Separation of [³H]-cAMP)

- The Dowex columns were placed over the alumina columns (grade I; type WN-3: Neutral) and 6 ml double distilled water added and allowed to run through both columns.
- The alumina columns were placed on top of the 2nd set of scintillation vials, and 4 ml of 0.1 M imidazole was added to each column and the elute collected.
> 12 ml of Ready Gel scintillation cocktail was added to the scintillation vials and the radioactivity counted in a scintillation counter.

**[3H]-adenine uptake studies**

The aim of this assay was to examine the possible effect of the sildenafil pre-treatment on [3H]-adenine uptake in SH-SY5Y cells.

Three wells for each drug pre-treatment condition were reserved for the measurement of [3H]-adenine uptake. The labelling medium was aspirated as for the cAMP assay, but 1 ml ice-cold lyses solution (5% TCA) was added directly. The lyses solution (containing the cell supernatant) from each well was then transferred directly to scintillation vials. Again 7 ml of Ultima Gold XR scintillation fluid was added to each scintillation vial and the radioactivity was counted in a scintillation counter.
(All abbreviations are listed in Appendix A)
(All statistical analyses are listed in Appendix B)

This chapter focuses on all the results as obtained from the experiments and associated assays (Trypan blue, propidium iodide, MTT, IP\(_x\)-accumulation & cAMP-accumulation) described in Chapter 3. A comprehensive discussion of each set of results is included, while the final conclusions and recommendation are given in Chapter 5.

As mentioned in section 1.2 and as illustrated in Figure 3-1, the experiments focussed on the investigation of two aspects of the mechanism of action of sildenafil, namely its effect on cell viability and on receptor function. A schematic layout of the order of how the results will be presented and discussed is given in Figure 4-1.

![Schematic layout of experimental results to be discussed](image-url)
All the experiments were conducted in the cell culture laboratory of the North West University — (School of Pharmacy (Pharmacology) at the Potchefstroom campus).

4.1 Serum Deprivation and Cell Viability

Andoh et al., (2004) indicated that 24-hour serum-deprivation in SH-SY5Y cells causes severe oxidative stress, leading to an increase in free radical formation and eventually cell death (for literature review see § 2.4.2 Mechanisms of Neurodegeneration and Protection). In the current study cell loss during the 24-hour pre-treatments in serum-free medium was also noticed under the experimental conditions employed. This observation needed further exploration and the results obtained from these experiments are presented in Figure 4-2.

![Figure 4-2](image)

**Figure 4-2** Comparisons of neuronal (SH-SY5Y) cells pre-treated for 24 hours with and without serum, as determined by the Trypan blue test for cell viability (intact cell counts only).

From figure 4-2 it can be seen that the positive control (+FBS + Lyses) damaged approx 95% (i.e. cell survival of $6.4 \pm 1.5\%$ relative to the negative control) of all the
viable cells relative to the negative control (+FBS; 100 ± 6.1%). Cell damage in serum deprived cells (-FBS) were approximately 58% (i.e. cell survival of 41.8 ± 4.4% relative to the negative control). All differences were statistically significant (P<0.0001\(^1\)) and statistical significance is maintained also after Bonferroni correction.

Please note that cell viability from Trypan blue tests in this study represents intact cell (white cells) counts only. It was observed constantly that intact counts and total counts corresponded proportionally (data not shown), so that these results suggest that lower intact cell counts represent proportionally lower total cells remaining attached to the well bottoms.

The results suggest that serum-deprivation damages about half of the cells, but that a significant number of cells remain intact. It is therefore possible to implement the pre-treatments in serum-free medium and to perform functional assays thereafter on the remaining intact cells.

### 4.2 Anti-oxidants/oxidant and cell viability

In order to establish whether reactive oxidative species (ROS) formation or oxidative stress is responsible for the effect of serum-deprivation on cell viability (Figure 4-2), studies on serum deprivation and cell viability was again executed, but with the addition of the anti-oxidant ascorbic acid at various concentrations, as well as of the oxidative stress inhibitor sodium salicylate.

The results can be seen in Figure 4-3 and Figure 4-4 respectively.

\(^1\) Student t-test
4.2.1 Anti-oxidant pre-treatment on cell viability

The effect of different concentrations of ascorbic acid as anti-oxidant on the serum deprivation-induced oxidative stress in SH-SY5Y cells was investigated and the results are displayed in Fig. 4-3.

![Graph showing cell viability after different concentrations of ascorbic acid pre-treatment.]

**Figure 4-3** The effect of anti-oxidant (ascorbic acid) pre-treatments on cell viability after serum deprivation, as measured by the Trypan blue test (intact cell counts only).

