

The modulatory effects of sildenafil and the cholinergic system on antidepressant action in a rat model of depression

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Abstract

Sildenafil, a selective phosphodiesterase type 5 (PDE5) inhibitor, is registered for the treatment of male erectile dysfunction (Viagra®) and pulmonary hypertension (Revatio®) in the United States. PDE5 is found in the endothelium of blood vessels in the penile corpus cavernosum, pulmonary vessels and also brain and other peripheral tissue. Sildenafil crosses the blood brain barrier, leading to side-effects such as headache and dizziness, as well as behavioural manifestations including depression, anxiety and aggression (Milman & Arnold, 2002). According to the Food and Drug Administration (2001), 12378 adverse events were reported after the use of sildenafil and 274 of these reports implicated sildenafil in neurologic, emotional, or psychological disturbances between January 1998 and 21 February 2001. In addition, *in vivo* studies in rats indicate that sildenafil has anxiogenic and stressogenic actions (Harvey *et al.*, 2005; Volke *et al.*, 2003). This is a clear indication that sildenafil influences neurological processes in the brain and may influence various signalling systems, which play major roles in the neural circuitry of the above-mentioned disturbances.

Recent *in vitro* studies in our laboratory suggest that sildenafil may potentiate cholinergic muscarinic receptor signalling (Eager, 2004). These results suggest potential depressogenic actions, since an increase in acetylcholine is associated with depression-like symptoms (El-Yousef *et al.*, 1973). It was therefore postulated that sildenafil may in fact possess antidepressant activity that is masked by a cholinergic-driven depressogenic activity.

In a study conducted by Müller and Benkert in 2000, patients reported a decrease in depression-like symptoms when treated with sildenafil for erectile dysfunction. This implied that sildenafil not only had a direct effect on erectile function in about 50-80% of men with erectile dysfunction (Langtry and Markham, 1999; Padma-Nathan, 1999) but might also improve anhedonia and depression. The substantial correlation between the International Index of Erectile Function and Epidemiologic Studies-Depression Scale scores supported this assumption (Müller & Benkert, 2000). In addition, Raffaele *et al.* (2002) reported an indirect improvement in depressive-like symptoms in patients treated for erectile dysfunction with idiopathic Parkinson's disease.

Aims: The current study investigated the behavioural and neuroreceptor properties of sildenafil in a rat model of depression. We also investigated a hypothesis that sildenafil displays

antidepressant-like properties, but which are masked by its potentiation of the cholinergic system.

Methods: The experimental layout was divided into three pilot studies. Pilot Study 1 validated the FST under our laboratory conditions, Sprague-Dawley rats received saline intraperitoneally (i.p.) for 7 days, whereafter half of the rats were pre-exposed to a 15 minute swim trial, while the remaining rats were not pre-exposed. All rats were then evaluated 24 hours later in the 5 minute scored swim trial. In Pilot Study 2 Sprague-Dawley rats were treated for 3, 7 or 11 days with vehicle (control) or 20 mg/kg fluoxetine to establish the time-dependency of the onset of antidepressant-like effects in a rat model of depression. We measured immobility in the rat forced swim test (FST), as well as changes in β -adrenergic receptor (β -AR) concentration in rat frontal cortex. In pilot study 3, rats were treated for 7 days with vehicle (control), 20 mg/kg fluoxetine, 10 mg/kg sildenafil, 1 mg/kg atropine or various combinations of these drugs. Again we employed the FST and measured cortical β -AR concentration.

Results: In the FST pre-exposure to a 15 minute swim trial 24 hours before the scored swim trial significantly increased immobility. Fluoxetine inhibited this development of increased immobility in FST and decreased β -AR concentration after 7 and 11 days of treatment with fluoxetine, but not after 3 days. Seven days of treatment with atropine and sildenafil alone did not exert any changes in immobility in the FST or changes in β -AR concentration. However, a combination of atropine and sildenafil exerted a significant antidepressant-like behavioural effect, comparable with fluoxetine. Moreover, the combination of atropine and fluoxetine as well as the a triple combination of fluoxetine, sildenafil and atropine was superior to fluoxetine alone.

Conclusion: Muscarinic cholinergic mechanisms mask the antidepressant-like properties of sildenafil in a rat model of depression. The antidepressant properties of the combination of sildenafil and atropine are comparable to that of fluoxetine in an animal model of depression. The combination of fluoxetine with atropine, and atropine and sildenafil enhances the antidepressant-like properties of fluoxetine.

Keywords: Sildenafil, depression, atropine, muscarinic cholinergic pathway, forced swim test, phosphodiesterase type 5 inhibition, nitric oxide/cGMP pathway.

Opsomming

Sildenafil, 'n selektiewe tipe 5 fosfodiësterase inhibeerder, is geregistreer vir die behandeling van manlike erektilie disfunksie (Viagra®) en pulmonêre hipertensie (Revatio®) in die Verenigde State van Amerika. Fosfodiësterase tipe 5 inhibeerders word gevind in die endoteelselle van bloedvate in die corpus cavernosum, pulmonêre bloedvate asook die brein en ander perifere weefsel. Sildenafil besit die vermoë om die bloedbreinskans te kruis, wat dan ook tot verskeie newe-effekte kan lei onder andere, hoofpyn, lighoofdigheid, asook gedragsmanifestasies, wat depressie, angstigheid en aggressie insluit (Milman & Arnold, 2002). Volgens die Voedsel en Geneesmiddel Adminstrasie van die VSA (2001) het 12 378 pasiënte ongewensde effekte gerapporteer na die gebruik van sildenafil vir die periode, 1 Januarie 1998 tot einde Februarie 2001, waarvan 274 hiervan ongewensde neurologiese, emosionele en psigologiese effekte insluit. Bykomend tot bogenoemde, het Harvey *et al.* (2005) en Volke *et al.* (2003) aangedui dat sildenafil ansiogeniese asook depressogeniese effekte in *in vivo* studies op rotte geopenbaar het. Dit is daarom 'n duidelike aanduiding dat sildenafil neurologiese prosesse in die brein beïnvloed en dat sildenafil 'n invloed op verskeie boodskappersisteme, wat van belang is in bogenoemde, het.

In vitro studies in ons laboratorium het aangedui dat sildenafil oor die vermoë beskik om cholinergiese muskariniese seinsisteme te potensieer (Eager, 2004). Dit dui op 'n definitiewe depressogeniese effek, omdat n toename in asetielcholien gewoonlik met depressiewe simptome gepaard gaan (El-Yousef *et al.*, 1973). Daarom, is dit gepostuleer dat sildenafil wel antidepressiewe werking besit, maar dat hierdie werking deur 'n cholinergies gedrewe depressogeniese aktiwiteit gemaskeer word.

'n Studie, uitgevoer deur Müller en Benkert (2000) het onthul dat self-gerapporteerde depressie gedaal het in pasiënte wat behandel is met Sildenafil vir impotensie. Dit is 'n aanduiding dat sildenafil nie net 'n direkte effek op erectile disfunksie in ongeveer 50-80% van mans met erektilie disfunksie gehad het nie (Langtry en Markham, 1999; Padma-Nathan, 1999), maar dat sildenafil moontlik oof anhedonia en depressie kan verbeter. Hierdie afleiding word sterk ondersteun deur die korrelasie wat getrek kan word tussen die *International Index of Erectile Function* en die *Epidemiologic Studies-Depression Scale Scores* (Müller en Benkert, 2000).

Bykomend het Raffaele *et al.* (2002) 'n indirekte verbetering in depressie simptome in pasiënte, met idiopatiese Parkinson's se siekte, wat behandel is met sildenafil, gerapporteer.

Doelwitte: Die huidige studie het die gedrags- en neuroreseptor eienskappe van sildenafil in 'n rot model van depressie ondersoek. Die hipotese dat sildenafil oor antidepressiewe eienskappe beskik, maar wat deur die geneesmiddel se vermoë om die cholnergiese sisteem te potensieer gemaskeer word, is ondersoek.

Metodes: Die eksperimentele uitleg is verdeel in 2 proefstudies asook 'n eksperimentële studie. In Proefstudie 1 is die forseer swem toets in ons laboratorium gevalideer. *Sprague-dawley* rotte is met saline of fluoksetien intraperitoneaal behandel vir 7 dae, daarna is die helfte van die rotte onderwerp aan 'n swem periode van 15 minute, terwyl die oorblywende rotte nie daaraan blootgestel is nie. Na 'n periode van 24 uur, is al die rotte gedurende 'n 5 minuut swem periode geëvalueer. In proefstudie 2 is die *Sprague-dawley* rotte met die kontrole (saline) of 20mg/kg fluoksetien behandel om sodoende die aanvang van antidepressiewe effekte in 'n rot model van depressie te bepaal. Immobilititeit is gedurende die geforseerde swem toets gemeet asook veranderinge in β -andrenergiese reseptor konsentrasies in die frontale korteks van die rotte is bepaal. In die eksperimentele studie is die rotte vir 7 dae behandel met die kontrole (saline), 20mg/kg fluoksetien, 10mg/kg sildenafil, 1mg/kg atropien of verskeie kombinasies van die geneesmiddels. Immobilititeit en β -andrenergiese reseptor konsentrasies is weereens bepaal soos bo bepsreek.

Resultate: Gedurende die geforseerde swem toets het die rotte wat aan die 15 minuut swem periode, 24 uur voor die werklike toets swem periode, deelgeneem het betekenisvolle afnames in immobilititeit getoon. Fluoksetien het die ontstaan van toenemende immobilititeit en afnames in β -andrenergiese reseptor konsentrasies na 7 en 11 dae van behandeling veroorsaak, maar nie na 3 dae van behandeling nie. Behandeling met atropien en sildenafil onderskeidelik, vir 7 dae, het geen effek op immobilititeit en β -andrenergiese reseptor konsentrasies getoon nie. Die kombinasie van sildenafil en atropien het egter betekenisvolle antidepressiewe gedrag getoon, wat vergelykbaar was met die effekte wat fluoksetien tot gevolg gehad het. Verder het die kombinasie van fluoksetien en atropien asook die kombinasie van atropien, fluoksetien en sildenafil, behandeling met fluoksetien alleen oortref.

Gevolgtrekking: Muskariniese cholinergiese meganismes maskeer die antidepressiewe eienskappe van sildenafil, in 'n rot model van depressie. Die antidepressiewe eienskappe van die kombinasie van sildenafil en atropien is vergelykbaar met dié van fluoksetien alleen in 'n dierlike model van depressie. Die kombinasie van fluoksetien met atropien, asook atropien en sildenafil verbeter die antidepressiewe eienskappe van fluoksetien.

Sleutelwoorde: Sildenafil, depressie, atropien, muskariniese cholinergiese meganismes, geforseerde swem toets, fosfodiësterase tipe 5 inhibisie.

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In this chapter the problem statement, research objectives, research methodology and chapter division will be discussed.

1.1 Problem statement

The World Health Organization (2001) estimated that 121 million people are currently suffering from depression, making depression the fourth most debilitating disease in the world. An estimated 5.8% of men and 9.5% of women will experience a depressive episode in any given year. Furthermore, 25% of the general population will present symptoms consistent with a major depressive episode at some time in their lives. Depression is a potentially fatal disorder (Disalver *et al.*, 1994; Strakowsky *et al.*, 1996) and can have a significant economic burden (Price, 2004). Depression is often underdiagnosed and undertreated (Baldessarini, 2001). In order to address these problems in a more significant manner, we need to better understand the pathophysiological basis of depression. There is also a need for new drugs with reduced side effect profiles, faster onset of action and improved therapeutic efficacy. This includes cases that are currently resistant to treatment.

The current hypotheses of depression include not only the well-described monoaminergic hypothesis, but also the cholinergic hypothesis of depression as well as neurodegenerative hypothesis of depression. The N-methyl-D-aspartate/nitric oxide/cyclic guanosine monophosphate (NMDA/NO/cGMP) pathway plays an important part in the latter (Dawson *et al.*, 1991), thus warranting an investigation into the potential efficacy of inhibitors of the phosphodiesterase enzymes, such as sildenafil in the alleviation of depression.

The Food and Drug Administration (FDA) approved Sildenafil for the treatment of erectile dysfunction (Viagra®) and pulmonary hypertension (Revatio®). Sildenafil is available in 90 countries, and by 2001, more than 15 million men were treated with the drug for male erectile problems (Pfizer Labs, 2006a). The localisation of PDE5 to certain areas of the brain as well as the ability of sildenafil to cross the blood brain barrier provides the possibility of central nervous system (CNS) effects. Several direct effects of sildenafil administration on the CNS have been reported in both rodents and humans. For instance, oral administration of sildenafil to rats has been shown to increase in brain levels of cGMP as well as evoke neurogenesis (Zhang *et al.*,

2002). Another study performed by Schultheiss and co-workers (2001), examined the potential central effects of sildenafil on attention and memory functions in humans. Although no overt effects on behaviour were observed, sildenafil treatment caused an enhanced ability to focus attention. It has also been found that sildenafil causes significant increases in sympathetic nerve activity in humans (Giuliani *et al.*, 2002).

According to the FDA (2001), 12 378 adverse events were reported after the use of sildenafil of which 274 of these reports implicated sildenafil in neurologic, emotional, or psychological disturbances between January 1998 and February 2001. In addition, *in vivo* studies on rats indicated that sildenafil has anxiogenic as well as stressogenic actions (Harvey *et al.*, 2005; Volke *et al.*, 2003). This is a clear indication that sildenafil influences neurological processes in the brain and may influence various signalling systems, which play major roles in the neural circuitry of the above-mentioned disturbances.

In vitro studies in our laboratory suggested that sildenafil may potentiate cholinergic muscarinic receptor signalling (Eager, 2004). These results suggest potential depressogenic actions, since an increase in acetylcholine is associated with depression-like symptoms (El-Yousef *et al.*, 1973). It was, therefore, postulated that sildenafil may in fact possess antidepressant activity that is masked by a cholinergic-driven depressogenic activity.

In a study conducted by Müller and Benkert in 2000, patients reported a decrease in depression-like symptoms when treated with sildenafil for erectile dysfunction. This implied that sildenafil not only had a direct effect on erectile function in about 50-80% of men with erectile dysfunction (Langtry and Markham, 1999; Padma-Nathan, 1999) but might also improve anhedonia and depression. The substantial correlation between the International Index of Erectile Function and Epidemiologic Studies-Depression Scale scores supported this assumption (Müller & Benkert, 2000). In addition, Raffaele *et al.* (2002) reported an indirect improvement in depressive-like symptoms in patients treated for erectile dysfunction with idiopathic Parkinson's disease.

1.2 Research objectives

This research includes general and specific objectives:

1.2.1 General objective

The current study investigates the behavioural and neuroreceptor modulating properties of sildenafil in a rat model of depression, in particular antidepressant-like properties and cortical β -adrenoceptor concentration. Secondly the study investigates the hypothesis that sildenafil displays antidepressant-like properties masked by cholinergic potentiation.

1.2.2 Specific objectives

The specific research objectives of the study are as follows:

- To investigate the effect of sildenafil pre-treatment on immobility time during the rat forced swim test (FST), as compared to the prototype antidepressant fluoxetine
- To investigate the effect of sildenafil pre-treatment in rats on cortical β -adrenoreceptor concentration, as compared to the prototype antidepressant fluoxetine.
- To investigate whether any putative antidepressant-like properties of sildenafil are masked by cholinergic muscarinic receptor potentiation.
- To investigate the possible behavioural and neuroreceptor effects of sildenafil in various combinations with fluoxetine and atropine in rats.

1.3 Study Layout

The experiments that were used in this study to investigate the possible antidepressant-like properties of sildenafil included the FST as well as radioligand binding studies.

In order to investigate the antidepressant-like properties of sildenafil in a rat model of depression, the traditional forced swim test (first described by Porsolt in 1978) was used, consisting of the induction of behavioural despair in rats after a preconditioning swim period of 15-minutes; followed 24 hours later, by a 5-minute test swimming session. To enhance the sensitivity of the traditional FST to be more responsive to serotonin reuptake inhibitors, the scoring technique of the modified FST was implemented (Decke *et al.*, 1995). The test swimming session was scored in immobility, swimming and climbing. An increase in immobility time was associated with an increase in behavioural despair. According to the principles of the FST behavioural despair can be reversed with the administration of antidepressants.

The radioligand binding studies performed during this study, investigated the neuromodulatory effects of the various chronic drug treatments on the β -adrenoreceptor density in the frontal cortex of the test rats.

The experimental layout was divided into two pilot studies as well as an experimental study.

- Pilot Study 1 validated the FST under our laboratory conditions. Sprague-Dawley rats received saline intraperitoneally (i.p.) for 7 days, whereafter half of the rats were pre-exposed to a 15 minute swim trial, while the remaining rats were not pre-exposed. All rats were then evaluated 24 hours later in the 5 minute scored swim trial.

- Pilot Study 2 Sprague-Dawley rats were treated for 3, 7 or 11 days with vehicle (control) or 20 mg/kg fluoxetine to establish the time-dependency of the onset of antidepressant-like effects in a rat model of depression. We measured immobility in the rat FST, as well as changes in β -adrenergic receptor (β -AR) concentration in rat frontal cortex.
- In the experimental study, rats were treated for 7 days with vehicle (control), 20 mg/kg fluoxetine, 10 mg/kg sildenafil, 1 mg/kg atropine or various combinations of these drugs. Again we employed the FST and measured cortical β -AR concentration.

In this chapter the proposed important role of the NO- cyclic guanosine monophosphate (cGMP) signal transduction pathway in depression and the involvement of sildenafil in this pathway will be discussed.

This chapter will also review the following:

- The pharmacology of sildenafil
- The bio-chemistry of the NO-cGMP pathway
- The association between depression and the NO-cGMP pathway
- Depression and the various hypotheses of the bio-molecular mechanisms underlying depression
- The forced swim test for investigating antidepressant-like responses in rats and β -adrenoreceptor down-regulation as markers of antidepressant-like effects in rats

2.1 Phosphodiesterase 5 inhibitors

Three phosphodiesterase type 5 (PDE5) inhibitors are currently marketed for clinical use, namely sildenafil citrate, vardenafil monohydrochloride trihydrate and tadalafil.

Sildenafil, a selective PDE5 inhibitor, is marketed by Pfizer as Viagra® and Revatio® respectively, for the treatment of male erectile dysfunction (MED) or pulmonary hypertension.

Bayer and GlaxoSmithKline introduced the PDE5 inhibitor vardenafil in 2003 and tadalafil was co-developed by Icos Corporation and Eli Lilly in 2002. Both are for the treatment of MED.

2.1.1 Physical and chemical properties

Sildenafil citrate is designated chemically as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine citrate. It has a molecular weight of 666.7 g/mol (Pfizer Labs, 2006a) and has a structural formula as depicted in Figure 2-1 (Pfizer Labs, 2006a).

The empirical formula for vardenafil is $C_{22}H_{19}N_3O_4$, and its official organic name is (6R,12aR)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl-pyrazino[1,2:1,6]pyrido[3,4-b]indole-1,4-dione and the drug has a molecular weight of 389.41 g/mol (European Medicine Agency, 2006b).

The official organic name of tadalafil is 4-[2-ethoxy-5-(4-ethylpiperazin-1-yl)sulfonyl-phenyl]-9-methyl-7-propyl-3,5,6,8-tetrazabicyclo[4.3.0]nona-3,7,9-trien-2-one. The drug has a molecular weight of 488.604 g/mol (European Medicine Agency, 2006a).

Figure 2-1 depicts the structural similarities between cGMP and the various PDE5 inhibitors. The structures of the PDE5 inhibitors must be similar to that of cGMP for it to be able to bind to the catalytic sites on the PDE5 isozyme.