In Figure 4-3 it can be seen that even low concentrations of ascorbic acid (0.01%) in the pre-treatment medium for 24 hours greatly damaged the cells (93% cell damage, i.e. 7.2 ± 2.6% cell survival relative to control; p < 0.0001). At concentrations of 0.05% and 0.1% the cells were virtually all lifted from the well bottoms (visual microscopic observation – data not shown), which is reflected in the very low or zero cell counts from the Trypan blue test.

It is clear that the anti-oxidant ascorbic acid at the concentrations employed is not able to protect the SH-SY5Y cells from oxidative stress induced by serum-deprivation, but that it is rather cytotoxic. This was not expected and is contradictory to the
mechanism proposed by Behl (1998), whereby cell death results from the formation of oxidants and free radicals. Although a concentration of 0.02% (m/v) ascorbic acid is routinely used for anti-oxidant properties in many biological assays (including functional assays in live cells, typically for 1-3 hours), this may have been an inappropriate concentration for a longer duration of 24-hour pre-treatment of the cells.

4.2.2 Sodium Salicylate and Cell Viability

Figure 4-4 illustrates the effect of an oxidative stress inhibitor sodium salicylate on cell viability during 24-hour serum deprivation:

Figure 4-4 The effect of an oxidative stress inhibitor (10 mM sodium salicylate) pre-treatment on cell viability during 24-hour serum deprivation, as measured by the Trypan blue test (intact cell counts only).

In Figure 4-4 it can be seen that the inhibitor of oxidative stress, sodium salicylate, does not protect the cells from the oxidative stress induced by serum deprivation. The
small increase in cell viability after sodium salicylate (from 100 ±10.4% to 117.4 ± 17.6%) did not reach statistical significance (p = 0.42). These results are contradictory to those of Andoh et al. (2004), who reported that 1 mM sodium salicylate is able to inhibit oxidative stress in SH-SY5Y cells, as induced by 24-hour serum deprivation. The results from the current study suggest that the cell damage may be due to a mechanism different from oxidative stress that involves the formation of oxidants or reactive oxygen species ROS species.

4.2.3 Different Drugs Pre-treated on SH-SY5Y Cells (Cell Survival after Oxidative Stress)

In order to investigate whether the NO/cGMP pathway plays any modulatory role in the neuronal damage observed after 24-hour serum deprivation, several PDE 5 inhibitors (sildenafil, dipiramidole, zaprinast), a non-selective PDE inhibitor (IBMX), a cGMP analogue (N²,2'-O-dibutyrylguanosine 3'-5'-cyclic monophosphate) and a NOS inhibitor (ODQ) were identified and added to the pre-treatments.

4.2.3.1 Trypan blue test

Results obtained from the Trypan blue test are given below:

![Cell viability graph](image)

Figure 4-5 Cell viability as determined with the Trypan blue test after pre-treatments on SH-SY5Y cells. Different drug pre-treatments on SH-SY5Y cells to determine cell survival after oxidative stress induction by serum deprivation. * = p < 0.05; ** = p < 0.01; *** = p < 0.001. For drug concentrations see section 3.3.1
From figure 4-5 it can be seen that a 24-hour pre-treatment with sildenafil showed a tendency to reduce cell viability, but this did not reach statistical significance (78.3 ± 18.3 % of control; p = 0.575)\(^2\) However, at a concentration of 450nM, sildenafil yielded 56.2 ± 6.5% cell survival as compared to that of the control (p = 0.00337), showing a statistically and practically significant decrease in cell viability. Dipiramidole, also a PDE 5 inhibitor, also significantly decrease cell viability (17.4 ± 1.5% of control; p = 0.000019). To the contrary, however, the PDE5 inhibitor zaprinast does not show a decrease of statistical significance (77.7 ± 10.6% of control; p = 0.543). ODQ, a NOS inhibitor, does not show any effect on cell viability. However, ODQ plus 450 nM sildenafil shows a remarkable decrease in cell viability (34.2 ± 8% of control; p = 0.00062). A statistical significance of the small difference between the sildenafil alone and the sildenafil + ODQ treatment groups was not reached. The cGMP analogue (db-cGMP) pre-treatment was not able to modulate cell viability.

4.2.3.2 Propidium Iodide

Results on cell viability as obtained from the propidium iodide test after different drug pre-treatments are given in Figure 4-6 below:

\(^2\) Dunnet's Post Hoc test.
Figure 4-6 Cell viability as determined by the propidium iodide test on 24-hour pre-treated SH-SY5Y cells. (A) Different drugs pre-treated on SH-SY5Y cells to determine cell survival after oxidative stress induction by serum deprivation. (B) Experimental comparison between sildenafil 450 nM in different experiments in order to determine if data retrieved can be interpreted after mycoplasmic infection.