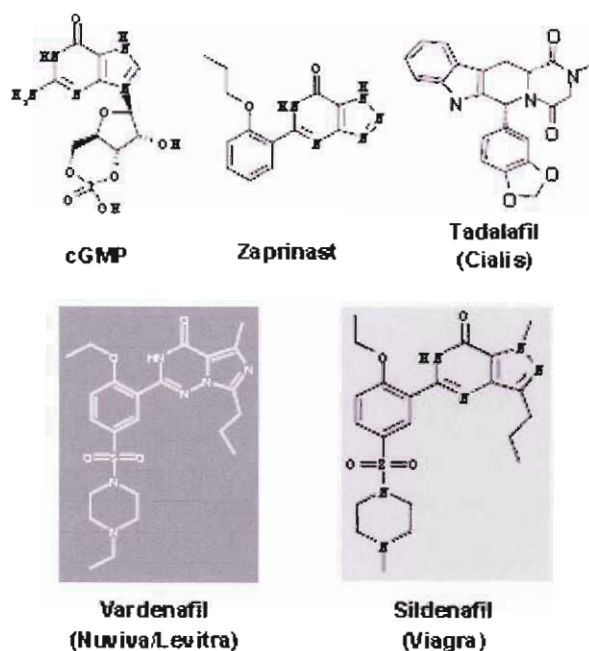


Figure 2-1 Comparison of the structure of PDE5 inhibitors with the native substrate, cGMP (Kukreja et al., 2005). The vardenafil and sildenafil structures are highlighted to indicate the similarity in structures.

2.1.2 Medicinal Formulation

Viagra® is formulated as a blue, film-coated rounded-diamond-shaped tablet equivalent to 25 mg, 50 mg and 100 mg of sildenafil for oral administration. Revatio® is formulated as a white, film-coated round tablet equivalent to 20 mg of sildenafil for oral administration (Snyman, 2006).

Cialis® is formulated as a yellow film-coated almond shaped tablet marked on the side with a "T 20" or "C 20". Each film-coated tablet contains 20 mg of tadalafil (Snyman, 2006).

Levitra® is formulated as orange (light-orange to grey-orange) film-coated round tablets with Bayer embossed on the one side. Each tablet contains vardenafil monohydrochloride trihydrate to the equivalent to 5 mg, 10 mg or 20 mg (Snyman, 2006).

2.1.3 Pharmacokinetic properties

In 1999, the pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man revealed the following (Walker *et al.*, 1999):

- In man, absorption from the gastrointestinal tract is essentially complete. With the exception of the male rat, T_{max} occurs at approximately 1 hour or less. Bioavailability is attenuated by presystemic hepatic metabolism in all species.
- The volume of distribution is similar in rodents and humans (1-2 l/kg).
- High clearance is the principle determinant of short elimination half-lives in rodents ($t_{1/2} = 0.4$ to 1.3 hours), whereas moderate clearance in dog and man results in longer half-lives ($t_{1/2} = 6.1$ and 3.7 hours respectively). Clearances are in agreement with *in vitro* metabolism rates by liver microsomes from the various species.

According to Pfizer Labs (2006a) Viagra® is rapidly absorbed after oral administration, with absolute bioavailability of roughly 40%. It is furthermore predominantly eliminated by hepatic metabolism (mainly cytochrome P450 3A4) and is then converted to an active metabolite with properties similar to the parent, sildenafil. According to Gibbon (2003), sildenafil has a terminal half-life of 3 to 5 hours, with plasma protein binding of 96%.

Vardenafil has a t_{max} of 0.9 and 0.7 hours for 10- and 20-mg doses, respectively, and a half-life of ≈ 4 hours for 10 and 20 mg (Klotz *et al.*, 2001). Klotz and colleagues (2001) found that single 10- and 20-mg doses of vardenafil led to a rapid increase in plasma concentrations and a mean C_{max} of 9.05 μ g/L and 20.9 μ g/L, respectively. Absorption rate is reduced when vardenafil is taken with a high-fat meal (European Medicine Agency, 2006b).

Tadalafil is rapidly absorbed and extensively metabolised by the liver (CYP3A4) (European Medicine Agency, 2006a). The drug has a t_{max} of 2.0 hours and a half-life of 1.75 hours (Patterson *et al.*, 2001a). At the 20mg dose, $C_{max} = 378\mu$ g/L the pharmacokinetics of tadalafil are unaffected by food, age, the presence of diabetes, or mild or moderate hepatic insufficiency (Patterson *et al.*, 2001b)

2.1.4 Phosphodiesterase 5 inhibition

The PDE isozyme is a homodimer and consists of a monomer that contains a carboxy-terminal catalytic domain, a highly conserved zinc-binding motif, two allosteric binding pockets for cGMP and, in the amino-terminal region, a phosphorylation site at serine 92 (Ser⁹²). Either protein kinase A (PKA) or protein kinase G (PKG) can phosphorylate PDE5, resulting in a 50-70% increase in enzyme activity (Corbin *et al.*, 2000). Each of the sub-units also contains two allosteric (non-catalytic) binding sites for cGMP and occupation of these sites is necessary for Ser⁹² phosphorylation (inactivation of cGMP), further binding of cGMP to the allosteric sites is enhanced following occupation of the catalytic sites (Corben & Francis, 1999).

PDE5 inhibitors, such as sildenafil, vardenafil and tadalafil, compete with cGMP for binding to the catalytic site but not the allosteric sites (Corbin and Francis, 1999). Occupation of the catalytic sites by PDE5 inhibitors inhibits binding of cGMP to the allosteric sites; and Ser⁹² phosphorylation has no effect on inhibitor binding (Corbin *et al.*, 2000). The data, therefore, suggest that the mode of antagonism is due to the fact that the PDE5 inhibitors inhibit the binding of cGMP to PDE5 as well as to the fact that PDE5 inhibitors are not phosphorylated when bound to the catalytic domain, which leads to an increase in cGMP concentration.

2.1.5 Therapeutical uses

The following sections will discuss the therapeutic uses of the above-mentioned PDE5 inhibitors

2.1.5.1 Male erectile dysfunction

Sildenafil, vardenafil and tadalafil are all PDE5 inhibitors and are indicated for the treatment of MED (Rosen & Kostis, 2003).

Sildenafil was the first in its class to be approved by the Food and Drug Administration (FDA) in the United States of America for the treatment of male erectile dysfunction (MED) (Goldenberg, 1998). The discovery of this drug in 1989 was the result of extensive research on chemical agents that might potentially be useful in the treatment of coronary heart disease. Initial clinical studies on sildenafil in the early 1990s were not promising with respect to its antianginal potential. However, a remarkable side effect was reported by a number of volunteers participating in these investigations; sildenafil seemed to enhance penile erections, which soon thereafter became the main focus of further studies (Kukreja *et al.*, 2005).

Sildenafil quickly gained acceptance by the medical community and the public because of its broad efficacy for different types of erectile dysfunction and its ease of use. Sildenafil is now

available in 90 countries, and by 2001, more than 15 million men had taken the drug for the treatment of MED (Pfizer Labs, 2006a).

Bayer and GlaxoSmithKline introduced the PDE5 inhibitor vardenafil in 2003 for the treatment of MED. Vardenafil has a similar duration of action as sildenafil, but is more potent and biochemically selective (Kukreja *et al.*, 2005). The added advantages of vardenafil in the treatment of MED is, a faster onset of action, extended half-life time, improved pharmacological profile and minimal interactions with food and alcohol when compared to sildenafil (Stief *et al.*, 2005) as well as a high efficacy and low adverse-event profile in a population with mixed erectile dysfunction etiologies (Prost *et al.*, 2001).

Tadalafil was co-developed by Icos Corporation and Eli Lilly in 2001. Tadalafil is a long-acting PDE5 inhibitor and is an option in men suffering from mild to severe MED. Due to the long half-life time, and extended period of responsiveness (up to 36 hours) and the convenience of taking the drug together with food and alcohol, tadalafil provides couples with a degree of spontaneity for sexual activities (Stief *et al.*, 2005).

2.1.5.2 Pulmonary arterial hypertension

Revatio® was developed by Pfizer Labs in 2006, and is an oral therapy for pulmonary arterial hypertension to improve exercise ability. Revatio is the citrate salt of sildenafil, a selective inhibitor of cyclic guanosine monophosphate-specific PDE 5 and is also marketed as Viagra® for male erectile dysfunction (Pfizer Labs, 2006b).

2.1.6 The mechanism of action

The physiologic mechanism of erection of the penis involves release of NO from non-cholinergic, non-adrenergic neurons, as well as from endothelial cells in the corpus cavernosum during sexual stimulation. NO diffuses into the trabecular smooth muscle cells, where it activates soluble guanylyl cyclase, the enzyme that converts GTP to cGMP. The cyclic nucleotide then stimulates PKG, which initiates a protein phosphorylation cascade. This leads to the subsequent phosphorylation of actin-myosin system as well as Ca²⁺ channels located in the outer cell membrane and in the membrane of the sarcoplasmic reticulum. This results in a decrease in intracellular levels of calcium ions, leading ultimately to dilation of the arteries allowing the inflow of blood into the penis and compression of the spongy corpus cavernosum (Kukreja *et al.*, 2005; Stief *et al.*, 2005).

Sildenafil has no direct relaxant effect on isolated human corpus cavernosum, but enhances the effect of nitric oxide by inhibiting PDE 5, which is responsible for degradation of cGMP in the corpus cavernosum. When sexual stimulation causes local release of NO, inhibition of PDE5 by

sildenafil causes increased levels of cGMP in the corpus cavernosum, resulting in smooth muscle relaxation and inflow of blood to the corpus cavernosum (Kukreja *et al.*, 2005; Stief *et al.*, 2005).

Vardenafil and tadalafil follow a similar mechanism of action to alleviate MED.

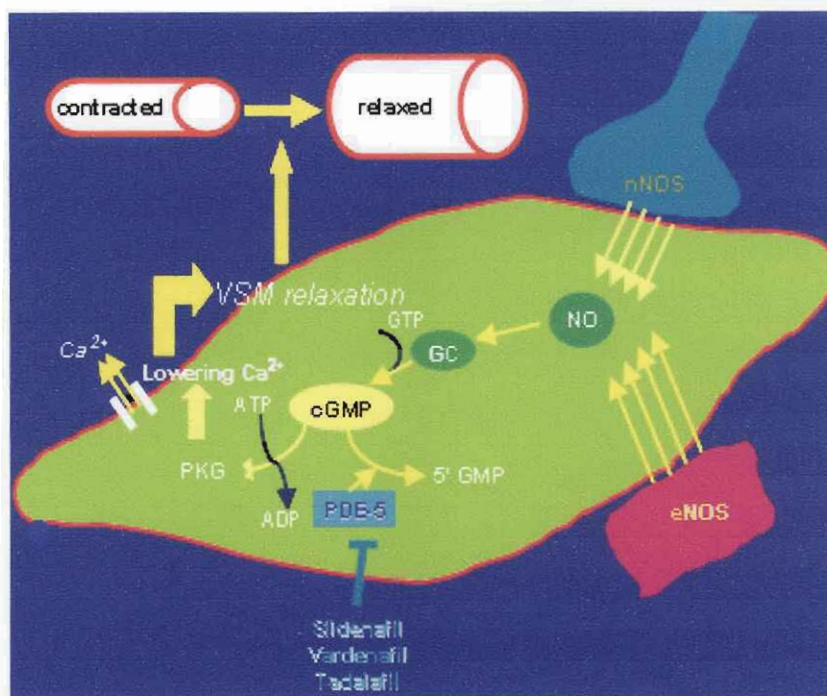


Figure 2-2 Mechanism of penile erection: Sexual stimulation releases NO from non-cholinergic, non-adrenergic neurons in the penis, as well as from endothelial cells. NO diffuses into the cells and activates soluble guanylyl cyclase, which converts GTP to cGMP. The cyclic nucleotide then stimulates protein kinase G (PKG), which initiates a protein phosphorylation cascade, thereby decreasing intracellular levels of calcium ions, leading ultimately to dilatation of the arteries allowing the inflow of blood into the penis and compression of the spongy corpus cavernosum (Kukreja *et al.*, 2005)

The same effect is achieved in the smooth muscle of the pulmonary vasculature. Sildenafil increases cGMP within pulmonary vascular smooth muscle cells resulting in relaxation. In patients with pulmonary hypertension, this can lead to vasodilatation of the pulmonary vascular bed and, to a lesser degree, vasodilatation in systemic circulation (Pfizer Labs, 2006b).

2.1.7 Adverse effects

Sildenafil

Various adverse effects (i.e., headache 16 %, facial flushing 10 %, dyspepsia 7 %, nasal congestion 4 %, and visual disturbances 3 %) have been attributed to the inhibition of PDE5 by sildenafil in the smooth muscle of cerebral or other vascular vessels, oesophageal sphincter, and nasal mucosa, and by inhibition of PDE6 in smooth muscle of the retina (Osterloh *et al.*, 1999; Goldstein, 1999; Young, 1999; Hotchkiss *et al.*, 2004).

The adverse effects of tadalafil and vardenafil are identical to that of sildenafil mentioned above, and the majority of the adverse effects are due to PDE5 inhibition. Tadalafil, however, shows an improvement in the occurrence of visual disturbances with only 0.9 % (n = 1561) reported cases compared to the 3 % of sildenafil (Snyman, 2006).

2.1.8 The selectivity of PDE5 inhibitors

The concentration at which a given drug inhibits the activity of a PDE by 50% is expressed as IC_{50} , a measurement of pharmacologic activity. Sildenafil is more selective for PDE5 (IC_{50} = 3.5 to 3.9 nmol/L) (Boolell *et al.*, 1996) than for other PDEs (> 80-fold more than for PDE1; >1,000-fold more than for PDE2 to PDE4; and about 10-fold more than for PDE6, an enzyme found in the retina) (Rosen & Kostis, 2003)

Vardenafil is also selective for PDE5 *in vitro* (IC_{50} = 0.7nmol/L) and more selective for PDE5 than for PDE1 to PDE4. Vardenafil is > 15-fold more selective for PDE5 than for PDE6 (Sáenz de Tejada *et al.*, 2001; European Medicine Agency, 2006b).

Tadalafil is highly selective for PDE5 (IC_{50} = 0.94 nmol/L) over other enzymes (Angulo *et al.*, 2001). *In vitro* studies with tadalafil have demonstrated a > 10.000-fold greater selectivity for PDE5 versus PDE1 to PDE4 and PDE7 to PDE10, as well as approximately 700-fold greater selectivity for PDE5 than for PDE6 (Angulo *et al.*, 2001; European Medicine Agency, 2006a).

2.1.9 Sildenafil and the central nervous system

The localisation of PDE5 to areas of the brain as well as the ability of sildenafil to cross the blood brain barrier provides for the possibility of central nervous system (CNS) effects. Several direct effects of sildenafil administration have been reported in both rodents and humans (Philips *et al.*, 2000; Schultheiss *et al.*, 2001).

Rodents - Oral sildenafil administration to rats has been shown to increase brain levels of cGMP and to evoke neurogenesis (Zhang *et al.*, 2002). In addition, sildenafil is capable of

centrally altering copulatory behaviour in rats, perhaps via a dopaminergic pathway (Phillips *et al.*, 2000; Ferrari *et al.*, 2002). Volke *et al.* (2003) reported that an augmentation of the NO-cGMP pathway, with sildenafil, induced angiogenic-like effects in mice.

Prikearts *et al.* (2006) reported that PDE 5 was involved in the early memory consolidation process. Furthermore, Rutten and co-workers (2007) observed that PDE 5 inhibitors, when used in rodents, showed a marked improvement in object memory.

Humans - Schulthesis and co-workers (2001) examined potential central effects of sildenafil on attention and memory function in humans. Although no overt effects on behaviour were observed, sildenafil treatment caused an enhanced ability to focus attention. Finally, sildenafil administration significantly increased sympathetic nerve activity in humans (Giuliani *et al.*, 2002; Hotchkiss *et al.*, 2004).

Table 2-1 depicts the various neurologic, emotional, or psychological effects and psychological disorders reported by patients to the FDA after the use of sildenafil (Milman & Arnold, 2002).

Table 2-1 Adverse event reports filed with the FDA listing sildenafil as the primary suspect for neurologic, emotional, or psychological disturbances (Milman & Arnold, 2002)

Adverse effects	No. of Reports
Neurologic, emotional, or psychological effects; psychological disorders; amnesia or loss of consciousness; or aggressive behaviour	274
Neurologic effects such as confusion, dizziness, anxiety, agitation, nervousness, attention disturbance, disorientation, or irritability	70
Emotional or psychological effects such as emotional disturbance, abnormal thinking, depression, abnormal behaviour, euphoria, abnormal dreams, delirium, or other mental or neurologic disturbances	71
Psychological disorders such as depersonalisation, hallucination, personality disorder, mania, delusion, other psychotic disorders, or paranoia	72
Amnesia or loss of consciousness	44
Aggressive behaviour such as aggression, hostility, or physical assault	37
Deaths due to suicide or murder	27
Specific effect	
Abnormal dreams	6
Abnormal thinking	11
Aggression	22
Agitation	10
Amnesia	35
Anxiety	14
Attempted suicide	22
Attention disturbances	4
Confusion	31
Delirium	5
Delusion	8
Depersonalisation	23
Depression	9
Disorientation	3
Dizziness	22
Emotional disturbances	19
Euphoria	8
Hostility	17
Hallucination	17
Irritability	1
Loss of consciousness	12
Mania	11
Murder	6
Nervousness	6
Other mental or neurologic disturbances	7
Other psychotic disorders	8
Paranoia	3
Personality disorders	14
Physical assault	5
Rape	13
Suicide	21
Suicide ideation	2

According to the FDA (2001), 12378 adverse events were reported after the use of sildenafil of which 274 of these reports implicated sildenafil in neurologic, emotional, or psychological disturbances between January 1998 and 21 February 2001(Milman & Arnold, 2002). This is a clear indication that sildenafil influences neurological processes in the brain and may influence various signalling systems, which play major roles in the neural circuitry of the above-mentioned

disturbances. It can be seen in the table that 70 of the reported adverse effects were due to neurologic disturbances, which included confusion, dizziness, anxiety, agitation, nervousness, attention disturbance, disorientation, or irritability. Anxiety on its own is also known to elevate acetylcholine release in the hippocampus, implicating the cholinergic muscarinic receptors in the aetiology of anxiety (Meany *et al.*, 1993).

This correlates with previous findings in our laboratory, which stated that sildenafil might potentiate the cholinergic muscarinic signalling system, which may explain why sildenafil causes anxiety in some patients (Eager, 2004).

Furthermore, Kurt and co-workers (2004) reported that, the results retrieved from experiments with mice in the plus-maze, might implicate the NO-cGMP pathway in the sildenafil induced anxiogenic-like effects that was observed.

Seventy-one of the reported cases of adverse effects of sildenafil implicated emotional or psychological effects. This included emotional disturbances, abnormal thinking, depression, abnormal behaviour, euphoria, abnormal dreams, delirium, or other mental or neurologic disturbances. The ability of sildenafil to potentiate the cholinergic muscarinic system might also be responsible for the depression-like symptoms reported by the FDA, due to the fact that patients with major depression have super-sensitive cholinergic systems when compared to patients without depression (Daws & Overstreet, 1999).

2.2 The NO-cGMP pathway

Figure 2-3 gives a schematic representation of the NO/cGMP pathway.

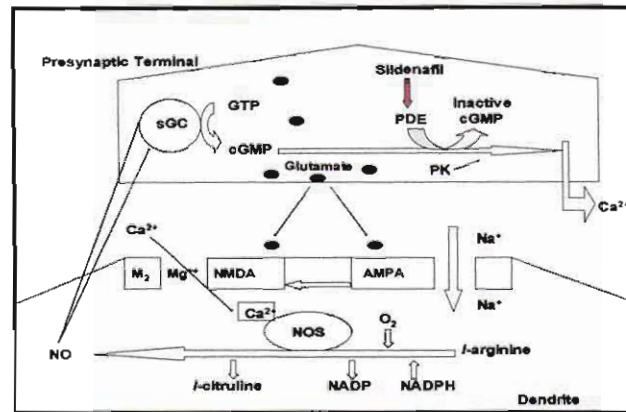


Figure 2-3 Schematic representation of the NO/cGMP biochemical pathway. After the activation of N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptors on the dendrite/endothelium by glutamate, calcium (Ca^{2+}) enters the cell and activates nitric oxide synthase (NOS). 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*-Citruiline and NADP are formed as by-products. The NO then diffuses to the presynaptic terminal and activates soluble guanylyl cyclase (sGC), which converts guanosine triphosphate (GTP) to cGMP. This increases intracellular cGMP. (Ballard *et al.*, 1998; Boolell *et al.*, 1996). Phosphodiesterase (PDE) enzymes that convert the cGMP to inactive GMP (adapted by Eager, 2004) terminate this process.

Nitric oxide synthase (NOS) has widespread distribution in the central nervous system (CNS) (Bredt *et al.*, 1990). In the hippocampus, two constitutive isoforms of NOS have been described, i.e. the neuronal NOS (nNOS) and the endothelial NOS (eNOS). In this brain area, both isoforms are activated through calcium/calmodulin dependent pathways, which are triggered by the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (Garthwaite & Boulton, 1995). NO diffuses from the site of synthesis to the target structures, which are neurons and astrocytes close to the site of the NO production (De Vente *et al.*, 1998; Garthwaite, 1991). The soluble guanylyl cyclase (sGC) presents an important target molecule for NO (Bredt & Snyder, 1989; Knowles *et al.*; 1989). NO activates sGC by binding to the heme group of the enzyme, which leads to an increase in cGMP synthesis. cGMP and the other second messenger cAMP are inactivated by hydrolytic cleavage of their 3'-phosphoester bonds to form 5'-GMP and 5'-AMP, catalysed by the superfamily of enzymes known as the cyclic nucleotide phosphodiesterase (PDEs) (Staveren *et al.*, 2000).

The signal transduction pathway of NO-cyclic GMP will be discussed below in terms of the excitatory amino acid receptors, nitric oxide (NO), soluble guanylyl cyclase (sGC), cGMP and phosphodiesterases (PDEs).

2.2.1 Excitatory Amino-Acid receptors:

Almost all neurons in the brain are influenced by the excitatory amino acid glutamate. Excitatory glutamate receptors are widely distributed in the prefrontal cortex, the striatum, and, to a lesser extent, the thalamus. Ionotropic glutamate receptors form cation-selective channels, with permeability for Na^{2+} and K^+ , and differing degrees of permeability to Ca^{2+} and Mg^{2+} . They are categorised into three classes, namely α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate or kainic acid (KA), and *N*-methyl-D-aspartate (NMDA). These receptors assemble as homo- or hetero-oligomeric complexes of protein subunits (Galvan *et al.*, 2006).

2.2.1.1 N-methyl-D-aspartate (NMDA)

The NMDA receptors are composed of three subunits, designated NR1, NR2A-NR2D and NR3A-NR3B (Madden, 2002; Mayer & Armstrong, 2004; Kew & Kemp, 2005). The NMDA receptor is a ligand-gated, voltage-sensitive ionophore which gates Ca^{2+} and, to a lesser extent, Na^+ and K^+ (Meguro *et al.*, 1992). Stimulation of the receptor and opening of the ionophore results in Ca^{2+} entry into the receptive neuron. The Ca^{2+} binds to, and stimulates a calcium-calmodulin complex that, in turn, stimulates nitric oxide synthesis (Southnam & Garthwaite, 1993; Andrew *et al.*, 1999). NMDA receptors mediate slower, long-lasting responses (Ozawa *et al.*, 1998). In addition to its agonist glutamate these receptors require glycine as a co-agonist.

The currents conducted by NMDA receptors are blocked by extracellular Mg^{2+} in a voltage-dependent mode. At resting membrane potential (-70mV) activation of the channel will result only in low current, because entry of Mg^{2+} ions into the channel will block the current. The affinity for Mg^{2+} will decrease at less negative membrane potentials as the electric driving force for Mg^{2+} is reduced and the block becomes ineffective. The NMDA receptors are co-localised with the AMPA receptors on many synapses, but the slow kinetics of the NMDA receptor minimise the receptor activation after a single presynaptic glutamate release where the neuron quickly repolarises, resulting in Mg^{2+} block of the NMDA receptor. However, the NMDA receptor is fully activated 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 Mg^{2+} block. This use-dependent Ca^{2+} influx has been interpreted to be one of the underlying mechanisms for many different neuronal processes, including learning and memory (Foreman & Johansen, 2003).

2.2.1.2 Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)

The selective agonist AMPA activates a fast desensitising current, and low Ca^{2+} permeability (as does glutamate) in the majority of these excitatory amino-acid receptors. Consequently, this subtype is referred to as AMPA receptors. The AMPA receptor family include subunits GluR1-GluR4 and co-assemble with one another, but not with subunits from other classes (Madden, 2002; Mayer & Armstrong, 2004; Kew & Kemp, 2005). AMPA receptors are co-localised with NMDA receptors on many synapses throughout the brain. AMPA receptors are activated during synaptic transmission and the rapid kinetics and the low Ca^{2+} permeability make these receptors ideal for fast neurotransmission without sufficient changes in the intracellular calcium concentration to activate Ca^{2+} -dependent processes (Foreman & Johansen, 2003).

2.2.1.3 Kianate (KA)

The interaction of kianate with AMPA receptors lead to a non-desensitising current, however, when kianate interacts with another receptor (the Kianate receptor type) it activates a fast desensitising current which leads to glutamatergic neurotransmission. These receptors are subdivided into GluR5-GluR7 and KA1/KA2 subunits (Madden, 2002; Mayer & Armstrong, 2004; Kew & Kemp, 2005) and exert a high affinity for kianate (Foreman & Johansen, 2003). Although AMPA and NMDA receptors are the primary mediators of glutamatergic transmission in the central nervous system, KA receptors also contribute to neuronal excitability (Frerking and Nicol, 2000; Huettner, 2003; Lerma, 2003).

2.2.2 Nitric oxide

In recent years, our understanding of the nitric oxide-cyclic GMP signal transduction system has grown remarkably. This was due to the fact that the focus of investigation has shifted from the cAMP pathway to the NO-cGMP pathway. Nitric oxide is a simple, but unique, gaseous molecule and free radical that can serve many diverse functions, including a function as an intracellular second messenger and as an intercellular messenger to regulate neighbouring and perhaps distant cells. Knowledge of the important interrelationships of nitric oxide and cGMP has led to our present understanding of this fundamentally ubiquitous and important signal transduction pathway (Murad, 1995).

Nitric oxide is formed by most, but not all, cells. Its formation and release by central and peripheral neurons permits the molecule to function as a neurotransmitter at "nitergic" neurons. Thus, nitric oxide may function as an intracellular second messenger and intercellular messenger (Murad, 1995).

2.2.2.1 The synthesis of nitric oxide

In the mammalian organism, NO is synthesised in several types of cells, such as neurons, endothelial cells, and macrophages by a family of three isoenzymes termed nitric oxide synthase (NOS). nNOS is present in neurons, it is constitutively expressed and its activity is regulated by Ca^{2+} . Another constitutive and Ca^{2+} -dependent type of NOS is present in the endothelial cells (eNOS) of the vasculature. Macrophages contain an inducible, Ca^{2+} -dependent NOS (iNOS) which is expressed upon induction by cytokines (Föstermann & Kleinert, 1995; Mayer & Andrew, 1998). Neurons of hippocampus and other brain regions also contain eNOS (Dinerman *et al.*, 1994). All three isoenzymes produce NO by oxidising a guanidino nitrogen of L-arginine utilising molecular oxygen and NADPH as co-substrates (Mayer *et al.*, 1991; Lohse *et al.*, 1998).

In neurons, NO synthesis is stimulated by Ca^{2+} -influx that is induced by activation of glutamate receptors, preferentially NMDA receptors (Garthwaite *et al.*, 1989; Wood *et al.*, 1990). NO primarily operates as an intracellular messenger. It diffuses rapidly to, and influences NO-responsive target cells. Thus, though NO-producing cells are scarcely spread in many tissues, the NO released may influence neurons in a widely extended area. The selectivity of this messenger is achieved by NO-receptive characteristics of cell components in certain cells, thus being target cells of NO (Prast & Philippu, 2000).

It seems that many of the effects of NO are mediated by the activation of cGC, which leads to an increase in the formation of cGMP. Wood and co-workers reported that treatment of mice with NMDA or non-NMDA receptor agonists enhances cGMP levels in the brain, and that this process is inhibited by NOS inhibitors. In several brain areas, the outflow of cGMP in the extracellular space is sensitive to NOS inhibition. The outflow of cGMP is greatly increased by the activation of kainate/AMPA and NMDA receptors, as well as by electrical stimulation of pathways consisting of excitatory amino acid utilising neurons, while the enhanced cGMP outflow is inhibited by NOS inhibitors and inhibitors of sGC (Luo *et al.*, 1994; Fedele & Raiteri, 1996; Fedele *et al.*, 1996; Consolo *et al.*, 1999).

2.2.2.2 The effects of NO on the release of neurotransmitters

2.2.2.2.1 Acetylcholine

By using the push-pull technique or microdialysis it has been shown, under *in vivo* conditions, that inhibitors of NOS, decreasing NO synthesis, decrease acetylcholine release on basal forebrain (Prast & Philippu, 1992) and in the nucleus accumbens (Prast *et al.*, 1995; Prast, 1997; Prast *et al.*, 1998). These findings suggest that cholinergic transmission in basal forebrain and ventral striatum is tonically modulated by endogenous NO. As it may be

expected, NO donors such as diethylamine/nitric oxide (DEA/NO), S-nitroso-N-acetylpenicillamine (SNAP) and linsidomine enhance acetylcholine release in basal forebrain (Prast and Philippu 1992), the nucleus accumbens (Prast *et al.*, 1995, 1998) and in the dorsal striatum.

This facilitatory effect of NO on acetylcholine release was also found in the medial pontine reticular formation. In this brain region, the NOS inhibitor NLA decreases acetylcholine release and blocks effects of neostigmine microinjection on non-rapid eye movement, sleep and breathing frequency (Leonard & Lydic, 1995, 1997).

Similar to the *in vivo* approaches, the NO donor hydroxylamine increases the release of ³[H]acetylcholine from hippocampal slices in a concentration-dependent way after loading them with the radiolabelled neurotransmitter (Lonart *et al.*, 1992). NO donors also enhance the release of acetylcholine from primary cultured cerebral cortical neurons and the potassium-induced transmitter of electric organ (Ohkuma *et al.*, 1995a, b; Morot Gaudry-Talarmain *et al.*, 1997). In all these *in vivo* and *in vitro* preparations, a release-enhancing effect of NO was consistently found.

Prast and co-workers (Prast *et al.*, 1995) investigated the role of cGMP in the process of enhancing the releasing effect of acetylcholine. Superfusion experiments have shown that, in the basal forebrain, the release of acetylcholine by NO donors DEA/NO and linsidomine was abolished by presuperfusion with LY-83,583 (Prast *et al.*, 1995), an inhibitor of guanylyl cyclase (Schmidt *et al.*, 1985; Malta *et al.*, 1988). Since the inhibition of GC may decrease cGMP concentration, these data suggest that cGMP may be involved in the NO-mediated release of acetylcholine. In the nucleus accumbens, the release of acetylcholine elicited by DEA/NO and linsidomine is also abolished by the guanylyl cyclase inhibitors 1H-(1,2,4)oxadiazolo(4,3- α)guinoxalin-1-one (ODQ) and LY-83,583 (Prast *et al.*, 1998). Microdialysis experiments (Guevara-Guzman *et al.*, 1994) have shown that, in the striatum, the release of acetylcholine is stimulated in a concentration-dependent way by the cGMP analogues 8-bromo-cyclic GMP and N², 2-O-dibutylguanosine 3,5-cyclic monophosphate. Thus, there is little doubt that the NO-induced modulation of cholinergic neurons is mediated by cGMP (Prast & Philippu, 2000).

Previous studies in our laboratory (Eager, 2004) showed that sildenafil possesses the ability to potentiate cholinergic muscarinic receptor (mAChR) function during *in vitro* experiments. The results that will follow were obtained from the above-mentioned study.

Figure 2-4A illustrates the modulating effects of sildenafil on mAChR function in human neuroblastoma (SH-SY5Y) cells. This neuronal cell line expresses muscarinic acetylcholine receptors endogenously (Słowiejko *et al.*, 1996; Kukkonen *et al.*, 1992). Dose response curves

were constructed after 24 hour pre-treatment with and without 450 nM sildenafil. Figure 2-4B illustrates the modulating effect of sildenafil (100 nM and 450 nM) on mAChR function after a 24-hour pre-treatment period.

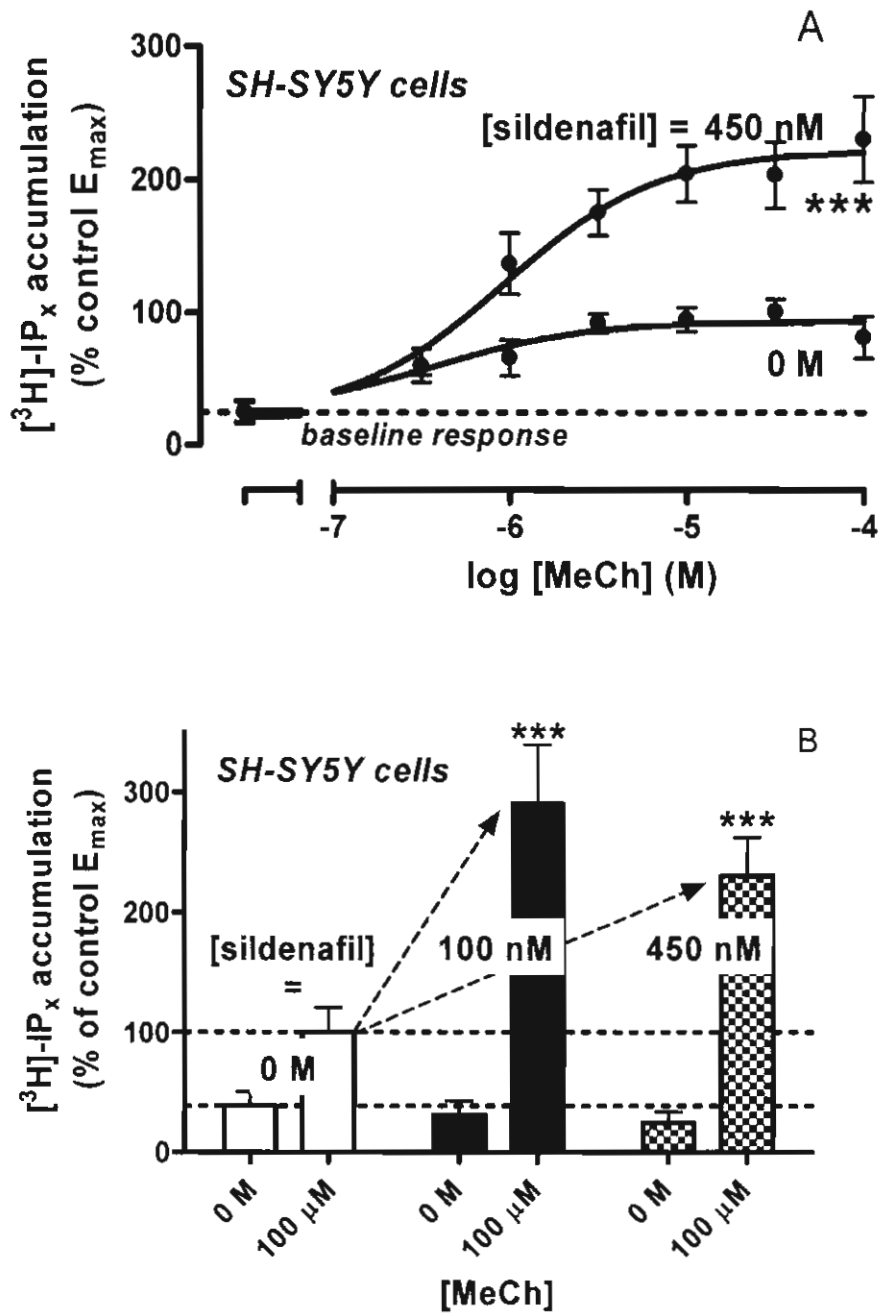


Figure 2-4 (A) Dose-response curves of MeCh after 24-hour pre-treatment of SH-SY5Y cells with 0 M or 450 nM sildenafil in serum medium. (B) The effect of treatment of SH-SY5Y cells (neuronal cells) with 100 nM and 450 nM sildenafil (Eagar, 2004).

In Figure 2-4 A it can be seen that 24 hours pre-treatment of human neuroblastoma cells with 450 nM (peak plasma levels of sildenafil in humans are 212 ng/ml, which is equal to 446 nM \approx 450 nM) (Pfizer Labs, 2006a) sildenafil greatly increased the E_{max} of the MeCh dose response curve. It is important to note that the baseline production of IP_x was not affected by the pre-treatment, suggesting that the increase in E_{max} is an mAChR signal transduction system-dependent effect (Eager, 2004). Eager (2004) reported that sildenafil possesses the ability to potentiate the mAChR function without affecting the baseline IP_x production or the EC_{50} value of the agonist metacholine. Note that whole-cell uptake of the radiolabel, [3H]-*myo*-inositol, was also not affected, validating the above-mentioned observations on the mAChR function. The result therefore suggests that sildenafil enhances muscarinic-receptor signalling capacity, since the maximal effect of muscarinic receptors is three times larger after sildenafil pre-treatment. In Figure 2-4B it can be seen that the maximal concentrations of IP_x accumulation have been increased dramatically after 100 nM and 450 nM pre-treatment with sildenafil. The data therefore suggest that a much lower concentration than the peak concentration also stimulates mAChR-signalling capacity. The mAChR modulating effect of sildenafil is, therefore observed at pharmacologically relevant concentrations (Eager, 2004).

In combination, these data suggest that sildenafil potentiates the signalling mechanism of mAChRs and/or mAChR concentration. In the same model, Brink *et al.* (2004) reported that pre-treatments with the antidepressants fluoxetine and imipramine inhibited mAChR function. This may explain the antidepressant actions of the drugs caused by a decrease in Ach function (Eager, 2004). Central cholinomimetics display antimanic properties as opposed to antidepressant effects of anticholinergics (Fritze & Beckmann, 1998). It has also been shown to exacerbate behavioural depression in an animal model of depression, while antimuscarinic drugs and several antidepressants inhibited this response (Chau *et al.*, 2001). The cholinergic system of depressed patients is supersensitive when compared to subjects without depression (Daws & Overstreet, 1999). The above-mentioned data illustrate the importance of the cholinergic system and the increase of mAChR function in depression. Thus, the potentiation of the cholinergic system by sildenafil may exacerbate depression.

Anxiety alone 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 activation of the hypothalamic-pituitary-adrenal (HPA) axis (Mizuno *et al.*, 1991), which is also implicated in the aetiology of depression. 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).

2.2.2.2.2 *l*-Norepinephrine

NO donors increase the release of noradrenalin in the hippocampus under *in vitro* and *in vivo* conditions (Lonart *et al.*, 1992; Satoh *et al.*, 1996, 1997). Additionally, the *in vitro* release of *l*-norepinephrine, stimulated by NMDA or 3, 4-diaminopyridine is enhanced by NO donors or *l*-arginine (Lauth *et al.*, 1993; Stout & Woodward, 1994; Jones *et al.*, 1995), while NOS inhibitors exert the opposite effect (Jones *et al.*, 1995). The results support the idea that NO modulates *l*-norepinephrine release from the brain.

2.2.2.2.3 Serotonin

In the medial preoptic area (Lorrain & Hull, 1993) and the striatum (Guevara-Guzman *et al.*, 1994), serotonin release is enhanced by *l*-arginine and NO donors, respectively. It has been reported that NO donors influence the release of serotonin in a biphasic way. Hypothalamic superfusion with low concentrations of linsidomine, DEA/NO, SNAP, SNOG or SNP decreases serotonin outflow, whereas higher concentrations of these NO donors enhance the release of monoamine. Moreover, both the inhibitory and the enhancing effects elicited by low and high linsidomine concentration respectively are abolished when cGMP synthesis is inhibited by ODQ, a sGC inhibitor. Thus, in the hypothalamus, NO-induced modulation of serotonin release is mediated by cGMP (Kaehler *et al.*, 1999), as was found for the release of acetylcholine (see above § 2.2.2.2.1). In the locus coeruleus, both NO donors and the NO precursor *L*-arginine elevate the release of serotonin, but superfusion with *N*^ε-nitro-*L*-arginine methyl ester (*L*-NAME), an NOS inhibitor, is ineffective. It seems that, in this brain structure, endogenous NO facilitates the outflow of serotonin, while NO release under resting conditions does not modulate the activity of serotonergic neurons (Sinner *et al.*, 2000).