Two way ANOVA statistical analysis of differences between different experimental pre-treatment drugs (as seen in Figure 4-6A) revealed that no significant variation can be found (p = 0.057). However, results were not repeatable, as can be seen in Figure 4-6B. Inter-day variation was large (total inter-day experimental variation percentage = 4.4%; p = 0.038) and total pre-treatment variation was also large (percentage = 11.3%; p = 0.0119). It is therefore clear that the results in Figure 4-6 cannot be interpreted and that the propidium iodide test could not be implemented in a way that is suitable for the current study.

One possible explanation for the large inter-day experimental variations may be the fact that the SH-SY5Y cells were infected by a possible mycoplasmic infection which

---

3 Organism not verified, but altered cell growth and behaviour were observed and granular cell surfaces were visible under a confocal microscope, suggesting mycoplasma.
could not be contained. This infection may have modified the cells response to pre-treatments.

4.2.3.3 MTT

Results on cell viability as obtained from the MTT test after different drug pre-treatments are in Figure 4-7 given below:

![MTT Results Plot]

*Figure 4-7 Cell viability as determined by the MTT test on pre-treated SH-SY5Y cells. Different drugs pre-treated on SH-SY5Y cells to determine cell survival after oxidative stress induction by serum deprivation - non-interpretable.*

It can be seen in Figure 4-7 that all pre-treatments showed no formation of formazan (typically formed from the MTT reagent in mitochondria of viable cells). The lack of formazan-product formation alters the optic density needed for spectrophotometric reading (as can be seen by the negative values when compared to that of the control, therefore rendering the obtained values invalid for interpretation.

Visual microscopic investigations did not show total cell death for the treatment groups, as suggested by Figure 4-7. This also does not correspond with the results employing the Trypan blue test, as shown in Figure 4-5. These results therefore suggest that the pre-treatment drugs interfere with the cells ability to synthesise formazan from
the MTT reagent. Again, the mycoplasmic infections (see § 4.1.3.2) may have contributed to the results.

4.3 IP₃- production

4.3.1 mAChR function

Dose response curves (DRC) of the full mAChR agonist methacholine (MeCh) was constructed after 24-hour pretreatment with and without 450 nM sildenafil in serum-free medium to investigate any modulating effects of sildenafil pre-treatments on mAChR function. The data are presented in Figure 4-8.

![Dose-response curves of MeCh after 24-hour pretreatment of SH-SYSY cells with 0 M or 450 nM sildenafil in serum-free medium.](image)

From Figure 4-8A it can be seen that 24-hour pre-treatment with 450 nM sildenafil greatly increased the Eₘₐₓ of the MeCh dose-response curve (283.5 ± 39.7 versus 100.0
± 19.5 of control; p = 0.0008⁴). Importantly, the baseline production of IP₆ (0 M MeCh) was not affected by the pre-treatment, suggesting that the increase in Eₘₐₓ is a mAChR and/or signal-transduction system-dependent effect. The results therefore suggest increased mAChR function after 24-hour pre-treatment with 450 nM sildenafil.

From figure 4-8B no statistical significant difference could be detected between the uptake values for 0 M and 450 nM sildenafil pre-treatments (p = 0.571). It is therefore important to note that 450 nM sildenafil pre-treatment does not alter the average uptake of [³H]-myo-inositol, affirming the validity of the results presented in Figure 4-5.

When comparing the EC₅₀ values of the two different pre-treatments, no statistically significant difference was found (p = 0.6747). The sildenafil pre-treatment therefore altered the maximal signalling capacity of mAChRs, without affecting the EC₅₀ value (half maximal concentration).

Preceding studies from our lab (Brink et al. 2004) have shown that similar pre-treatments of neuronal SH-SY5Y cells with the antidepressants fluoxetine and imipramine decreases mAChR-mediated IP₆ production. The results form the current study, however, suggest an opposite effect by sildenafil. This may suggest that sildenafil may exert an opposite clinical response to typical anti-depressants and that it may potentiate or worsen affective illness. Indeed, work in our lab (Bothma, 2004) has shown it to exuberate stress induced activation of NOS while most anti-depressants have been found to inhibit NOS activity (Wegener et al., 2003). It is also noteworthy that a recent case report doubts the exuberation of mania in a bipolar depressed patient (drop) after taking sildenafil (Boggot & Singh, 2004)

---

⁴ Student t-test
4.3.2 Comparison SH-SY5Y vs HeLa

A comparison between the modulating effects of sildenafil on mAChR function in neuronal (SH-SY5Y) and non-neuronal (HeLa) cells is provided in Figure 4-9.