Since *in vivo* (Singewald *et al.*, 1998) and *in vitro* (Kendrick *et al.*, 1996) the release of serotonin is under modulatory control of NMDA and AMPA/kainite receptors (Kendrick *et al.*, 1996), it seems likely that NO-induced release of glutamate significantly contributes to the modulation of serotonin by NO.

The findings are of interest in the context that NO donors in the hypothalamus and locus coeruleus enhance the release of serotonin (Kaehler *et al.*, 1999).

2.2.3 Soluble guanylate cyclase

Soluble guanylate cyclase (sGC) is the only conclusively proven receptor for nitric oxide (NO), a signalling agent produced by the enzyme nitric oxide synthase (NOS) from the amino acid *L*-arginine. sGC catalyses the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3',5'-monophosphate (cGMP).

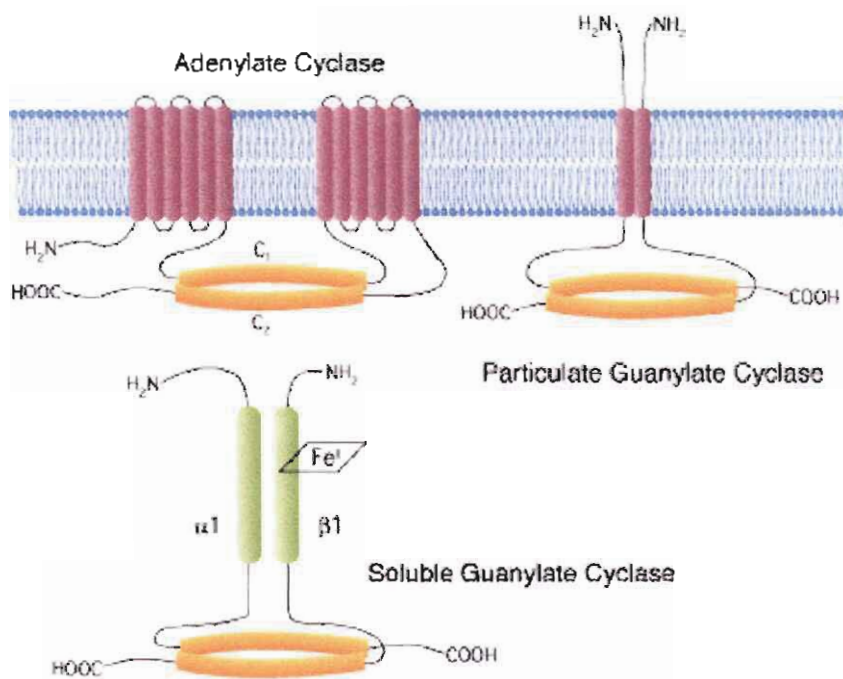


Figure 2-5 Schematic representation of sGC, pGC and AC. Transmembrane helices are shown in purple, the conserved sGC, pGC and AC catalytic regions are shown in orange and the conserved NH_2 -terminal domains of the sGC $\alpha 1$ and $\beta 1$ subunits are shown in green. The sGC $\beta 1$ subunit also binds prosthetic heme, which is ligated by histidine 105 (Denninger & Marletta, 1998).

sGC is one of two enzymes, the other is the membrane-bound peptide-receptor guanylate cyclase (particulate guanylate cyclase) that produces cGMP, and it is the only definitive receptor for NO. sGC is intimately involved in many signal transduction pathways, most notably in the cardiovascular system (e.g. in the regulation of vascular tone and platelet function) and in the nervous system (e.g. in neurotransmission and, possibly long-term potentiation and depression). Soluble guanylate cyclase is a heterodimeric protein composed of α and β subunits; in addition, it is a hemoprotein. NO binds to the sGC heme; this binding event activates the enzyme. The activation of sGC and the subsequent rise in cGMP concentration are what allow sGC to transmit an NO signal to the downstream elements of the signalling cascade-cGMP-dependent protein kinase, cGMP gated cation channels and cGMP-regulated phosphodiesterase (Denninger & Marletta, 1998).

Just as sGC is a component of the larger NO/cGMP signalling pathway, so is it a member of a larger group of related enzymes: the nucleotide cyclases. This family of enzymes converts nucleotide triphosphates (GTP and ATP) to cyclic nucleotide monophosphates (cGMP and cAMP) and includes adenylate cyclase (AC), particulate guanylate cyclase (pGC) and soluble

guanylate cyclase. As a family, these enzymes are involved in a broad array of signal transduction pathways mediated by the cyclic nucleotides cAMP and cGMP, involved in vision, blood pressure regulation, response to hormones and many others. An evolutionarily conserved catalytic domain defines these three groups of enzymes as a single family; in other words, the genes that code for the catalytic portions of these enzymes are derived from a common ancestor. This is unsurprising, given that they catalyse virtually identical reactions. Moreover, because of their relatedness and similar function, insights into the structure, catalytic mechanism and possibly even regulation of one member of this family may be generalisable to other members (Denninger & Marletta, 1998).

AC, for example, has been studied for nearly four decades. As a result, there is a rich literature of findings with AC that may, because of similarity of AC to sGC, prove useful in understanding sGC. Unlike GC, which contains both receptor and catalytic functions within the same protein, ACs is stimulated by hormone receptors acting through heterotrimeric G-proteins (Wedel & Garbers, 1997). AC is an integral membrane protein and is assumed to be monomeric. All the known mammalian isoforms are activated by $G_s\alpha$, while other activators are isoform specific (Hanoune *et al.*, 1997).

Particulate guanylate cyclase has also provided insight into the structure and function of sGC. After the purification of cGMP and guanylate cyclase, it was observed that guanylate cyclase activity could be separated into a membrane-bound (particulate) and cytosolic (soluble) fractions. As the name implies, particulate guanylate cyclase is the membrane-bound guanylate cyclase. In a sense, pGC is a prototypic cell-surface receptor enzyme: it contains an extracellular peptide receptor domain and an intracellular catalytic domain separated by a single transmembrane domain (Denninger & Marletta, 1998).

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 & Papapetropulo, 2000).

2.2.4 Cyclic guanosine monophosphate (cGMP)

The GC/cGMP cell signalling system was identified soon after its related nucleotide system AC/cAMP (Waldman & Murad, 1987; Hobbs, 1997; Wedel & Garbers, 1998). However, while advances in our understanding of the cellular functions of cAMP followed on quickly from this discovery, the same was not true for cGMP. The main reasons for this delay were the lower (about 10 times) concentrations of cGMP in cells compared with cAMP, the diverse forms

(particulate and soluble) of the cGMP synthesising enzyme GC, and the problem of identifying the endogenous substances utilising the GC/cGMP pathway as a cellular signal. During the 1980s however, it became clear that the multiple particulate isoforms of GC were receptors for a variety of endogenous peptides, while soluble GC was the target for nitric oxide released from either vascular endothelium or from certain types of non-adrenergic, non-cholinergic nerves (Gibson, 2001).

cGMP acts directly with downstream effectors such as the family of cGMP-dependent protein kinases (PKGs), cyclic nucleotide-gated channels (CNGs), and cGMP-regulated phosphodiesterases (PDEs) (Krumenacker & Murad, 2005).

cGMP acts as a second messenger in neuronal cell-cell signalling from post- to presynaptic elements and *vice versa*, as well as between presynaptic fibres or between postsynaptic structures (Garthwaite *et al.*, 1993; Hartell *et al.*, 2001; Morris *et al.*, 1992; Milman & Arnold, 2002).

2.2.5 The phosphodiesterase family

The PDEs have been classified into 11 different families *i.e.* PDE1 to PDE11, based on their relative selectivity in hydrolysing 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 (Conti & Jin, 1999; Wallis *et al.*, 1999; Soderling & Beavo, 2000).

Within the PDE family multiple splice variants exist, which makes the number of PDE isozymes that are known more than 30 (Beavo, 1995; Houslay, 1996). A number of these enzymes are localised regionally (Billingsley *et al.*, 1990; Iwahashi *et al.*, 1996) and may be expressed to different degrees, even within one cell type (Beavo, 1995; Juilfis *et al.*, 1997). The cAMP-specific enzymes include phosphodiesterase 4, -7 and -8. The cGMP specific PDEs are PDE5, -6 and -9, whereas PDE1, -2, -3, -10 and -11 deactivate both nucleotides (Rotella, 2002).

Table 2-2 depicts the various PDE isozymes as well as the relevant substrates, effect of inhibition of the specific PDE, the tissues where the specific PDEs are localised and the assumed role that the specific PDE plays throughout the human body. The IC_{50} indicates the affinity of sildenafil to the various PDE isozymes.

Table 2-2 The Phosphodiesterase family (adapted from Rosen & Kostis, 2003; Wallis *et al.*, 1999)

Gene products	Substrate	Effect of inhibition	Tissue	IC ₅₀ (nM)	Assumed role
1 A-C	cGMP, cAMP	Hypotension	Brain, heart, peripheral nerves, smooth muscle, skeletal muscle, liver, kidney	280	CNS modulation, vasodilation
2 A	cAMP > cGMP	Unknown	Brain, heart, corpus cavernosum, skeletal muscle, smooth muscle, adrenal cortex	68,000	Uncertain
3 A-B	cAMP > cGMP	Inotrope, arrhythmia	Corpus cavernosum, smooth muscle, platelets. Liver, kidney, heart	16,200	Positive inotropism, vascular and airway dilatation, platelet inhibition
4 A-D	cAMP	Emesis, anti-inflammatory	Kidney, smooth muscle, heart, lung lymphocytes, skeletal muscle	7200	Airway dilatation, inhibition, CNS modulation, sperm and egg maturation
5 A	cGMP	Vascular relaxation	Corpus cavernosum, vascular smooth muscle, platelet	3.5	Penile detumescence, vasodilation, platelet inhibition
6 A-C	cGMP	Visual disturbances, retinitis pigmentosa	Retina (cone) Retina (rod)	34 39	Phototransduction
7 A	cAMP	Inhibition of T-cell activation	Skeletal muscle		
8 A-B	cAMP	Unknown			
9 A	cGMP	Unknown	Kidney, spleen, lung, brain		
10 A	cAMP > cGMP	Unknown			
11 A	cGMP, cAMP	Unknown			

PDE5 was purified by Francis and co-workers in 1980 from rat lung, and was first cloned by McAllister-Lucas and co-workers in 1993. The enzyme is active as a homodimer, which has a molecular mass of approximately 200 kDa. Each monomer contains a carboxy-terminal catalytic domain, a highly conserved zinc-binding motif, two allosteric binding pockets for cGMP and (in the amino-terminal region) a phosphorylation site at serine 92 (Ser⁹²). Either protein kinase A (PKA) or PKG can phosphorylate PDE5, resulting in a 50-70% increase in enzyme activity (Corbin *et al.*, 2000).

Each of the sub-units also contains two allosteric (non-catalytic) binding sites for cGMP and occupation of these sites is necessary for phosphorylation of Ser⁹²; further, binding of cGMP to the allosteric sites is enhanced following occupation of the catalytic sites (Corbin & Francis, 1999) (also see § 2.1.4).

The catalytic domain is located at the carboxy terminus. The sequence of enzyme activation and regulation begins with elevated levels of cGMP in the cytoplasm, resulting in enhanced binding to the catalytic site; this, in turn, promotes binding of cGMP to the allosteric sites, allowing phosphorylation of Ser⁹² and increased enzyme activity (Corbin & Francis, 1999). This provides a highly regulated mechanism by which the cell can control cytoplasmic concentrations of cGMP. The protein is widely distributed throughout the smooth muscle of the human body and is also found in platelets (Kukreja *et al.*, 2005)

PDE5 is one of the members of this family that specifically binds to cGMP. It is composed of 875 amino acids and was first identified in lung vascular tracheal smooth muscle, and platelets. PDE5 is selectively inhibited by the drugs sildenafil, vardenafil and tadalafil, and less selectively by zaprinast and dipyridamole. The tissue distribution of the PDE5 family is relatively restricted compared to other PDEs. Still, recent immunohistochemical and reverse transcript polymerase chain reaction analyses have demonstrated the presence of PDE5 in rat cerebellum, kidney, pancreas, aortic smooth muscle cells, heart, placenta, skeletal muscle, and, to a much lesser extent, in other regions of the brain, liver and lungs (Kulkarni & Patil, 2004).

As can be seen in Table 2-2 from the geometric mean IC₅₀, sildenafil has a substantially lower affinity for other PDE isozymes, manifested by the much higher concentrations needed to inhibit 50% of their activities (Wallis *et al.*, 1999).

2.3 Depression

The American Psychiatric Association (2000) defines depression as a heterogeneous disorder, often manifested with symptoms at the physiological, behavioural and physiological level. Depressive states have been, by tradition, classified on the basis of the acute clinical picture

such as “retarded,” “agitated,” “anxious,” “atypical,” “hostile,” etc. (Akiskal, 1983). These constructs, in turn, were often dichotomised into overlapping, though not necessarily synonymous, contrasting terminology such as melancholic versus neurasthenic, psychotic versus neurotic, endogenous versus reactive, and primary versus secondary (Akiskal, 1983). Implicit in these dichotomies is biologic vs. psychological causation, the former due to genetic factors impinging on a more or less normal or “obssoid” personality, the latter arising from environmental adversity in the setting of an abnormal personality with “neurotic,” “immature” or “psychopathic” traits, except for, the stability of the psychotic subtypes across episodes (Coryell *et al.*, 1994). Prospective observations have generally failed to support the longitudinal validity of these dualistic nosologic schemas.

The most damaging evidence against the foregoing schemas derives from prospective observations on the transformation of “neurotic depressions” into its antitheses (Akiskal *et al.*, 1978; Bronisch *et al.*, 1985). Despite these inconsistencies, elements of these dualistic concepts have survived within the general framework of the bipolar-unipolar dichotomy favoured by two official current classification systems of mental disorders—the DSM-IV and ICD-10. In both manuals, the larger and heterogeneous universe of unipolar major depression receives qualification, e.g., “major depressive” vs. “dysthymic” or “melancholic” vs. “atypical.” (Hagop, Akiskal, 1995)

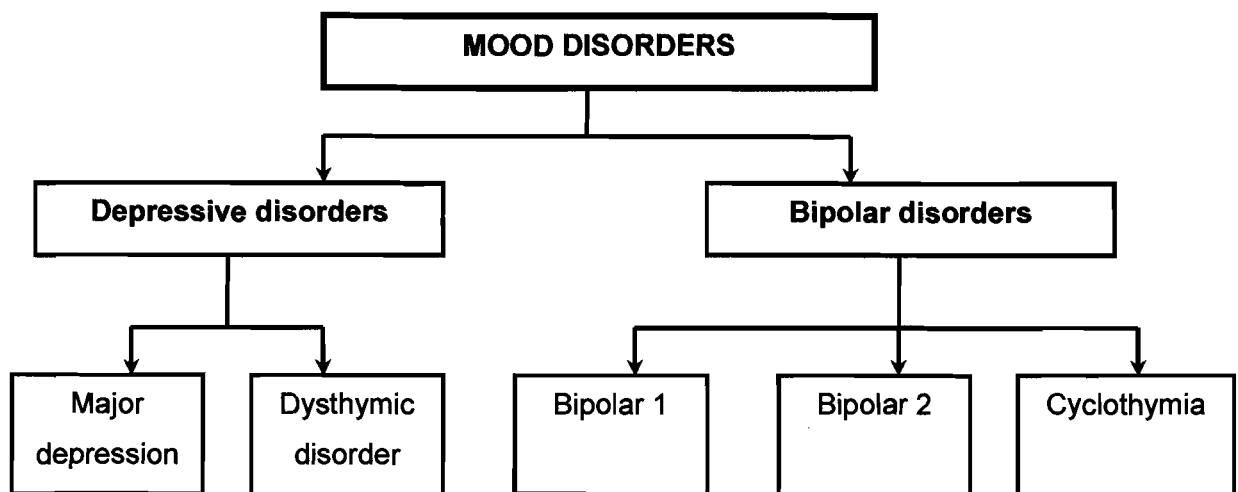


Figure 2-6 The mood disorders (adapted from Barlow & Durand, 1995).

Major depression

Major depression is a serious illness that can have devastating effects on a person's everyday life, including social relationships and the ability to work. It is characterised by sad mood, change in appetite or weight, difficulty in sleeping, physical slowing or agitation, fatigue, feeling of worthlessness, and recurrent thoughts of death or suicide. However, in contrast to the normal experiences of sadness, clinical depression is a chronic disease and can interfere significantly with an individual's ability to function (Páez-Pereda, 2005).

Dysthymic disorder

Dysthymic disorder often begins in childhood, adolescence or early adulthood and is a more chronic but milder form of depression (National Institute of Mental Health, 2002). According to Bech (1999) dysthymic disorder is a mild, yet long-term depressed mood present more often than not for at least two years. This type of disorder often occurs in tandem with other psychiatric and physical conditions. Up to 70% of patients suffering from dysthymia have both major depressive disorder and dysthymic disorder, commonly referred to as double depression. Dysthymia is prevalent in patients with certain medical conditions including post-cardiac transplantation and diabetes (Rowland, 2001). Symptoms of dysthymia include:

- ❖ Poor appetite or overeating.
- ❖ Hypersomnia.
- ❖ Low self-esteem.
- ❖ Difficulty making decisions.
- ❖ Hopelessness.

Manic depressive illness

Manic depressive illness is known as bipolar illness and is the most distinct and dramatic of the depressive disorders (Ewart-Smith, 1994). The average age of onset is the mid-twenties and it is known to be inheritable (National Institute of Mental Health, 2000). Bipolar disorders are characterised by the presence of mania or hypomania in addition to depression. Mania is characterised by experiencing extreme feelings in every activity, whilst hypomania is below the level of a manic episode (Roy & Kroenke, 1999; Barlow & Durand, 1995). According to Roy and Kroenke (1999), a manic episode lasts up to a week, whilst hypomanic episodes may last as little as four days (Barlow & Durand, 1995).

2.3.1 Treatment

Current antidepressant treatments are efficacious. However, they work in only 70-80% of the patients and it often takes more than 5-8 weeks until the patients respond to the treatment (Páez-Pereda, 2005). In addition, several forms of psychotherapy (in particular, cognitive and behavioural therapies) can be effective for patients with mild to moderate cases, and the combination of medication and psychotherapy can exert a synergistic effect (Nestler *et al.*, 2002). Moreover, current treatment modalities are hindered by adverse effects and often produce only partial remission, which proved to be a risk factor for early relapse. The response to treatment varies considerably between individuals. These different responses are, to a certain extent, due to different genetic factors (Páez-Pereda, 2005).

The treatment of depression was revolutionised about 50 years ago, when two classes of agents were discovered to be effective antidepressants, the tricyclic antidepressants and the monoamine oxidase inhibitors. The original tricyclic agents (e.g., imipramine) arose from antihistamine research, whereas the early monoamine oxidase inhibitors (e.g., iproniazid) were derived from work on antitubercular drugs. The discovery that depression can be treated with these medications provided one of the first clues into the types of chemical changes in the brain that regulate depressive symptoms. Indeed, much depression research over the last century was based on the notion that understanding how these treatments work would reveal new insight into the causes of depression (Nestler *et al.*, 2002).

The acute mechanisms of action of antidepressant medications were identified:

- inhibition of serotonin or *l*-norepinephrine reuptake transporters by tricyclic antidepressants (Frazer, 1997), and
- inhibition of monoamine oxidase (a major catabolic enzyme for monoamine neurotransmitters) by monoamine oxidase inhibitors (Frazer, 1997).

These discoveries led to the development of numerous second generation medications (e.g., serotonin selective reuptake inhibitors [SSRIs] and *l*-norepinephrine-selective reuptake inhibitors) which are widely used today. The clinically active antidepressants also made it possible to develop and validate a wide range of behavioural tests with which to study depression-like phenotypes in animal models. Moreover, these medications and behavioural tests represent important tools with which to study brain function under normal conditions and to identify a range of proteins in the brain that might serve as targets for novel antidepressant treatments (Nestler *et al.*, 2002).

Inhibition of serotonin or *l*-norepinephrine reuptake or catabolism would be expected to result in enhanced actions of these transmitters. However, all available antidepressants exert their mood-elevating effects only after prolonged administration (several weeks to months), which means that enhanced serotonergic or noradrenergic neurotransmission, per se, is not responsible for the clinical actions of these drugs. Rather, some gradually developing adaptations to this enhanced neurotransmission would appear to mediate drug action (Nestler *et al.*, 2002).

Important progress has been made in the search for such drug-induced neuroplasticity. Moreover, several generations of research have failed to provide convincing evidence that depression is caused by abnormalities in the brain's serotonin or *l*-norepinephrine systems. This is consistent with the ability of "antidepressant" medications to treat a wide range of syndromes, far beyond depression, including anxiety disorders, post traumatic stress disorder (PTSD), obsessive-compulsive disorder, eating disorders, and chronic pain syndrome. It also is consistent with the fact that many medications used in general medicine work or act distantly from the molecular and cellular lesion underlying the disease (Nestler *et al.*, 2002).

2.3.2 Neural Circuitry of Depression

While many brain regions have been implicated in regulating emotions, we still have a rudimentary understanding of the neural circuitry underlying normal mood and the abnormalities in mood that are the hallmark of depression. This lack of knowledge is underscored by the fact that, even if it were possible to obtain brain biopsies from patients with depression, there is no consensus as to the central site(s) of the pathology, and hence the best brain region for the biopsy. This is in striking contrast to other neuropsychiatric disorders (e.g., Parkinson's disease, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis) where pathologic lesions have been identified in specific regions of the central nervous system (Nestler *et al.*, 2002).

It is likely that many brain regions mediate the diverse symptoms of depression. This is supported by human brain imaging studies (still in relatively early stages) which have demonstrated changes in blood flow or related measures in several brain areas, including regions of the prefrontal and cingulate cortex, hippocampus, striatum, amygdala, thalamus, to name a few (Drevets, 2001; Lotti & Mayberg, 2001). Similarly, anatomic studies of brains of depressed patients obtained at autopsy have reported abnormalities in many of the same brain regions (Zhu *et al.*, 1999; Manji *et al.*, 2001; Drevets, 2001; Rajkowska, 2000). Much work remains to be done, however, since some of the imaging and autopsy studies yielded contradictory findings (Nestler *et al.*, 2002).

Knowledge of the function of these brain regions under normal conditions suggests the aspects of depression to which they may contribute. Neocortex and hippocampus may mediate cognitive aspects of depression, such as memory impairments and feelings of worthlessness, hopelessness, guilt, doom, and suicidality. The striatum, particularly the ventral striatum or nucleus accumbens (NAc), and amygdala, and related brain areas, are important in emotional memory, and could as a result mediate the anhedonia (decreased drive and reward of pleasurable activities), anxiety, and reduced motivation that predominate in many patients. Given the prominence of so-called neurovegetative symptoms of depression, including too much or too little sleep, appetite, and energy, as well as a loss of interest in sex and other pleasurable activities, a role for the hypothalamus has also been speculated. These various brain regions operate in a series of highly interacting parallel circuits, which may define a neural circuitry involved in depression (Nestler *et al.*, 2002).

2.3.3 Theories and aetiology of depression

No single hypothesis appears to be adequate to explain the mechanisms of antidepressant action, given the complexity of the neural circuitry and all the systems involved in depression (Diagnostic and Statistical Manual of Mental Disorders, 2000). According to the Diagnostic and Statistical Manual of Mental Disorders (2000), it is naïve to assume that depression is caused by a synaptic deficiency in a single group of indolamine and/or catecholamine neurotransmitters. This assumption fails to explain why cerebral fluid, urinary and serum transmitter metabolites do not reveal any consistent pattern of abnormality in patients with depressive symptoms (Diagnostic and Statistical Manual of Mental Disorders, 2000).

The various hypotheses of depression:

- Monoamine hypothesis
- Muscarinic supersensitivity hypothesis
- GABA hypothesis
- Glutamate hypothesis
- Neuroplasticity hypothesis
- Neurodegenerative hypothesis

The following hypotheses will be discussed in more detail: The monoamine hypothesis, the muscarinic supersensitivity hypothesis and the glutamate hypothesis, because of their relevance to this study and the study objectives set out to be achieved.

2.3.3.1 The monoamine hypothesis

Initially, the idea that biogenic monoamines are involved in the aetiology of depression came from three main lines of evidence (Blier, 2003). Firstly, it was found that reserpine (an antihypertensive drug) was able to induce symptoms of depression in certain patients by depleting the brain monoamine stores of *l*-norepinephrine, dopamine and serotonin (Goodwin & Bunney, 1971). Secondly, depressed patients' cerebrospinal fluid could possibly have reduced levels of monoaminergic metabolites (Blier, 2003). Finally, the first tricyclic antidepressants and monoamine oxidase inhibitors empirically showed improvement of symptoms in depressive patients. The discovery of the pharmacological mechanism of action of these compounds led later to the monoamine hypothesis of depression and to the development of new antidepressants. Both types of antidepressants increase levels of monoamines either by inhibiting their uptake by monoamine transporters (tricyclic antidepressant) or by inhibiting their inactivation (monoamine oxidase inhibitors). More recently, the development of selective serotonin or *l*-norepinephrine reuptake inhibitors produced improved antidepressants, at least with regard to their side effect profiles. All these compounds produce an increase in the extracellular levels of serotonin, *l*-norepinephrine or dopamine. Most of these compounds work on both *l*-norepinephrine and serotonin (Páez-Pereda, 2005).

Nevertheless, this theory suffered several drawbacks and failed to explain certain facts, *inter alia* the following (Baldessarini, 1989):

- There are drugs that can increase brain monoaminergic activity such as cocaine and amphetamine that are not effective in the treatment of depression.
- Some novel atypical antidepressants, such as tianeptine, have been shown to enhance serotonin reuptake, thereby decreasing monoamine levels.
- Not all depressed patients respond equally to the same antidepressants.
- These changes in the monoamine levels at the synapse take place within hours after administration; however, the therapeutic response requires the continuous administration of these drugs for a number of weeks.

The modified amine theory suggests that the acute increase in the levels of the monoamines at the synapse could be an early step in a potentially complex cascade of events that ultimately result in antidepressant activity (Piñeyro & Blier, 1999). This acute increase in the amount of monoamine neurotransmitter at the synapse, has been found to induce desensitisation of the inhibitory auto- and heteroreceptors that are located in certain brain regions. The desensitisation of the aforementioned receptors would then result in higher central

monoaminergic activity that coincides with the appearance of the therapeutic response. These adaptive changes depend on the availability of the specific monoamine at the synapse, as depletion of monoamine will either reverse the antidepressant effect or cause a relapse in the state of a drug-free depressed patient previously treated with antidepressants. The blocking of the somatodendritic and nerve terminal autoreceptors increases the response rate in the treatment of major as well as treatment resistant depression, providing further support to the assumption that antidepressant effect results from the long-term adaptive changes in the monoamine auto- and heteroregulatory receptors (Elhwuegi, 2004).

Up-regulation of β -adrenergic receptors is a continuous finding in patients with depression, whilst down-regulation is regarded as a marker for antidepressant activity (Leonard, 1997). Numerous lines of experimental data support this hypothesis of antidepressant-induced down-regulation of postsynaptic receptors. This was illustrated in particular for β -adrenoceptors and serotonin receptors in animal experiments. Furthermore, this generally occurs along a similar time-course to the development of antidepressant response in patients. The down-regulation of post-synaptic β -adrenoceptors is indeed a common finding among classical antidepressants and is still often regarded as indicative of antidepressant potential for new drugs (Blier, 2003).

Monoamine receptors modulate the cAMP pathway. This pathway is mainly activated in the hippocampus and the forebrain by antidepressants. The increase in cAMP levels leads to activation of protein kinase type A (PKA) and the activation of transcription factor cyclic AMP response element-binding protein (CREB) (Nibuya *et al.*, 1996). The association of this signal transduction pathway in chronic antidepressant action, and its relevance for drug discovery, is further emphasised by the fact that phosphodiesterase (PDE) inhibitors that increase cyclic AMP levels (such as rolipram) may act as antidepressants (Zeller *et al.*, 1984; Nestler *et al.*, 2002a & b; Páez-Pereda, 2005).

2.3.3.2 Muscarinic supersensitivity hypothesis

Janowsky and colleagues (1972) postulated that an increased ratio of cholinergic to adrenergic activity might underlie depression, whereas the reverse occurs in mania. Although the adrenergic-cholinergic balance hypothesis appears incomplete in the face of increasing data on the pre-eminent role of alterations in serotonergic neurotransmission in major depression, the importance of cholinergic mechanisms in some forms of depression is supported by both preclinical and clinical observations (Janowsky *et al.*, 1972).

Animal models of depression (e.g., the forced swim test) are exacerbated by the administration of cholinesterase inhibitors such as physostigmine, whereas muscarinic receptor antagonists reverse the effects of such "procholinergic" agents (McKinney, 1984). More convincing

evidence of the importance of acetylcholine in the maintenance of mood, is the induction of depressed mood following the administration of physostigmine or cholinergic agonists (e.g., arecoline) in euthymic control subjects (El-Yousef *et al.*, 1973), unipolar depressed patients (Janowsky & Risch, 1984), and bipolar manic patients (Janowsky & Risch, 1984). Indeed, depressive symptoms, including psychomotor retardation and depressed mood, are often unwelcome sequelae of acetylcholinesterase inhibitor treatment of Alzheimer's disease. Such sensitivity to mood-lowering effects of cholinergic drugs appears dependent on the presence of underlying psychiatric disorder. This drug-induced syndrome fits the profile of major depressive disorder, which gave rise to the cholinergic or muscarinic hypothesis of depression (Dilsaver, 1986).

If cholinergic mechanisms contribute in some manner to the pathophysiology of major depression, then anticholinergic drugs should be effective antidepressants. Although physostigmine-induced dysphoria may be reversed with atropine (El-Yousef *et al.*, 1973), little evidence indicates that anticholinergic medications have significant antidepressant properties (Janowsky & Risch, 1984). Moreover, the more recent antidepressants, including the SSRIs, have little affinity for muscarinic cholinergic receptors yet remain effective antidepressants.

The following provide evidence, in support of the cholinergic involvement in affective disorders:

- Effectiveness of antimuscarinic drugs such as imipramine in the treatment of depression (Goldman & Erickson, 1983).
- The euphorogenic characteristics of antimuscarinic drugs (that are frequently abused) (Smith, 1980).
- Proof of cholinergic-monoamine antagonism in regulation of reward, punishment and hedonic capacity as well as the antimuscarinic agent withdrawal effects such as anxiety, sleep disturbances and rebound exacerbation of motor dysfunction (Dilsaver & Greden, 1984).

Preceding studies have also shown that the cholinergic system of depressed subjects is super-sensitive compared to subjects without depression (Janowsky & Risch, 1984). However, it is still uncertain whether this is a cause or result of depression (Daws & Overstreet, 1999). A normal subject's response to pharmacologically induced central cholinergic overdrive is not necessarily relevant to the question whether central cholinergic overdrive plays a role in the genesis of affective disorders like depression. Subjects that develop a depressed mood in reaction to cholinergic agents may possess pre-existing aberrations at central cholinergic loci, resembling those of patients with affective disorders. However, these aberrations may not be

specific to an affective disorder. Such disturbances may permissibly promote development of affective disorders; on the other hand, they could be a factor, or one of a group of factors, necessary to produce affective illness (Dilsaver, 1986).

Several cholinergic systems distributed in different parts of the brain seem to be involved in certain aspects of depression. The cholinergic system based in the basal forebrain has dense projections to the core limbic structures such as the hippocampus and the amygdala. These systems may be implicated in depression since they are imperative 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 stimulation. Dilsaver (1986) suggested that this system's interaction with the monoaminergic system might contribute to depression and mania.

Sleep abnormalities are also common in subjects that are suffering from depressive disorders. These abnormalities might also be produced by cholinoreceptor blockade (McCarley, 1982). Sitram and Gillin (1980) reported that anticholinesterases produce subjective disturbances in sleep continuity and are able to produce vivid terrifying dreams. This shows that the aforementioned pathway may be involved in depressive disorders.

Cholinergic interneurons are also situated in the nucleus accumbens, important in mediating aspects of motivation and reinforcement. These interneurons are well suited for controlling the transmission of limbic information to the accumbens' target nuclei, they are able to interact with mesencephalic dopamine as well as medial thalamus and limbic cortex glutaminergic inputs (Chau *et al.*, 2001).

Schatzberg and Mooney (1991) have offered a slightly different perspective on acetylcholine/catecholamine interactions in depression. Increased acetylcholine activity may result in increased catecholamine output as well as the commonly co-occurring elevation in cortical activity in a subgroup of depressed patients. The role of acetylcholine in the pathogenesis of depression awaits further clarification (Schatzberg & Mooney, 1991).

2.3.3.3 Glutamate hypothesis

When deregulated generation of NO reaches an excess level, it induces a neurotoxic cascade (Dawson *et al.*, 1992). Overactivation of NMDA receptors by excess glutamate mediates cell death in focal cerebral ischemia (Choi, 1988). Involvement of NO in glutamate neurotoxicity was first demonstrated in *in vitro* experiments using primary cortical cultures (Dawson *et al.*, 1991). NMDA applied only for a short period of time is able to elicit cell death in cortical cultures assessed 24 hours later. This type of cell death has been called delayed neurotoxicity, in which

irreversible processes are set in motion by the application of NMDA (Choi & Rothman, 1990). This type of cell death is exclusively dependant on calcium. Furthermore, calcium influx via NMDA receptors elicits more potent toxicity than other modes of calcium entry (Dawson *et al.*, 1991).

The nNOS null mouse provides further evidence of the role of NO in NMDA neurotoxicity. These mice are relatively resistant to NMDA neurotoxicity as well as combined oxygen and glucose deprivation (Dawson *et al.*, 1996). Neurotrophins, despite their role of attenuating excitotoxic neuronal injury, were recently shown to increase the number of nNOS neurons in cortical culture grown on glial feeder layers, and render neurons more sensitive to NMDA (Samdani *et al.*, 1997)

Glutamate neurotoxicity also contributes to some degree to the pathogenesis of neurodegenerative disease such as Huntington's disease and Alzheimer's disease (Choi, 1994), implicating NO in these disorders.

It has been found that structurally dissimilar antidepressants, including fluoxetine, imipramine as well as electro-convulsive therapy suppress glutamate activity at NMDA receptors by restraining allosteric coupling between glycine and glutamate recognition sites in the NMDA-ion channel (Skolnick, 1999).

Furthermore, Skolnick (1999) found that NMDA receptor antagonists possess antidepressant-like actions in various animal models. Regulation of the NMDA glutaminergic mechanism has been implicated in the behavioural and adaptive neuronal response to antidepressants (refer to § 2.3.4), suggesting that this pathway may play a key role in the neuropathology of affective disorders (Skolnick, 1999).

2.3.4 The NO-cGMP pathway and Depression

2.3.4.1 NMDA antagonists

Recent *in vivo* and *ex vivo* findings indicate that the NMDA receptor complex may be a locus of antidepressant action. Functional antagonists of the NMDA receptor complex, including a competitive NMDA receptor antagonist (2-amino-7-phosphonoheptanoic acid; AP-7) (Watkins & Olverman, 1987), a glycine partial agonist (1-aminocyclopropanecarboxylic acid; ACPC) (Marvizon *et al.*, 1989; Watson & Lanthorn, 1990), and a use dependent channel antagonist (dizocilpine) (Wong *et al.*, 1986) are as efficacious as tricyclic antidepressants in pre-clinical tests predictive of antidepressant activity (Trullas & Skolnick, 1990; Trullas *et al.*, 1991; Maj *et al.*, 1992; Skolnick *et al.*, 1992). Similarly, dizocilpine and NMDA receptor antagonist DL-(E)-2-amino-4-methyl-5-phosphono-3-pentonic acid (CGP-37849) block behavioural effects of two

putative animal models of depression, learned helplessness and chronic mild stress-induced deficits in sucrose consumption (Meloni *et al.*, 1993; Papp & Moryl, 1993a, b; Harkin *et al.*, 1999).

Memantine (1-amino-3,5-dimethyladamantane) is a moderate affinity uncompetitive NMDA receptor antagonist with voltage dependent binding characteristics (Parsons *et al.*, 1999; Sonkusare *et al.*, 2005; Witt *et al.*, 2003). It has been used in the treatment of patients with Parkinson's disease, dementia and neurogenic bladder dysfunction in spasticity (Jarvis & Figgit, 2003). Furthermore, it has been approved by the European Union and the United States of America for the treatment of moderate to severe Alzheimer's disease (Lipton, 2004; Parsons *et al.*, 1999; Sonkusare *et al.*, 2005; Witt *et al.*, 2003) and is widely accepted as very well tolerated (Parsons *et al.*, 1999). Memantine exerts an antidepressant-like effect in the Forced Swim Test after acute administration (at a dose that did not change the locomotor activity). This could be partly due to the inhibition of NO-cyclic GMP synthesis (Almeida *et al.*, 2006)

In addition, a chronic regimen of either 1-aminocyclopropanecarboxylic acid (ACPC) or dizocilpine produces a reduction in the density (downregulation) of cortical β -adrenoreceptors in mice, comparable to that produced by the prototypic tricyclic antidepressant, imipramine (Paul *et al.*, 1992). Similarly, Klimek and Papp (1994) reported that chronic dizocilpine treatment is responsible for the down-regulation of cortical β -adrenoreceptors, as well as 5-HT₂ receptors in rats. Thus, in both behavioural and biochemical screening procedures, antagonists at the NMDA receptor complex behave in a manner comparable to clinically active antidepressants (Harkin *et al.*, 1999).

2.3.4.2 Nitric Oxide Synthase inhibitors

Methylene blue

Methylene blue (MB), a non-toxic dye, has been intermittently used in psychiatry over the past century (Naylor *et al.*, 1986). MB is known to affect iron-containing enzymes (Kelner *et al.*, 1988). Stoichiometrical amounts of iron are present in both sGC and NOS (Gerzer *et al.*, 1981; Mayer *et al.*, 1991). The heme group of sGC has been suggested as a target site of MB, resulting in a reduced responsiveness to activation of NO (Grutter *et al.*, 1979). Accordingly, this compound has been extensively used as a selective inhibitor of sGC to demonstrate cGMP-mediated processes (Ignarro & Kadowytz, 1985). However, recently, Mayer and co-workers (1997) showed that MB acts as a direct inhibitor of NOS, another iron-containing enzyme. Moreover, NOS seems to be more sensitive than sGC to the inhibitor effect of MB, since NOS is more completely inactive at a concentration of MB that exerts no great effect on direct activation of sGC (Mayer *et al.*, 1993). Furthermore, MB can inactivate NO extracellularly probably

through generation of superoxide anions during auto-oxidation of reduced form of MB (Wolin *et al.*, 1990). Therefore, MB does not represent either a selective or a potent inhibitor of sGC as previously assumed (Bauer *et al.*, 1994; Young *et al.*, 1994). Nevertheless, the data quoted above imply that MB has an inhibitory effect on the NOS-NO-cGMP pathway. This prominent effect of MB on the NOS-NO-cGMP pathway appears to be antidepressant with an anxiolytic property at the same time (Eroğlu & Çağlayan, 1997).

1-(2-trifluoromethylphenyl) imidazole

1-(2-trifluoromethylphenyl) imidazole (TRIM) has been shown to be a relatively selective inhibitor of nNOS (Handy, 1996). TRIM decreases immobility of mice in the forced swim test. The magnitude of the antidepressant-like effect of TRIM is comparable with that of standard antidepressant imipramine. Thus, these effects may result from a change of 5-HT levels in the brain (Volke *et al.*, 2003).