<table>
<thead>
<tr>
<th>A) SH-SY5Y cells</th>
<th>B) HeLa cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>[MeCh]</td>
<td>[MeCh]</td>
</tr>
<tr>
<td>0 M 100 nM 100 nM</td>
<td>0 M 100 nM 100 nM</td>
</tr>
</tbody>
</table>

Figure 4-9 Comparison of sildenafil pre-treatment on (A) neuronal (SH-SY5Y) cells. (B) Non-neuronal (HeLa) cells.

In Figure 4-9A it can be seen that the maximal concentrations of MeCh (100 μM) is dramatically increased after 100 nM and 450 nM pre-treatment with sildenafil (from 100.0 ± 20.5% in control to 290.5 ± 48.7% (p = 0.0024) after 100 nM sildenafil and to 230 ± 32.2% (p < 0.05). Interestingly, the effect after 100 nM was not smaller that found with 450 nM sildenafil, suggesting that this effect on mAChR function is relevant at relatively low concentrations of sildenafil.

However, in human epithelial HeLa cells (non-neuronal) the effect of 100 nM sildenafil pre-treatment is not as pronounced as in neuronal SH-SY5Y cells. The MeCh-mediated effect increased from 100.0 ± 4% to 139.7 ± 13.4 (p = 0.0118) after 100 nM sildenafil.

When comparing Figure 4-9 (A) vs. B) one can see that sildenafil (100 nM) has a much greater potentiating effect on mAChR function in neuronal cells, as compared to those in non-neuronal cells. It is also noteworthy that phenomenon needs further investigation to determine the mechanism by which sildenafil exerts this neuroselective effect, it is however strange to notice that at a concentration of 450 nM, sildenafil
showed a decrease in cellular response when compared to that of the 100 nM sildenafil pre-treatment in the same neuronal SH-SY5Y cell line. A possible explanation for the decrease in $[^3H]$-IP$_x$ accumulation after 450nM sildenafil pre-treatment when compared to the 100nM sildenafil pre-treatment (in neuronal cells) may be because of a possible neurodegenerative effect that sildenafil is able to exert at high concentrations. However, further investigation into this seemingly concentration like degenerative effect of sildenafil still needs to be done. Another point worth mentioning is that no endothelial cell experiments were carried out in order to establish sildenafil's effect in this cell line (due to mycoplasmic infection) to be able to compare the distribution of PDE5 in the different kinds of cell lines and their effect after stimulation — see § 5.3 Recommendations.

### 4.3.3 cAMP production

The effect of sildenafil pre-treatment on cAMP production in neuronal (SH-SY5Y) cells is provided in Figure 4-10.

![Figure 4-10 Changes in cAMP production after 24 hour sildenafil pre-treatment.](image-url)

In order to eliminate any possible cAMP-production, anti-depressant like effects of sildenafil we conducted $[^3H]$-cAMP accumulation experiments in order to determine any cAMP increase after 450nM sildenafil pre-treatment. Neuronal mycoplasmic infections
made it impossible to further examine the possible modulatory effects of sildenafil pre-
treatment on anti-depressant like effects of β-stimulants.

It can be seen in Figure 4-10 that sildenafil pre-treatment does not affect cAMP production in SH-SY5Y cells ($p = 0.1689$)
5.1 Summary

The current study had two main objectives, namely to investigate the effect of sildenafil pre-treatment on cell viability and on muscarinic acetylcholine receptor (mACHR) and β-adrenoceptor (β-AR) function in neuronal and non-neuronal cultured cells.

Human neuroblastoma (SH-SY5Y) and human epithelial (HeLa) cells were pre-treated for 24-hours in serum-free medium with or without NO/cGMP pathway modifying drugs, whereafter cell viability, muscarinic acetylcholine receptor (mACHR) function and baseline cAMP production were determined.

Cell viability experiments utilising the Trypan blue method showed that 24-hour serum deprivation of SH-SY5Y cells caused damage to 58% of neuronal cells (decrease in cell viability). Neither ascorbic acid (an anti-oxidant) nor sodium salisylate (an oxidative stress inhibitor) were able to inhibit the negative effect of serum deprivation on SH-SY5Y cell survival (Figure 4-3 and Figure 4-4, respectively).