Harkin *et al.* (1999) reported that antagonists of NO synthase possess significant antidepressant-like features during the forced swim test that are dose-dependent, stereoselective and reversible by L-arginine.

Karolewicz *et al.* (1999) demonstrated that chronic, but not acute, treatment of mice with NO synthase inhibitor N^G -nitro-L-arginine down-regulates cortical β -adrenoreceptors with a magnitude comparable to that observed following chronic imipramine treatment. These findings are congruent with previous observations, which have demonstrated the antidepressant-like properties of NO synthase inhibitors in the forced swim test. The antagonists of NOS represent a novel class of potential therapeutic agents for the treatment of major depression. The interruption of the production of NO by NOS may be critical to the action of antidepressants (Harkin *et al.*, 1999).

2.3.4.3 NO-cGMP-pathway and neurogenesis

The formation of new neurons or neurogenesis is an important phenomenon implicated in the development of the nervous system. In the last years, it has been demonstrated that neurogenesis also occurs in the adult nervous system (Gage, 2000), and that it can be induced after brain injuries such as seizures or stroke (Kokaia & Lindvall, 2003; Parent, 2003). The discovery of adult neurogenesis has been considered as a possible new facet of recovery that may translate into new treatments for stroke (Nadareishvili & Hallenback, 2003). In addition, its inhibition has recently suggested to be involved in the pathogenesis of depression and Alzheimer disease (Reif *et al.*, 2004; Wen *et al.*, 2004; Cárdenas *et al.*, 2005).

By using pharmacological or genetic approaches, it has been demonstrated that NO synthesised either by iNOS in dentate gyrus after focal cerebral ischemia (Zhu *et al.*, 2003) or by eNOS in subventricular zone also after focal ischemia (Chen *et al.*, 2005) or in dentate gyrus in an adult model of depression (Reif *et al.*, 2004) activates neurogenesis. The neurogenesis effect of NO in adult brain could be mediated through an increase in the tissue levels of cGMP (Zhang *et al.*, 2001; Zhang *et al.*, 2003). Moreover, it must be considered that cGMP produced by NO is involved not only in increasing neurogenesis but also in promoting functional recovery, an effect that could be related to the implication of cGMP in the modulation of axonal guidance and neurite outgrowth. Indeed, it has been demonstrated that NO could stimulate neurite outgrowth from hippocampal neurons and PC12 cells exposed to nerve growth factor through a cGMP-dependent mechanism (Hindley *et al.*, 1997; Cárdenas *et al.*, 2005)

This may explain the effects of some pharmacological approaches to increase neurogenesis, such as administration of sildenafil, which is an inhibitor of PDE 5 and therefore causes intracellular accumulation of cGMP (Zhang *et al.*, 2003). The treatment with sildenafil significantly increased numbers of bromodeoxyuridine-immunoreactive cells in the subventricular zone and the dentate gyrus and numbers of immature neurons, as indicated by betaIII-tubulin (TuJ1) immunoreactivity in the ipsilateral subventricular zone and striatum (Zhang *et al.*, 2002). Atorvastatin, which upregulates the eNOS isoform, increases the cGMP levels (Chen *et al.*, 2003), or NO donors also increase cGMP levels (Zhang *et al.*, 2001; Cárdenas *et al.*, 2005).

Moreover, the effect of cGMP on neurogenesis could be related to the activation of cGMP-dependent protein kinase type I, which has been described to enhance sensory neuron precursor proliferation (Firestein & Bredt, 1998).

2.3.4.4 Sildenafil

In a placebo-controlled study to determine the effect of depression on the treatment of erectile dysfunction with sildenafil and whether the effective treatment of erectile dysfunction affects comorbid depression and related quality-of-life symptoms, led to a surprising result. It was established that patients treated with sildenafil and classified as treatment responders, showed a clinically significant improvement in depressive symptoms and quality-of-life measurements compared with subjects who did not respond to treatment. The magnitude of improvement observed in treatment-responsive subjects in the trial was comparable to that commonly observed in clinical trials of either drug or nondrug interventions for major depression (Seidman *et al.*, 2001). A study conducted by Müller and Benkert (2000), indicated lower self-reported depression in patients with erectile dysfunction treated with sildenafil when compared to patients left untreated. This implied that sildenafil not only had a direct effect on erectile

function in about 50-80% of men with erectile dysfunction (Langtry & Markham, 1999; Padma-Nathan, 1999) but might also have improved anhedonia and depression. The substantial correlation between the International Index of Erectile Function and Epidemiologic Studies-Depression Scale scores supported this assumption (Müller & Benkert, 2000).

Raffaele *et al.*, reported in 2002, an indirect improvement in depressive-like symptoms in patients treated for erectile dysfunction with idiopathic Parkinson's disease.

2.4 The forced swimming test

A major impediment in depression research is the lack of validated animal models. Many of the core symptoms of depression (e.g., depressed mood, feelings of worthlessness, suicidality) cannot be easily measured in laboratory animals. In addition, the lack of known depression vulnerable genes means that genetic causes of depression cannot be replicated in animals (Nestler *et al.*, 2002).

Numerous attempts have been made to design animal models of depression, or at least of the symptoms of depression, and criteria for their evaluation have been established. Some of the most widely cited criteria were developed by McKinney and Bunney (1969) more than 30 years ago. They proposed that the minimum requirements for an animal model of depression would include the following:

- It is reasonably analogous to the human disorder in its manifestations or symptomatology.
- Behaviour that can be monitored objectively.
- The behavioural changes observed should be reversed by the same treatment modalities that are effective in humans.
- It should be reproducible between investigators.

Various paradigms have been developed and are instrumental in detecting the antidepressant-like potential of novel compounds in preclinical settings. The models commonly used are diverse and were developed originally based on the behavioural consequences of stress, drug, lesion or genetic manipulations. Many of these models have undergone iterative improvements to keep pace with continuing advances in the development of drugs with an increasingly wide array of pharmacological actions. Moreover, such improvements to models continue to be necessary to detect antidepressant effects more precisely in genetically engineered animals and after modifications of cellular and molecular targets (Cryan *et al.*, 2002).

All available animal models of depression rely on one of two principles: actions of known antidepressants or responses to stress (Willner, 1995; Hitzemann, 2000; Porsolt, 2000; Lucki, 2001).

The following table depicts the various animal models used in depression research with a short description of the main features of each model.

Table 2-3 Examples of Animal Models Used in Depression Research (Nestler, *et al.*, 2002)

Model	Main Features
Forced swim test	Antidepressants acutely increase the time an animal struggles in a chamber of water; lack of struggling thought to represent a state of despair.
Tail suspension test	Antidepressants acutely increase the time an animal struggles when suspended by its tail; lack of struggling thought to represent a state of despair.
Learned helplessness	Animals exposed to inescapable foot shock take a longer time to escape, or fail to escape entirely, when subsequently exposed to escapable foot shock; antidepressants acutely decrease escape latency and failure.
Chronic mild stress	Animals exposed repeatedly to several unpredictable stresses (cold, disruption of light-dark cycle, foot shock, restraint, etc.) show reduced sucrose preference and sexual behaviour; however, these endpoints have been difficult to replicate, particularly in mice.
Social stress	Animals exposed to various types of social stress (proximity to dominant males, odours of natural predators) show behavioural abnormalities; however, such abnormalities have been difficult to replicate, particularly in mice.
Early life stress	Animals separated from their mothers at a young age show some persisting behavioural and HPA axis abnormalities as adults, some of which can be reversed by antidepressant treatment.
Olfactory bulbectomy	Chemical or surgical lesions of the olfactory bulb cause behavioural abnormalities, some of which can be reversed by antidepressant treatments
Fear conditioning	Animals show fear-like responses when exposed to previously neutral cues (e.g., tone) or context (cage) that has been associated with an aversive stimulus (e.g., shock)
Anxiety-based tests	The degree to which animals explore a particular environment (open space, brightly lit area, elevated area) is increased by anxiolytic drugs (e.g., benzodiazepines)
Reward-based tests	Animals show highly reproducible responses to drugs of abuse (or to natural rewards such as food or sex) in classical conditioning and operant conditioning assays
Cognition-based tests	The ability of animals to attend, learn, and recall is measured in a variety of circumstances.

Most of these tests are available in rats and mice; the tail suspension test is used in mice only.

^a Examples include open field, dark-light, and elevated plus maze test.

^b Examples include conditioned place preference, drug self-administration, conditioned reinforcement, and intra-cranial self-stimulation assays

^c Examples include test of spatial memory (Morris water maze, radial arm maze), working memory (T-maze), and attention (5 choices serial test)(Nestler *et al.*, 2002).

Some of these tests (in particular, the forced swim test) have been very effective at predicting the antidepressant efficacy of new medications. They also provide potentially useful models in which to study the neurobiologic and genetic mechanisms underlying stress and antidepressant responses (Nestler *et al.*, 2002).

The forced swim test (FST), described originally by Porsolt *et al.*, (1977; 1978) is the most widely used model for assessing pharmacological antidepressant activity. The test is based upon the observation that rodents eventually develop immobility when they are placed in a cylinder of water after they have stopped active escape behaviours, such as climbing or swimming. In this way, the higher the values of duration of immobility, the higher the levels of

behavioural despair, the higher the latency to immobility (and duration of climbing and swimming), the lower the levels of behavioural despair (Porsolt *et al.*, 1997; 1998).

Antidepressant treatments reduce the amount of immobility, or delay its onset, and increase or prolong active escape behaviours displayed during the FST (Cryan *et al.*, 2005). Clinically effective treatments for depression that are detected by the rat FST include: tricyclics, monoamine oxidase inhibitors, atypical antidepressants and somatic treatments, such as electroconvulsive shock, rapid eye movement, sleep deprivation, exercise and transcranial magnetic stimulation (Borsini & Meli, 1988). The forced swim test can also distinguish drugs that are not antidepressants, for example: drugs with anti-anxiety effects such as benzodiazepines are not active in the FST (Cryan *et al.*, 2005). Because of its sensitivity to antidepressants and to stimuli that provoke depressive behaviour, the FST seems to measure a behavioural dimension that is relevant to depression (Porsolt, 2000).

In 1995 a scoring system for the FST was introduced that uses a behaviour sampling procedure to quantify the frequency of behaviours (swimming and climbing) and immobility during the test session (Decke *et al.*, 1995). This scoring system was shown to be sensitive for detecting a behavioural pattern for serotonin uptake inhibitors in the FST, which decreased immobility and increased swimming. The system also distinguished serotonin from *l*-norepinephrine uptake inhibitors, which decreased immobility and increased climbing but not swimming (Detke *et al.*, 1995; Hemby *et al.*, 1997).

Figure 2-7 illustrates the scoring technique that was used during the FST in our study to determine whether sildenafil possessed any antidepressant-like properties in an animal model of depression.

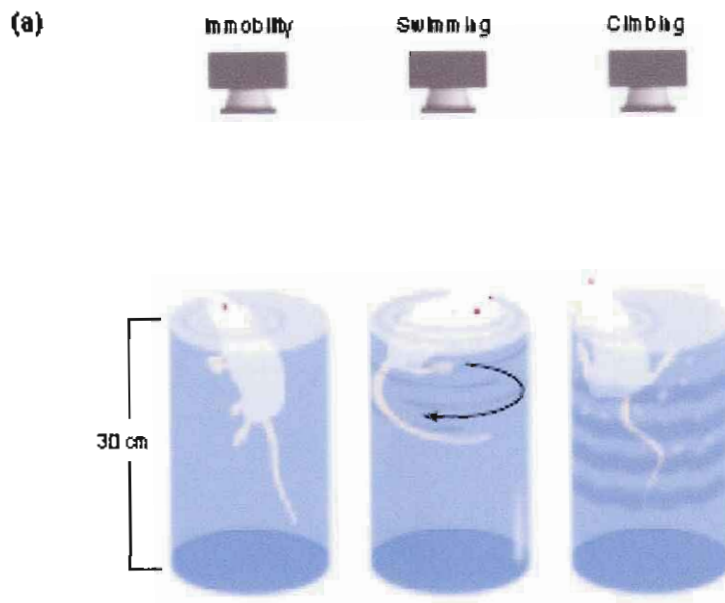


Figure 2-7 Rats undergoing forced swim test (FST) behaviours. The rats can engage in three different forms of behaviour: immobility, swimming and climbing. Immobility - measured when no additional activity was observed other than the required to keep the rat's head above water. Swimming - movement [usually horizontal] throughout the swim chamber which includes crossing across quadrants of the cylinder. Climbing - upward directed movements of the forepaws usually along the side of the swim chamber.

Some disagreement may occur between the original claims that the FST is a rodent model of human depression, which is difficult to support, and the more careful consideration and use of the FST as an objective marker for a behavioural state associated with depression (Cryan *et al.*, 2005). The original view of the FST offered by Porsolt (1977; 1978) was that of a model of depression with similar features to the learned helplessness model but technically easier to construct. The internal affective state of rodents after exposure to the initial swim in the FST was labelled as 'behaviour despair' (Porsolt *et al.*, 1977; 1978).

This procedure measures the development of behavioural immobility after a rodent has been placed in a tank of temperature maintained (23-25 °C) water to a depth of 18 cm for a 5-minute test session. The development of immobility is facilitated by a 15-minute pretest session 24 hours earlier. Antidepressant drugs administered between the pretest and test sessions decrease the duration of behavioural immobility in the FST, the principal dependant measure (Borsini & Meli, 1988).

The pretest swim induction procedure was similar procedurally to the initial session that induced learned helplessness by exposing rats to inescapable stress. Induction of learned helplessness produces broad-ranging behavioural deficits in affect, cognition, sleep and motor performance that closely are similar to many of the symptoms of depression (Weiss & Kilts, 1998).

Interpreting the FST as a model of depression required animals, exposed to the swim induction procedure, to have changes in gene expression or a similar broad spectrum of biological effects that resembled human depression, and this seemed rather unlikely from the effects of a single 15 minute induction session whose effects were measured only 24 hours later (Cryan *et al.*, 2005).

The importance of the behavioural despair model in understanding the pathology of depression builds from the argument that helplessness is a common characteristic of depression and that depression can improve if the clinician instils in the patient a sense of mastery and control over the environment (Hitzeman, 2000).

The predictive value (the hit-or-miss rate) refers to how accurately the test predicts the antidepressant activity of a novel substance, based on two concepts, false positives and false negatives (Porsolt *et al.*, 2000).

A false positive is a substance that reduces immobility in rodents but is not clinically effective in humans. The chief consistent false positive in the FST are psychomotor stimulants, some antihistamines and anticholinergics, and some antipsychotics, and a whole range of other compounds including angiotensin-converting enzyme inhibitors, calcium antagonists, gamma-aminobutyric acid (GABA) agonists, and several neuropeptides (Porsolt *et al.*, 1991), which reduce immobility in the FST and are not effective clinically as antidepressants. Several reports suggest that some compounds that potentiate monoaminergic transmission may also yield false-positive effects in the FST (Borsini & Meli, 1988). These potential false positives can be detected by evaluating their effects on locomotor activity (Hemby *et al.*, 1997).

Thus, most studies continue to employ tests of locomotor activity with the FST. Nearly all established reference antidepressants decrease locomotor activity. Although drugs that decrease immobility in the FST and increase locomotor activity may still be antidepressants, their antidepressant activity does not appear unambiguously unless accompanied by an absence of motor activating effects (Cryan *et al.*, 2005).

A false negative is a substance that shows no activity in the animal test but is active clinically. Several serotonin uptake inhibitors (clomipramine, citalopram and fluvoxamine) produce the

most important false negatives for the FST, although others (fluoxetine, indalpine and paroxetine) do display antidepressant-like activity (Porsolt *et al.*, 1991).

Though the FST does not adequately reflect the symptomatology of human depression, it appears to have a higher predictive validity compared to other animal models (Borsini & Meli, 1988; Willner, 1984). There is, in fact, a significant correlation between potency, which has not been demonstrated for any other model of depression (Willner, 1984). Additionally, it is sufficiently specific, since it discriminates antidepressants from neuroleptics and anxiolytics (Borsini & Meli, 1988; Willner, 1984). However, the compounds enhancing locomotor activity may give rise to a false positive effect in this test (Borsini & Meli, 1988; Porsolt *et al.*, 1977; 1978).

2.5 β -adrenoreceptor-downregulation

Down-regulation of the β -adrenoreceptor system following chronic antidepressant administration, was observed initially by Vetulani & Sulser (1975). They demonstrated that chronic administration of antidepressants resulted in a reduction in activity of adenylate cyclase and a reduction in the number of β -adrenoreceptors in the rat brain (Vetulani *et al.*, 1976; Banerjee *et al.*, 1977; Wolfe *et al.*, 1978; Okada *et al.*, 1986).

Chronic treatment of rodents with many clinically effective antidepressant therapies, which include tricyclics, monoamine oxidase inhibitors, atypical antidepressants, electroconvulsive shock, and rapid eye movement sleep deprivation, reduce the density of cortical β -adrenoreceptors without altering the affinity (Heninger & Charney, 1987). Conversely, procedures that result in antidepressant-sensitive behavioural changes are often accompanied by increased cortical β -adrenoreceptor density (Papp *et al.*, 1994). Finally, some laboratories have reported an increase in β -adrenoreceptor density in post-mortem cortical samples from humans diagnosed with major depressive disorders (Mann *et al.*, 1986; De Paermentier *et al.*, 1989, 1990, 1991; Karolewics *et al.*, 1999).

A consistent finding in learned helplessness animals is the up-regulation of hippocampal β -adrenoreceptors and increase in the sensitivity of adenylate cyclase to stimulation by *l*-norepinephrine (Martin *et al.*, 1990). The down-regulation of the β -adrenoreceptor has been shown to be a reliable marker of antidepressant activity in learned helplessness rats. The central role of β -adrenoreceptor down-regulation has been disputed due to the lack of effects of the serotonin reuptake inhibitors (SSRIs) and mianserin on the regulation of β -adrenoreceptor in wild mice and rats. Interestingly, all active antidepressants down-regulate the up-regulated β -adrenoreceptor in learned helplessness rats. The down-regulation of the β -adrenoreceptor was

also found to occur in cases where helplessness was reversed by behavioural training (Henn *et al.*, 2002; Vollmayr & Henn, 2003).

3.1 Introduction

In this chapter the experimental layout for investigating the antidepressant-like properties of sildenafil will be described. The description will include information on the materials and animals used and the animal model and experimental protocols employed.

In brief, the study is divided into three pilot studies:

- Pilot Study 1: Lab-validation of the forced swim test.
- Pilot Study 2: Determination of the shortest chronic treatment period necessary for fluoxetine to exert its antidepressant-like effect in the forced swim test, as well as to down-regulate β -adrenoceptors.
- Pilot Study 3: Investigation into the proposed antidepressant-like properties of sildenafil, as compared to fluoxetine, in a rat model of depression, and to establish the role of the muscarinic receptors in modulating the response of sildenafil.

The primary objective of this study was to investigate whether sildenafil displays any antidepressant-like properties in an animal model of depression. The antidepressant-like properties were screened by two approaches:

- **Behavioural changes** in treated animals compared to control drug-naïve animals were evaluated in the rat forced swim test (FST), testing also in parallel for locomotor activity to verify that any behavioural changes in the FST cannot be explained by altered locomotor activity.
- Specific **neuroreceptor changes** in frontal cortex brain region of treated rats compared to control drug-naïve animals were evaluated by measuring β -adrenoceptor concentration in the frontal cortex.

3.2 Experimental layout

As mentioned above, the current study was divided into three pilot studies. A schematic representation of the respective studies is shown in Figures 3-1, 3-2 and Figure 3-3.