Pre-treatment of the cultured cells with PDE inhibitors was not able to increase cell viability or exert a neuroprotective effect against serum deprivation. The same results were observed for IBMX, the cGMP analogue, guanosine and the NOS inhibitor ODQ. Dipiradimole, also a PDE inhibitor caused a statistical significant decrease in cell viability. Interestingly, sildenafil 450nM + ODQ showed a statistical significant decrease in cell survival, indicating that the inhibition of NOS wasn’t able to inhibit the effect of
sildenafil 450nM after pre-treatment with 450nM sildenafil. The latter indicates that NO production inhibition does not play any role in neuroprotection. The propidium iodide test data showed a too large experimental variation to be interpreted and lack of formazan formation, the production needed for spectrophotometric readings in the MTT test, yielded the MTT test void.

A 24-hour pre-treatment of neuroblastoma SH-SY5Y cells with 450 nM sildenafil potentiates mAChR function, as can be seen from an increase in the $E_{\text{max}}$ value from $100.0 \pm 19.5\%$ (control) to $283.5 \pm 39.7\%$ (450 nM sildenafil), without affecting the $EC_{50}$ value or baseline production of inositolphosphates. Importantly, a lower concentration of 100 nM sildenafil was able to induce a similar potentiation of mAChR function. This effect of sildenafil on mAChR was seen to a much smaller degree in epithelial HeLa cells, suggesting possible neuroselectivity.

Baseline cAMP production is not affected by 24-hour pre-treatment with 450 nM sildenafil in SH-SY5Y cells.

5.2 Conclusion

Results from the current study suggest that 24-hour serum deprivation has a neurodegenerative effect on neuronal SH-SY5Y cells. These results confirm the findings of Andoh et al. (2004), who reported that 24-hour serum deprivation of SH-SY5Y cells causes severe oxidative stress, leading to an increase in free radical formation and eventually cell death. This production of or excess peroxinitrates may be the result of excessive NO production (Geordge, 1998) However, the current study shows that ascorbic acid, which acts as an antioxidant, is not able to protect against the neurodegenerative effects of 24-hour serum deprivation of SH-SY5Y cells. To the contrary, ascorbic acid was severely cytotoxic, even at relatively low concentrations of 0.02% and 0.01% that are typically used during experiments with live cells (Brink et al., 2004). This unexpected effect of ascorbic acid may be explained by the relatively long incubation period, compared to literature reports of incubation lasting only several hours. From the current study it was also clear that the oxidative stress inhibitor, sodium
salicylate, does not reverse the degenerative effects of serum-deprivation. These results are different from those obtained by Andoh et al., (2004), who reported that 10 mM sodium salicylate is able to fully protect SH-SY5Y cells against oxidative stress during 24-hour serum deprivation. From the results of the current study it may be concludes that serum deprivation exert its cytotoxic effect in SH-SY5Y cells by a mechanism that is not preventable with sodium salicylate. This does not exclude diverse mechanisms of oxidative stress and the formation of reactive oxygen species (ROS – e.g. peroxynitrates and free radicals). For example, ROS are regularly formed via oxidative phosphorylation during ATP formation, which is responsible for the peroxidation of lipids, leading to the lyses of the cell in most cases (Behl, 1998).

When SH-SY5Y cells were pre-treated for 24 hours, it was observed that sildenafil exerts a concentration-dependent neurodegenerative effect (i.e. no significant effect at 100 nM and a significant effect at 450 nM relative to control). Sildenafil is responsible for the inhibition of PDE5 and cGMP breakdown, thus leading to an increase of cGMP concentration. cGMP has been implicated in neuroprotection, secondary to NO production (Kim et al., 1999). Importantly, NO exerts a neuroprotective effect at normal physiological concentrations, but may also cause peroxynitrate-induced membrane toxicity and eventually cell death (Geordge, 1998). According to Kim et al., (1999), the underlying mechanism for the survival promoting effect of NO appears to involve the activation of guanylyl cyclase (GC) and the generation of cGMP. These studies suggest that cyclic nucleotides, such as cGMP (and possibly cAMP), play an important role in promoting cell survival in oxidative stress models (Wirtz-Brugger and Giovanni, 2000). It was therefore expected that sildenafil may display neuroprotective properties.