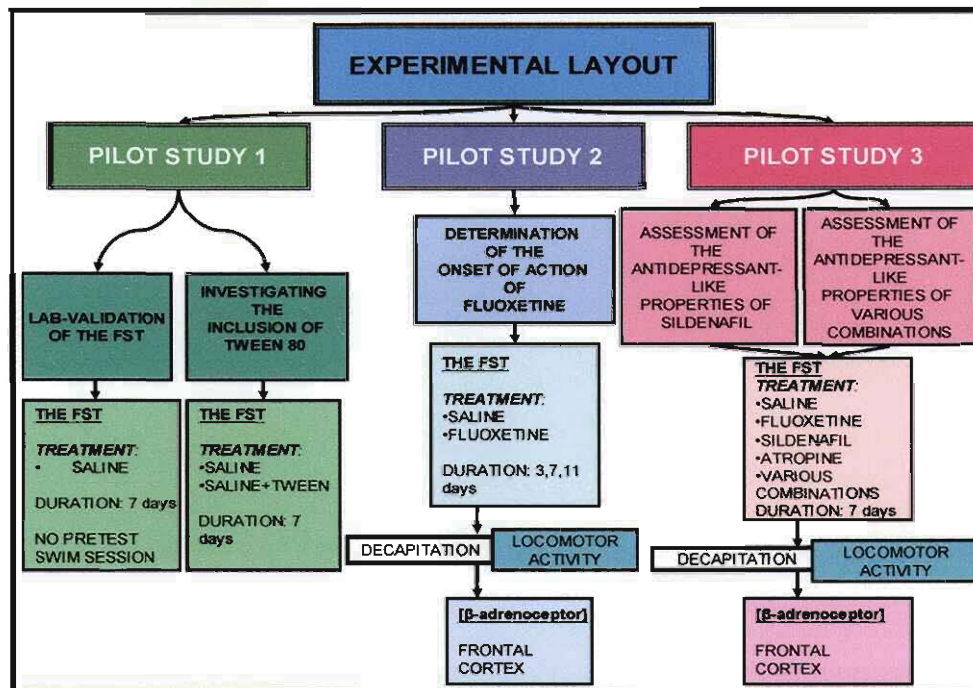


Figure 3-1 A schematic representation of the experimental design

Firstly, pilot study 1 was performed to validate the FST in our laboratory and to investigate the influence of tween 80 on immobility time during the FST. Secondly, pilot study 2 investigated the treatment period required with fluoxetine (SSRI) to induce an antidepressant-like response in the FST as well as β -adrenoreceptor downregulation. Lastly, pilot study 3 was conducted to investigate the antidepressant-like properties of sildenafil as well as various combinations of fluoxetine, atropine and sildenafil in the FST and radioligand binding studies.

3.3 Materials

3.3.1 Animals

The study protocol was approved and done in accordance with the guidelines stipulated by the Ethics Committee (Medical) (Evaluation Sub-committee for Experimental Animals) of the North-West University (Pothefstroom campus). Ethics approval number: 03D08.

Male Sprague-Dawley rats, initially weighing 140 - 160 g were used throughout the study and provided by the Animal Research Centre of the North-West University. At the end of the treatment period, the animals weighed 180 – 220 g. The rats were kept on a natural 12 hour

light/dark cycle with free access to food and water. The rats were housed in cages (width 28 cm, length 44.5 cm and height 12.5 cm), with a density of 7 rats per cage. The climate conditions in the animal centre were controlled at $21 \pm 0.5^\circ\text{C}$ and $50 \pm 5\%$ relative humidity. Full spectrum cold white light, with a light intensity of 350 - 400 lux was provided over a 12 hours light – 12 hours dark cycle. A positive air pressure was maintained in the laboratory with air filtration 99.7% effective for particle size of 2 micron and 99.9% for a particle size of 5 micron. Food and water were provided *ad libitum*. Rats received standard rats pellets with the following composition: 180 g/kg protein, 25 g/kg fat, 60 g/kg fibre, 18 g/kg calcium, 7 g/kg phosphor and 120 g/kg moisture. All animals were maintained according to a code of ethics in research, training, diagnosis and testing of drugs in South Africa. All behavioural studies were performed in the Animal research centre.

3.3.2 Drugs and chemicals

Drugs and reagents used were of appropriate quality, as available commercially. Sterile saline solution was obtained from Alpha Pharm (South Africa). NaOH, Tris (hydroxymethyl)-aminomethan and atropine were from Merck (Darmstadt, Germany). Bradford reagent (protein dye reagent, Sildenafil and Tween 80 were obtained from Sigma Aldrich (Johannesburg, South Africa). Fluoxetine Hydrochloride was from Aspen (Port Elizabeth, South Africa). Afrox (Johannesburg, South Africa) supplied liquid N₂. GE Healthcare (formerly Amersham Biosciences Buckinghamshire, England supplied the radioligand [³H]-propranolol hydrochloride (20Ci/mmol). The Propranolol, Sigmacote and fractioned BSA were from Sigma Aldrich (St. Louis, USA). PerkinElmer (Boston, USA supplied the Filtercount LSC-cocktail).

3.3.3 Choice of drugs used

3.3.3.1 Fluoxetine (20 mg/kg)

Fluoxetine can be regarded as well known and well-studied prototype antidepressant that has been used with success in the rat forced swim test (Detke & Lucki, 1995; Porsolt *et al.*, 2000). These antidepressants have been used at concentrations of 10 mg/kg or 20 mg/kg (Cryan & Lucki, 2000; Reneric *et al.*, 2001; Reneric *et al.*, 2002) in rat behavioural studies. In particular, a dose of 20 mg/kg was used in a study that distinguished between serotonergic and noradrenergic effects in the rat forced swim test (Detke *et al.*, 1996).

Previous studies have also indicated that a higher dosage of SSRIs is needed to achieve antidepressant behaviour in the traditional Forced Swim Test (Lucki *et al.*, 1994). As a result, from the above-mentioned data, I chose to use a dose of 20 mg/kg fluoxetine.

3.3.3.2 Sildenafil (10 mg/kg)

Sildenafil is a selective inhibitor of PDE5 that catalyses hydrolysis of cGMP. Prickaerts and co-workers (2002) used a maximal dose of 10 mg/kg to investigate the effects of sildenafil on object recognition memory in rats. Ottani and co-workers (2002) also used the same dose to investigate the behavioural modulating effects of sildenafil in a rat model. Volke and Vasar (2002) used a dose of 10 mg/kg sildenafil to investigate the anxiogenic-like properties of the above-mentioned PDE5 inhibitor in a mouse light-dark compartment test.

Therefore, It was decided that a dose of 10 mg/kg sildenafil will be used.

3.3.3.3 Atropine (1 mg/kg)

Atropine is a natural alkaloid that competitively antagonises acetylcholine activity at muscarinic receptors. It is lipophylic and crosses the blood brain barrier, giving rise to central effects.

O'Hare *et al.* (1996) reported that 3 – 55 mg/kg atropine slowed brain activity, impaired maze running, increased motor activity; however, using 1 mg/kg in Sprague Dawley rats was sufficient in the study to create a model for delerium.

In a pharmacological validation of the chronic mild stress model 1 mg/kg of atropine was used to determine the influence of the drug on the stress model. Papp and colleagues (1995) reported that atropine did not influence the model after a week of treatment, and indicated no antidepressant-like properties after a 7-day treatment period.

Therefore, It was decided that a dose of 1 mg/kg atropine will be used.

3.3.4 Instruments

Forced swim test

Plexiglass cylinders (diameter 18 cm; height 40 cm), Sony Digital Video Camera Recorder (model: DCR-TRV330E), Digiscan Animal Activity Monitor (DAAM, AccuScan Instruments, Columbus, OH, USA), Sartorius BP211D balance, Tempedair drier.

Radioligand binding studies

Consort P901 electrochemical analyzer (pH meter), Tri-Carb 2100 TR liquid scintillation analyzer (Packard, A.D.P. South Africa), Sorvall Discovery 90SE ultra-centrifuge, Consort P901 electrochemical analyzer (pH meter), Sartorius BP211D balance, 96 well plate reader and 560 nm filter (Labsystems multiskan RC), Polytron Homogeniser (Kinematica, Switzerland),

Eppendorf micropipettes (10 – 100 μ l 100 – 1000 μ l), 40-well Hoeffler filter manifold (locally manufactured).

3.3.5 Other materials

Whatman GF/B glass microfibre filters were obtained from Merck (Darmstadt, Germany). Consumables such as 50 ml polypropelene test tubes, serological pipettes and 96-well plates were obtained from Sigma Aldrich (Johannesburg, South Africa).

3.4 Methods

3.4.1.1 Pilot study 1 - Lab-validation of the forced swim test

It was important to validate and optimise the FST under our laboratory conditions, thus, pilot study 1 was included in this study to demonstrate the induction of behavioural despair during the FST as well as the reversal of immobility, caused by behavioural despair, with a known antidepressant (fluoxetine). Two groups, each consisting of 5 rats, were used (one group received only the vehicle while the other group received fluoxetine) and were treated for 7 days. These two groups were exposed to a pre-conditioning swim of 15 minutes followed 24 hours later by the test swimming session of 5 minutes. This was done to determine whether treatment with a known antidepressant (fluoxetine) can reduce immobility during the FST and so reduce the development of behavioural despair. A third group, consisting of 5 rats, was not exposed to the pre-conditioning swim session, thus, these rats were not habituated to the test situation and were also treated for 7 days with saline (control). This was done to establish whether the pre-conditioning swim 24 hours before the test session could induce a state of behavioural despair (Harvey *et al.*, 2002; Xu *et al.*, 2005).

3.4.1.2 Pilot study 2 – Determining the onset of action of fluoxetine

Pilot study 2 was conducted to determine of the shortest chronic treatment period necessary for fluoxetine to exert its antidepressant-like effect in the forced swim test, as well as to down-regulate β -adrenoceptors. The rats taking part in this experiment were divided into two groups each with 5 rats (one group received only the vehicle while the other group received fluoxetine). The different groups were treated in parallel for 3, 7, or 11 days (also see Figure 3-2). This was repeated twice to ensure repeatability of the experiments. Two naïve (did not take part in FST) rats from each group were chosen at random to take part in the locomotor activity experiment. This was done to establish whether the reduction in immobility time observed during the FST was not due to an increase in locomotor activity.

The following table illustrates the treatment regimes for pilot studies 1 and 2. The rats were treated for 3, 7 and 11 days with either fluoxetine (T1, T2 and T3) or saline (C1, C2, C3 and

C4). Each group consisted of 7 rats of which 5 took part in the FST and 2 in the locomotor activity experiment. Each experiment was repeated twice.

Table 3-1 The treatment regimes for pilot studies 1 and 2

Group	Drug(s)	Days	Rats / group	Groups	Total
C1	Saline	3	5+2	3	21
T1	Fluoxetine	3	5+2	3	21
C2	Saline	7	5+2	3	21
T2	Fluoxetine	7	5+2	3	21
C3	Saline	11	5+2	3	21
T3	Fluoxetine	11	5+2	3	21
					126
					21
C4	Saline	7	5+2	3	21
					21

The rats received their respective treatments in the morning for the respective time periods.

The following figure demonstrates the treatment periods for pilot study 1 in order to determine the onset of action of fluoxetine. The rats were treated for 3, 7 and 11 days, with the pre-conditioning swim period on the penultimate day of the experiment followed 24 hours later with the test swimming session after which the rats were decapitated and the frontal cortexes removed.

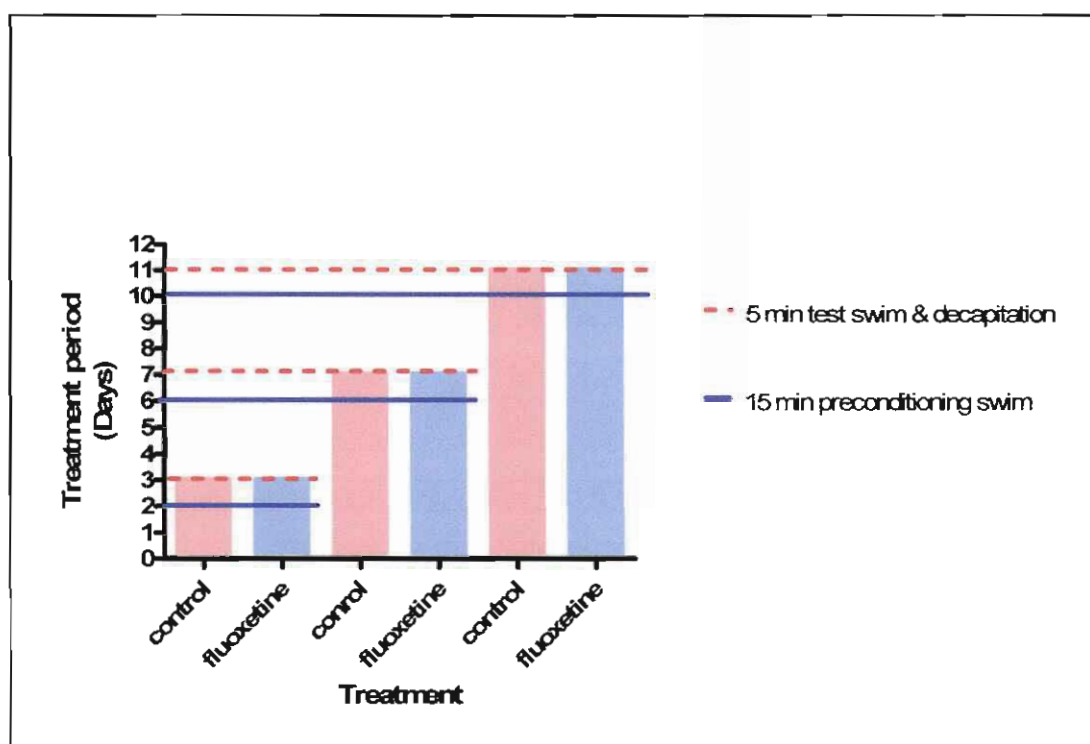


Figure 3-2 Treatment periods for pilot study 2

3.4.1.3 Pilot study 3 - Investigation of the proposed antidepressant-like properties of sildenafil

Pilot study 2 was based on the data from pilot study 1, the shortest treatment period that induced behavioural, and biochemical changes were used during pilot study 3. The data from phase 1 showed that significant behavioural and neuroreceptor changes were evident after 7 days of treatment, but not after 3 days. Consequently pilot study 3 was conducted by using 7 day treatment periods (see results in § 4.3.1). Various drugs were used throughout this particular experiment, which included sildenafil, atropine and fluoxetine. The above mentioned drugs were also used in various combinations (also see Table 3-2) to determine whether these drugs and the various combinations may possess any antidepressant-like properties during the FST.

The following table illustrates the treatment regimes for pilot study 3. The rats were treated for 7 days with either fluoxetine, saline and tween 80, saline, sildenafil, atropine and various combinations of the above-mentioned drugs. Each group consisted of 7 rats of which 5 took part in the FST and 2 in the locomotor activity experiment.

Table 3-2 Treatment regimes for pilot study 3

Group	Drug(s)	Days	Rats / group	Groups	Total
C5	saline + tween 80	7	5+2	3	21
T5	fluoxetine	7	5+2	3	21
T6	sildenafil	7	5+2	3	21
T7	fluoxetine + sildenafil	7	5+2	3	21
C8	saline + tween	7	5+2	3	21
T9	atropine	7	5+2	3	21
C10	saline	7	5+2	3	21
T11	sildenafil + atropine	7	5+2	3	21
T12	fluoxetine+atropine	7	5+2	3	21
T13	fluoxetine+atropine+sildenafil	7	5+2	3	21
					210

Note: Tween 80 is not included in C10

Due to the poor solubility of sildenafil in water and saline (used as vehicle for test drugs), tween 80 was used to increase the solubility of sildenafil in saline to give a uniform solution. Tween 80 was included throughout the study to ensure uniformity, except group C10, which was used as a control to ensure that the tween 80 inclusion did not have an effect on the FST and radioligand binding studies compared to saline control without tween 80, as used in phase 1.

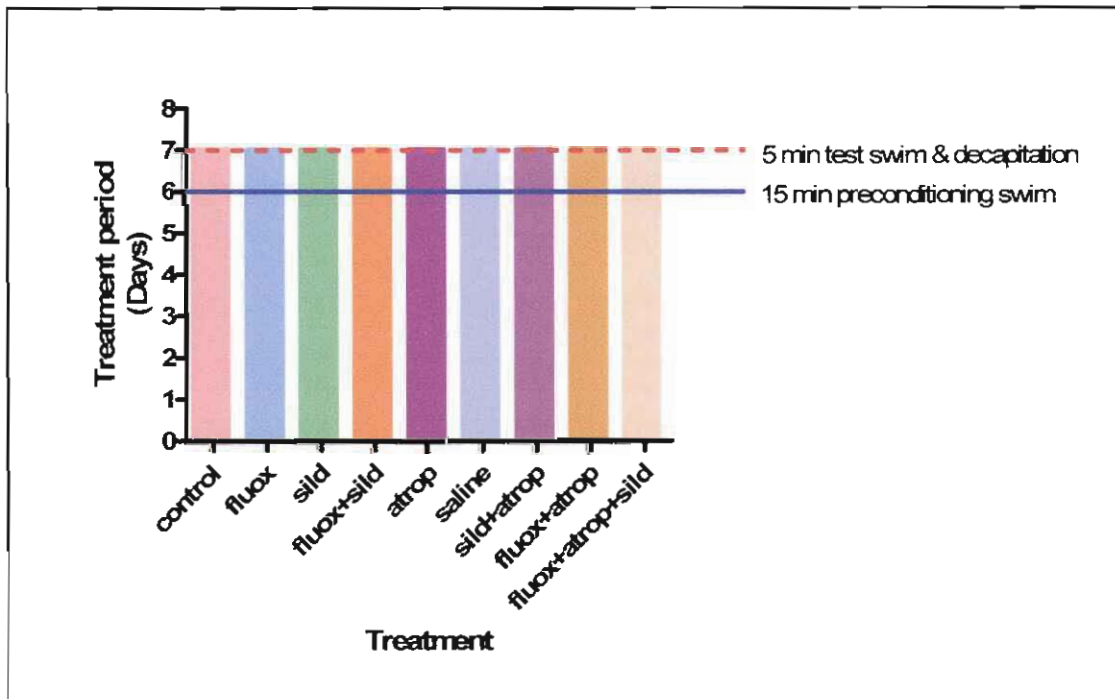


Figure 3-3 Treatment periods for pilot study 3

3.4.1.4 Drug administration

The animals were weighed each morning, whereafter the dosages were calculated and drug solutions prepared. Drugs were dissolved in a 0.9% saline solution containing tween 80 (3% v/v). Depending on the treatment group, animals were treated with drug(s) or vehicle for 3, 7 or 11 days during pilot study 2, and for 7 days during pilot study 3.

3.4.2 The forced swim test

The test is based on a learned helplessness model that bears a striking similarity to decreased drive, apathy, lack of self-worth and -esteem and locomotor retardation seen in depression. Effective antidepressants counter the stress-induced immobility response in the test.

The forced swim test originally described by Porsolt and co-workers in 1978 was used, but strictly modified in that the pre-conditioning swim was instated on the penultimate day of the designated chronic treatment period. This approach has been successfully used in chronic drug studies (Harvey *et al.*, 2002; Xu *et al.*, 2005). The pre-conditioning swim session was performed to habituate the rats to the test session, thereby providing a stable, high level of immobile behaviour during the 5-minute test session 24 hours later.

On the penultimate day of the elected treatment period, the animals were placed in the testing room for 60 minutes to habituate, whereafter they were placed in the Perspex cylinders for 15 minutes. The cylinders contained 18 cm of clean water and were maintained at a constant temperature of 25 °C. The cylinders were separated by opaque screens and the water deep enough to keep the rats from touching the bottom of the cylinder with their hind paws. Thereafter, the animals were transferred to the tempedair drier maintained at 35 °C to dry for 15 minutes. Immediately after drying, the rats were returned to their home cages.

On the final day of the treatment period the rats were placed in the testing room to habituate for 60 minutes, whereafter they were placed in the Perspex cylinders (with conditions as described earlier) for 5 minutes. The behaviour of the rats was recorded on video from the side. After the test, the rats were allowed to dry in the dryer for 15 minutes.

3.4.2.1 Scoring technique

The scoring of behavioural changes during the FST was done by a person blind to the treatment group that was being scored.

The following specific behavioural components were distinguished and measured from the videotapes:

- Climbing behaviour (also known as thrashing), which is defined as upward directed movements of the forepaws along the side of the cylinder.
- Swimming behaviour, which is defined as swimming movements (usually horizontal) throughout the cylinder.
- Immobility, which is defined, as in the original Porsolt test, when no additional activity is observed other than that required to keep the rat's head above the water.