Interestingly, in the current study another selective PDE5 inhibitor, dipiridamole, and the non-selective PDE inhibitor, IBMX, also caused neurodegeneration of SH-SY5Y cells. However, the selective PDE5 inhibitor, zaprinast, and the cell permeable cGMP analogue, db-cGMP, did not result in any neurodegenerative effects. These results suggest that the neurodegenerative effect by sildenafil may be mediated by a mechanism different from its PDE5 inhibition (i.e. not related to increased cGMP levels). The mechanism may involve a common action of sildenafil, dipiridamole and IBMX.
Other tests for cell viability, namely the propidium iodide and MTT tests, were not successfully implemented in the current study. Experimental variations in the propidium iodide test can possibly be contributed to a drug-resistant mycoplasma infection which rendered our SH-SY5Y cell line unsuitable for future studies; therefore, all results obtained in these experiments were rendered un-interpretable. Results obtained by the MTT test yielded negative values when compared to the control. This suggests a lack of formazan formation by the cellular mitochondria, which may be attributed to a possible mitochondrial effect of the NO-cGMP modifying drugs or to the possible mycoplasma infections.

Although several researches (Bremner et al., 2000; Mervaala et al., 2000; Sheline et al., 1996) did not find any changes in hippocamal volume in patients suffering from depression, reliable evidence (Berkov and Fletcher, 1992) suggest that major depression is accompanied by structural abnormalities in the limbic-system (which includes the hippocampus). These structural changes include a decrease in adult neurogenesis in the hippocampus, atrophy of pyramidal neurons in the hippocampus etc. (see section 2.3.1.3.6. for detailed explanation). It is for this reason that neurodegeneration has been implicated in depression. In the current study it has been shown that sildenafil has a neurodegenerative effect on neuronal tissues (SH-SY5Y cells), which is not suggestive of any supportive role in the treatment of depression in fact, it may worsen it.

Sildenafil has the ability to potentiate mAChR function (measuring IP$_x$ production), without affecting the baseline IP$_x$ production or the EC$_{50}$ value of the agonist methacholine. Importantly, whole-cell uptake of the radiolabel, $[^3]H$-myo-inositol, was also not affected, validating the above mentioned observations on mAChR function. Taken together these data suggests that sildenafil potentiates the signalling mechanism of mAChRs and/or mAChR concentration. In the same cell model Brink et al. (2004) reported that pre-treatments with the antidepressants fluoxetine and imipramine inhibits mAChR and 5HT$_{2A}$-receptor function. For the latter receptor type the mechanism appears to involve a reduction of receptor-G protein coupling. The current study did, however, not investigate the mechanism whereby sildenafil exerts its potentiating effect on mAChR function (i.e. via signalling capacity and/or receptor concentration).
Nevertheless, it heightens the possible depressiogenic actions of an increase in cGMP sildenafil.

Evidence from the current study also suggest that the mAChR modulating effect of sildenafil may be neuroselective, exerting a much greater modulating effect on mAChR function in neuronal (SH-SY5Y) cells than in non-neuronal (HeLa) cells. This may be useful in understanding sildenafil’s central actions and also suggests that chronic sildenafil may exert a greater effect on central than peripheral mAChRs.

Central cholinomimetics display antimanic properties as opposed to antidepressant effects of anticholinergics (Fritze and Beckmann, 1998) and has also been shown to exacerbate behavioural depression in animals subjected to the Porsolt swimming test, while antimuscarinic agents and several antidepressants inhibited this response. (Chau et al., 2001). Previous studies have also shown that the cholinergic system of depressed subjects is super-sensitive compared to those without depression (Daws and Overstreet, 1999). These data taken together show the great importance of the cholinergic system and mAChR function in mood disorders like depression. Potentiation of the central cholinergic system by sildenafil may potentially exacerbate depression. However, it has not been established whether this is a general effect or whether it will affect specific neurons and mAChRs only. It is therefore premature to speculate from the data about the effect of chronic sildenafil on central mAChRs in vivo and also to speculate about its effect on mood. Anxiety on its own is also known to elevate ACh release in the hippocampus, implicating mAChRs in the aetiology of anxiety (Meany et al., 1993). Stressful stimuli are also known to produce a thoroughly characterized activation of the hypothalamic-pituitary-adrenal (HPA) axis (Mizuno et al., 1991), which is also implicated in the aetiology of depression (see section 2.4.2.). It is therefore possible that sildenafil can exacerbate anxiety, possibly contributing to the reports of adverse events after the use of sildenafil, including aggression, as filed by the FDA (Milman & Arnold, 2002). Exacerbation of anxiety after the use of sildenafil in pre-clinical models has also been reported by Volke et al., (2003) and Kurt et al. (2004).

In conclusion, this study shows that sildenafil shows a neurodegenerative effect and has no protective effect on neuronal cell viability – see § 5.3 Recommendations. This effect does not seem to involve the formation or inhibition of cGMP in any way, but may
be the result of a still unknown mechanism. Sildenafil is also able to potentiate mAChR function (IP$_x$ - production) by a still unknown mechanism which may be neuroselective.

5.3 Recommendations

Questions that remained unanswered in the current study include the mechanism(s) whereby sildenafil exert its effect on cell viability and on mAChR function. Especially the role of the possible mycoplasma infection during a late stage of the study may have influenced the results on cell viability. In addition, we did not investigate the possible modulating effect of sildenafil on β-AR function in SH-SY5Y cells, due to the infection of cells that rendered them unresponsive. The clinical significance of these findings, especially on mAChR function, is not clear.

For this reason it can be recommended that future studies focus on investigating these remaining questions:

1. Studies on cell viability may be repeated with uninfected cells and may even be extended to other neuronal and non-neuronal cell lines.
2. The mechanism whereby sildenafil exerts its mAChR potentiating effect may be investigated by performing receptor binding studies in order to determine receptor concentration before and after treatment and may also include investigations into the expression of relevant cellular mRNA and proteins (such as enzymes in the signal-transduction system of mAChRs), G protein activation (e.g., GTPγS binding) and related studies.
3. The concentration dependant mAChR potentiating effect of sildenafil (the connection between sildenafil concentration and mAChR response)
4. The modulating effect of sildenafil on β-adrenergic receptors may also be investigated, especially as this receptor type is associated with antidepressant potential of drugs.
5. The investigations into receptor modulating effects of sildenafil may be extended to other central receptor systems, for example serotonergic, dopaminergic, NMDA and GABAergic receptors.
6. Animal models of depression (e.g. rodent models) may be utilised to determine the effect of chronic sildenafil treatment on animal behaviour and also to verify the in vivo relevance of in vitro findings on receptor functions.
7. Further cell viability test in neuronal cells to verify the neurodegenerative properties of sildenafil as seen in this thesis.

Future studies may very well explain observed central effects and also open new possible clinical applications for sildenafil or other PDE5 inhibitors.


DSM-IV-TR., 2000. See DIAGNOSTIC AND STASTISTICAL MANUEL OF MENTAL DISORDERS.


FAZELI, E., VARNIER, G. & RAITERI, M. 1992. In vivo microdialysis study of GABA\textsubscript{A} and GABA\textsubscript{B} receptors modulating the receptor/NO/cyclic GMP pathway in the rat hippocampus. Neuropharmacology, 36:1405-1415.


References


http://circ.ahajournals.org/cgi/content/full/105/20/2398 [Date of access: 15 Nov. 2004]


PRAST, H. & PHILIPPU, A. 2000. Nitric oxide as modulator of neuronal function. Institute of Pharmacy, Department of Pharmacology and Toxicology, University of Innsbruck, Peter-Mayr-Str. 1, A-6020 Innsbruck, Austria.


WALSH, A.A. (http://inside.salve.edu/walsh/newpsypages/newpsyhome.html). Introduction to Psychology: Unit 3. [E-mail to:] Walsh. A.A. (walsh@salve.edu) [Date of access: 10 Jul 2004]


### Table of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Agonist</td>
</tr>
<tr>
<td>AAD</td>
<td>Aromatic L-amino acid decarboxylase</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Ach or ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Atrp</td>
<td>Atropine</td>
</tr>
<tr>
<td>$\beta$-AR</td>
<td>$\beta$-adrenoreceptor</td>
</tr>
<tr>
<td>BK$_{Ca}$</td>
<td>Large-conductance $Ca^{2+}$-activated channels</td>
</tr>
<tr>
<td>$Ca^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>$Ca^{2+}$ calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CK1a</td>
<td>Casein kinase 1a</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>$CO_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotrophin releasing factor</td>
</tr>
<tr>
<td>CVT</td>
<td>Cell viability test</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>db-cGMP</td>
<td>$N^2,2'$-O-Dibutyrylguanosine 3'-5'-cyclic monophosphate</td>
</tr>
<tr>
<td>DD/dd</td>
<td>Double distilled</td>
</tr>
<tr>
<td>Dip</td>
<td>Dipiridamole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagles Medium</td>
</tr>
<tr>
<td>DRC</td>
<td>Dose response curve</td>
</tr>
<tr>
<td>DRCs</td>
<td>Dose response curves</td>
</tr>
<tr>
<td>DSM-IV-TR</td>
<td>See References</td>
</tr>
<tr>
<td>EAAR</td>
<td>Excitatory amino acid receptors</td>
</tr>
<tr>
<td>EMEM</td>
<td>Minimum Essential Media with Earle's Base</td>
</tr>
<tr>
<td>ECT</td>
<td>Electro-convulsive therapy</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelium nitric oxide synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Feb</td>
<td>February</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GABA</td>
<td>y-amino butyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Generalized anxiety disorder</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GC</td>
<td>Guanylyl cyclase</td>
</tr>
<tr>
<td>GCA</td>
<td>Guanylyl cyclase A</td>
</tr>
<tr>
<td>cGC</td>
<td>Soluble Guanylyl cyclase</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRKs</td>
<td>GPCR kinases (see GPCRs)</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Guan</td>
<td>Guanosine</td>
</tr>
<tr>
<td>HeLa</td>
<td>Type of cell line (ATCC Number: CRL-2266)</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamus–pituitary–adrenal</td>
</tr>
<tr>
<td>Jan</td>
<td>January</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin or 5-hydroxy-tryptamine</td>
</tr>
<tr>
<td>5-HT-Rs</td>
<td>Serotonergic receptors</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-Isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IC50</td>
<td>Macrophagial nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inositol (1,4,5)-trisphosphate</td>
</tr>
<tr>
<td>[Ins(1,4,5)P3]</td>
<td>Inositolphosphate</td>
</tr>
<tr>
<td>IPx</td>
<td>Inositol-multiphosphates</td>
</tr>
<tr>
<td>IP2</td>
<td>Inositol-4,5-biphosphate</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>K+</td>
<td>Sodium</td>
</tr>
<tr>
<td>Lam</td>
<td>Laminin</td>
</tr>
<tr>
<td>LH</td>
<td>Leuteinizing hormone</td>
</tr>
<tr>
<td>LHPA</td>
<td>Limbic-hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>Li2+</td>
<td>Lithium</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium Chloride</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LTD</td>
<td>Long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>l-NE</td>
<td>l-norepinephrine</td>
</tr>
<tr>
<td>MAOIs</td>
<td>Monoamine oxidase inhibitors</td>
</tr>
<tr>
<td>MAO – A</td>
<td>Monoamine oxidase A</td>
</tr>
<tr>
<td>MAO – B</td>
<td>Monoamine oxidase B</td>
</tr>
<tr>
<td>Mg2+</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>MDE</td>
<td>Major depressive episodes</td>
</tr>
<tr>
<td>mACHR</td>
<td>Cholinergic muscarinic receptor</td>
</tr>
<tr>
<td>MED</td>
<td>Male erecty dysfunction</td>
</tr>
<tr>
<td>MeCh</td>
<td>Metacholine</td>
</tr>
<tr>
<td>MI</td>
<td>Myo-inositol</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>mV</td>
<td>milli Volt</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NANC</td>
<td>Nonadrenergic-noncholinergic neurons</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
</tbody>
</table>
### Appendix A - Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NO-guanylyl cyclase</td>
<td>Nitric oxide – guanylyl cyclase</td>
</tr>
<tr>
<td>NO/cGMP</td>
<td>Nitric oxide / cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NMDA</td>
<td>M-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDA-NO-cGMP</td>
<td>M-methyl-D-aspartate – nitric oxide – cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive compulsive disorder</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDE1</td>
<td>Phosphodiesterase type 1</td>
</tr>
<tr>
<td>PDE2</td>
<td>Phosphodiesterase type 2</td>
</tr>
<tr>
<td>PDE3</td>
<td>Phosphodiesterase type 3</td>
</tr>
<tr>
<td>PDE4</td>
<td>Phosphodiesterase type 4</td>
</tr>
<tr>
<td>PDE5</td>
<td>Phosphodiesterase type 5</td>
</tr>
<tr>
<td>PDE6</td>
<td>Phosphodiesterase type 6</td>
</tr>
<tr>
<td>pGC</td>
<td>membrane Guanylyl cyclase</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-triphosphate</td>
</tr>
<tr>
<td>PK</td>
<td>Protein kinases</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PSF</td>
<td>Penicilllin/Streptomycin/Fungizone</td>
</tr>
<tr>
<td>[PtdIns(3,4,5)P3]</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PTSD</td>
<td>Post traumatic stress disorder</td>
</tr>
<tr>
<td>PUK</td>
<td>Potchefstroomse Universiteit Kampus</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
<tr>
<td>RAS</td>
<td>Reticular activating system</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulators of G-protein signalling</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SS</td>
<td>Sodium Salysilate</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>Type of cell line (ATCC Number: CCL-2)</td>
</tr>
<tr>
<td>Sild</td>
<td>Sildenafil</td>
</tr>
<tr>
<td>SSRIs</td>
<td>Selective serotonin reuptake inhibitors</td>
</tr>
<tr>
<td>TB</td>
<td>Trypan blue</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>T/V</td>
<td>Trypsin/versene</td>
</tr>
</tbody>
</table>