The animals were scored separately and the individual behavioural components were identified and measured in terms of the amount of time (seconds) that specific behavioural component was observed for a 5 minute period, *i.e.* the combined time of the three components added up to 300 seconds.

3.4.2.2 Decapitation

After the FST had been completed the rats were decapitated and the brains removed. The brains were quickly dissected on ice and the frontal cortex regions were snap-frozen and stored at -86 °C for a maximum of 3 months.

3.4.3 Locomotor activity

A decrease in immobility time as measured in the FST in response to the drug may reflect an increase in motor activity. Thus, an improved response to the FST may not necessarily reflect an improvement in psychological response to environmental adversity, such as increase in drive and diminished helplessness, the main determinants measured in the FST. To avoid this confounding variable, total horizontal locomotor activity is measured in parallel to the FST to ensure that changes in swim motivation are based only on an antidepressant response, and not due to an indirect effect of the drug on locomotor activity.

The automated method provides continual computerised monitoring of the animal and is more sensitive than simple observation and is without risks of investigator bias (Sanberg *et al.*, 1983; 1987). The cages are surrounded by a series of horizontal infrared light beams (16 beams spaced 2.5 cm apart), with one set of beams at ground level and a second set 10cm above the first. This array of infrared beams enables the computerised collection of all locomotor activity by a digital analyser that effectively determines the position of the animal 100 times per second. This high-speed analysis provides a dynamic picture of all aspects of the animal's activity throughout the observation period (AccuScan Instruments Incorporated, 2000). The interruption of any beam is recorded as an activity score while an interruption of two or more consecutive beams is a movement score.

Two rats were chosen at random from the total of seven rats per treatment group to take part in the locomotor activity experiment (and not the FST). The rats were termed naïve, due to the fact that the rats received the same treatment as the other rats, but did not take part in the FST.

On the last day of the study, the naïve rats were placed in the monitoring cages for a period of 30 minutes. The first 20 minutes of this period was to let the rats become more familiar with their surroundings without influencing the results of the experiments, the habituation period. This was followed by a 10-minute period, in which the horizontal activity of the naïve rats, was determined. This was done in triplicate per treatment group.

All behavioural studies were performed between 08:00 and 12:00 to reduce the possible influence of different times in the diurnal cycle.

3.4.4 Assessment of biochemical change: Radio-ligand saturation binding studies

3.4.4.1 Preparation of membrane suspensions from brain tissue

The objective of this experiment was to prepare membranes from the rat brain frontal cortex for the purpose of determining β -adrenoceptor concentration, employing appropriate radio-ligand saturation binding studies.

- On the day of the radioligand binding studies the frontal cortex brain regions were removed from the -86 °C freezer and thawed on ice (five brains were pooled for one experiment).
- A beaker containing 25 ml of ice cold 50 mM Tris HCl buffer (pH = 7.4) was weighed, the brains added, the beaker weighed again and the wet weight of the tissue calculated and recorded.
- The tissue was homogenised with the Polytron homogeniser on setting 7 for 10 seconds.
- The homogenate was centrifuged in an ultra-centrifuge at 48,000 × g (4 °C) for 10 minutes, after which the supernatant had been decanted.
- The pellet was resuspended in 25 ml of ice cold 50 mM Tris buffer and the homogenation and centrifugation repeated and the supernatant decanted.
- The final pellet was resuspended in an appropriate volume ice-cold 50 mM Tris buffer to give a concentration of 16 mg wet weight per ml. The resuspension was done using the Polytron homogeniser on setting 7 for 10 seconds.
- The membrane suspension was kept on ice until use.

3.4.4.2 Measurement of protein concentration

The Bradford method (Bradford, 1976)

The objective of this experiment was to quantify the protein concentration in the prepared membrane suspension to make the necessary dilutions to produce a suspension with a specific required protein concentration to be used in radio-ligand saturation binding studies.

- The Bradford reagent was gently shaken in the bottle, 5 ml withdrawn, and placed in a dark environment to reach room temperature.
- Protein standards were prepared by dissolving 2 mg BSA in 1 ml double-distilled water (produces a 2 mg/ml solution) and then making a series of 100 μ l dilutions as indicated in Table 3-1:

Table 3-3 Protein concentration dilutions

Protein concentration	Dilution in test tubes	
	Volume of 2 mg/ml BSA	Volume of 50 mM Tris buffer
0 mg/ml	0 μ l	100 μ l
0.1 mg/ml	5 μ l	95 μ l
0.4 mg/ml	20 μ l	80 μ l
0.7 mg/ml	35 μ l	65 μ l
1.0 mg/ml	50 μ l	50 μ l
1.4 mg/ml	70 μ l	30 μ l

- 2 \times 5 μ l of each dilution, as well as the membrane suspension, was added to separate wells of a 96-well plate, i.e. all in duplicate.
- 250 μ l of Bradford reagent was added to each well containing the 5 μ l of membrane suspension as well as the various BSA solutions, and immediately shaken with the mixing facility of the plate reader for 30 seconds. The plate was incubated for 15 minutes at room temperature.
- The absorbance in each well was determined in the plate reader using the 560 nm filter. The protein concentration of the membrane suspension was then calculated from the plotted net absorbance against protein concentration of the standards. This was done by using the

formula for a straight line $y = mx + c$ ($y = y$ intercept, $m =$ gradient, $x =$ variable (the absorbance of the membrane suspension), and $c =$ constant).

- The membrane suspension was then diluted with ice-cold 50 mM Tris buffer to give a protein concentration of 500 $\mu\text{g}/\text{ml}$. The membrane suspension was kept on ice until use.

3.4.4.3 Measurement of β -adrenoceptor density

The aim of this experiment was to determine the concentration of β -adrenoceptors (B_{max}) in the frontal cortex regions of the rat brains, by using the prepared membrane suspensions. An eight point concentration series that ranged from 0.5 nM to 25 nM of [^3H]-propranolol (“hot propranolol”) was used to define total binding, while the same series that also contained 60 μM propranolol (“cold propranolol”) was used to define non-specific binding. Specific binding was determined by subtracting non-specific binding from total binding, which was then plotted to calculate the B_{max} .

- Sigmacote® was used to coat eight polypropylene test tubes with relatively inert silica atoms to minimise adsorption of the radio-ligand to the tube. These tubes were used to prepare the radio-ligand concentration series.
- The following dilutions of [^3H]-propranolol were prepared in 50 mM Tris buffer: 0, 0.375, 0.75, 1.5, 3, 6, 12, 25 nM.
- Another 20 polypropylene were placed on ice (8 tubes, in duplicate, for measurement of total binding; 8 tubes, in duplicate, for measurement of non-specific binding), and appropriately marked. To the 16 tubes for measurement of total binding 960 μl of membrane suspension was added, as well as 20 μl of buffer, and to the 16 tubes for measurement of non-specific binding 960 μl of membrane suspension was added, as well as 20 μl of a 60 μM propranolol solution.
- 20 μl of each radio-ligand concentration was then added to the 4 corresponding test tubes (2 tubes for measurement of total binding and 2 tubes for measurement of non-specific binding).
- Each tube was vortexed and placed in a shaking water bath for 15 minutes at 25°C.
- The contents of each tube was transferred to a Whatman GF/B filter on the manifold and washed twice with 5 ml ice cold 50 mM Tris buffer.

- The filters were placed in scintillation vials, 3 ml scintillation cocktail added, and capped, and allowed to stand overnight.
- They were then gently shaken and placed in the Tri-Carb TR liquid scintillation counter to be counted, to determine Bmax.

3.5 Data analysis

Data from the forced swim test were obtained from three separate, comparable experiments, each with 5 rats. The data from the FST consisted of individual observations for each rat, while the tissues from the frontal cortexes of a group of 5 rats were pooled for radioligand binding studies. Parallel locomotor control studies were routinely evaluated from 3 separate, comparable experiments, each with 2 rats.

The Student's t test (two tailed) was implemented for the comparison of two means. Multiple comparisons were analysed using a one-way analysis of variance (ANOVA), followed by the appropriate post-test. For multiple comparisons of test groups to the control, the Dunnett's post test was used and for multiple comparisons between treatment groups the Tukey-Kramer post test. In all cases, a confidence interval of 95% was applied and $p < 0.05$ was taken as statistically significant.

When appropriate, significant outliers were identified and excluded, using the Grubbs' test as made available by GraphPad Software (<http://www.graphpad.com/quickcalcs/grubbs2.cfm>).

The computer software GraphPad Prism (version 4.01 for Microsoft Windows, GraphPad Software, San Diego, CA, U.S.A) was used for all statistical analyses of all data.

4.1 Layout

In this chapter, the results obtained from the experiments performed will be depicted and discussed.

4.2 Pilot Study 1

4.2.1 Laboratory validation of the Forced Swim Test

Pilot Study 1 was a lab-validation of the FST, since it had not yet been performed on rats in the current laboratory setup and conditions. The aims were to determine whether behavioural despair could be induced in rats, as well as to determine whether a known antidepressant (fluoxetine) could reverse the behavioural despair.

Figure 4-1A illustrates the effect on immobility during the 5-minute scoring swim trial, 24 hours after a 0 or 15-minute preconditioning swim trial. Likewise, Figure 4-1B demonstrates the effect of a 7-day treatment period with fluoxetine on immobility in the FST.

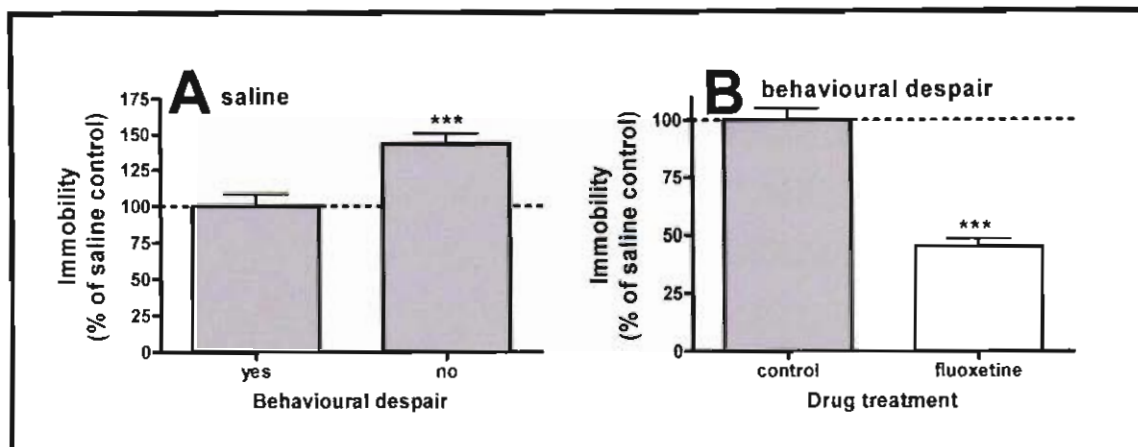


Figure 4-1A and B: Lab-validation of the FST. (A) The induction of behavioural despair by the 15-minute preconditioning swim trials. (B) The reversal of behavioural despair by fluoxetine. Immobility is expressed as a percentage of the control (saline). Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. Data were analysed statistically by performing a one-way ANOVA and the Tukey-Kramer post-test, with * $p < 0.001$.**

In A it can be seen that immobility was increased after the second swimming session from $100 \pm 8.2\%$ to $143 \pm 7.1\%$ ($n = 15$; $p < 0.001$). In addition, 7 days fluoxetine-treated rats demonstrated significant decrease in immobility when compared to control rats ($143 \pm 7.1\%$ versus $64 \pm 4.6\%$, $n = 15$; $p < 0.001$).

The data therefore confirm that the FST, as outlined in the methodology and applied under our laboratory conditions, induced behavioural despair that was reversible after a chronic treatment programme with fluoxetine. The FST, therefore, presents with sufficient sensitivity to detect the antidepressant-like actions of the positive control, the SSRI fluoxetine.

4.2.2 Investigating the inclusion of Tween 80 with control

Drugs to be used in the experimental section include fluoxetine HCl, atropine sulphate, and sildenafil citrate. Of these drugs, the solubility of sildenafil in water was too low for the preparation of daily injections for the rats. In order to prepare a homogenous suspension, it was therefore necessary to include Tween[®] 80 to enhance the solubility of sildenafil. Since Tween[®] 80 was not included in Pilot Study 1; we had to determine whether the inclusion of Tween[®] 80, during pilot study 3, would have any effect on immobility time in the FST.

Figure 4-2 illustrates the effect of the combination of saline and Tween[®] 80 (the control during pilot study 3) on immobility time versus saline (used as control in Pilot Studies 1 and 2).

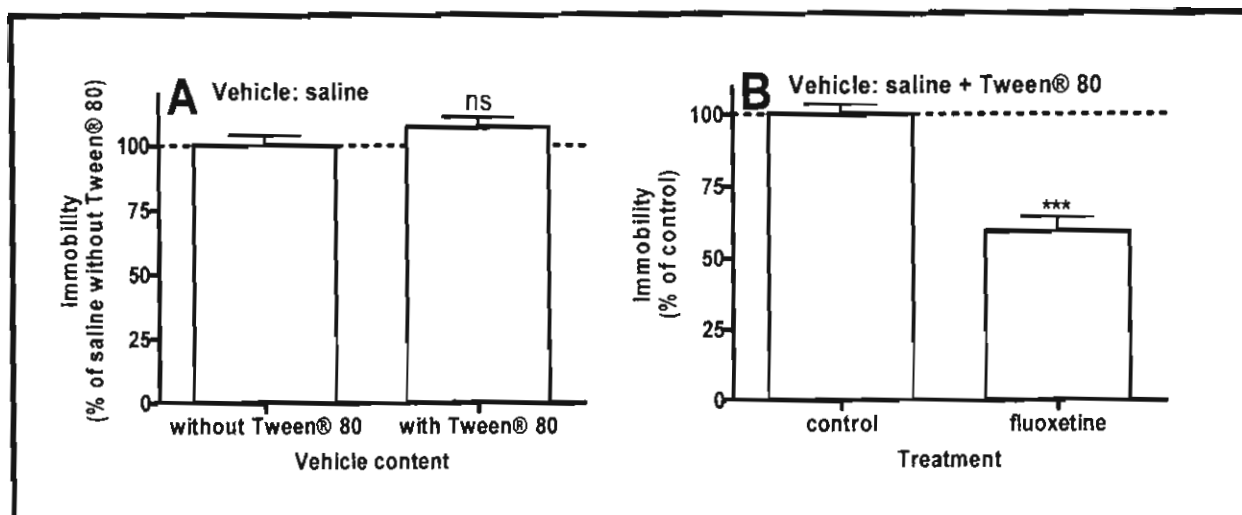


Figure 4-2: The effect of the inclusion of Tween® 80 with saline on the immobility of Sprague Dawley rats during the forced swim test. As compared to (A) saline and (B) fluoxetine (20 mg/kg). Immobility is expressed as a percentage of the control (saline). Data points represent averages ± standard error of the mean (S.E.M) from three independent experiments, each with five rats. Data were analysed statistically by performing a one-way ANOVA and the Tukey-Kramer post-test, with * $p < 0.001$.**

Figure 4-2 illustrates no significant reduction in immobility time when the combination of saline and Tween® 80 was compared to saline ($107.1 \pm 3.8\%$ versus $100.0 \pm 3.9\%$; $n = 15$; $p > 0.05$). A statistical significant reduction in immobility time was observed when fluoxetine was compared to the saline and Tween® 80 combination ($100 \pm 3.5\%$ versus $59.3 \pm 5.1\%$; $n = 15$; $p < 0.001$).

The results of Figure 4-2 indicate that the inclusion of Tween® 80 with saline, did not alter immobility in the FST in a significant manner, when compared to the vehicle (saline without Tween® 80). This also indicates that the results from Pilot Studies 1, 2 (Tween® 80 not included) and pilot study 3 (Tween® 80 included) are comparable to each other, due to the fact that no statistically significant difference in immobility time was observed between rats that received saline with Tween® 80 and those that were treated with saline alone.

4.3 Pilot study 2: Time to onset of action of fluoxetine

In Pilot Study 2 the time-dependency of the onset of antidepressant-like response caused by fluoxetine was determined.

4.3.1 Rat forced swim test

Figure 4-3 depicts the effect of the various chronic treatment durations with fluoxetine on immobility during the FST.

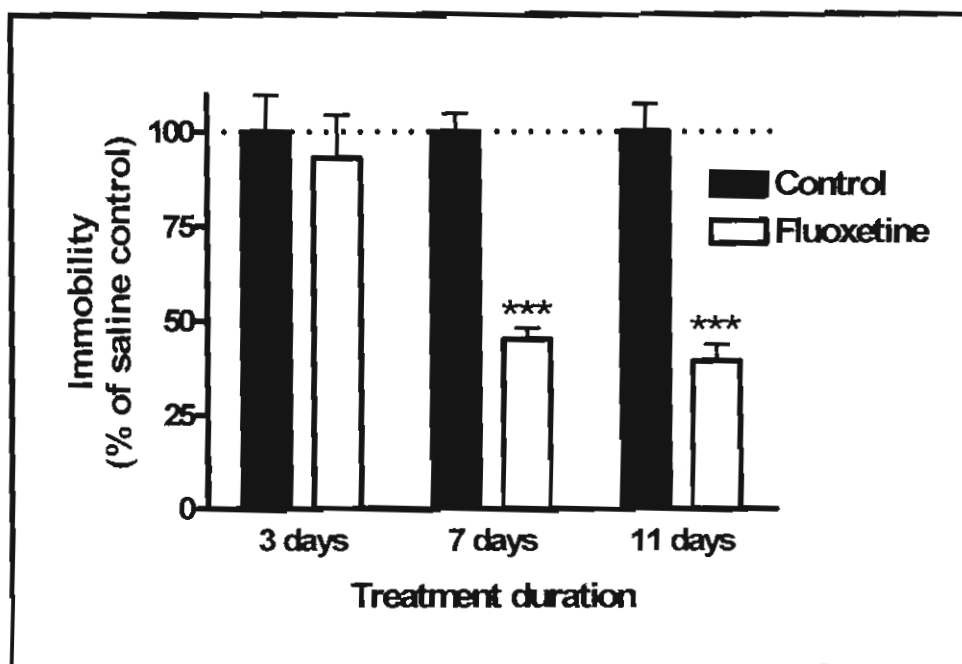


Figure 4-3: The effects of 3, 7 and 11 day treatments with fluoxetine on the immobility of Sprague Dawley rats in the forced swim test. Immobility is expressed as a percentage of the control (saline). Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. Data were analysed statistically by performing a two tailed Student's t-test with * indicating $p < 0.001$.**

Immobility was significantly reduced by fluoxetine when compared to the vehicle-treated control in the FST after 7 days ($100 \pm 4.8\%$ versus $45 \pm 2.9\%$; $n = 15$; $p < 0.001$) and 11 days (100 ± 7.4 versus $39 \pm 4.4\%$; $n = 15$; $p < 0.0001$), but not after 3 days ($100 \pm 9.7\%$ versus $93 \pm 11.3\%$; $n = 15$; $p > 0.05$).

These data correlate with previous findings that fluoxetine and other SSRIs reduce immobility during the forced swim test (Detke *et al.*, 1995; Hemby *et al.*, 1997). Porsolt and co-workers (1991) reported that certain SSRIs (fluoxetine, indalpine and paroxetine) possess antidepressant-like properties in the FST. However some clinically effective SSRIs such as clomipramine, citalopram and fluvoxamine, give false negative results in the FST.. The scoring technique implemented also increases the sensitivity of the FST for SSRIs.

In addition, the data suggest that the onset of antidepressant action of fluoxetine is time-dependent in the FST as currently employed. This clearly simulates the delayed onset of antidepressant action observed in patients with depression.

A 7-day treatment period would therefore be sufficient to determine whether sildenafil and other drug treatment regimes display antidepressant-like properties in the FST in the current study.

4.3.2 Influence of Fluoxetine on Locomotor Activity

Figure 4-4 illustrates the effect of fluoxetine on locomotor activity after the 3, 7 and 11 day treatment periods.

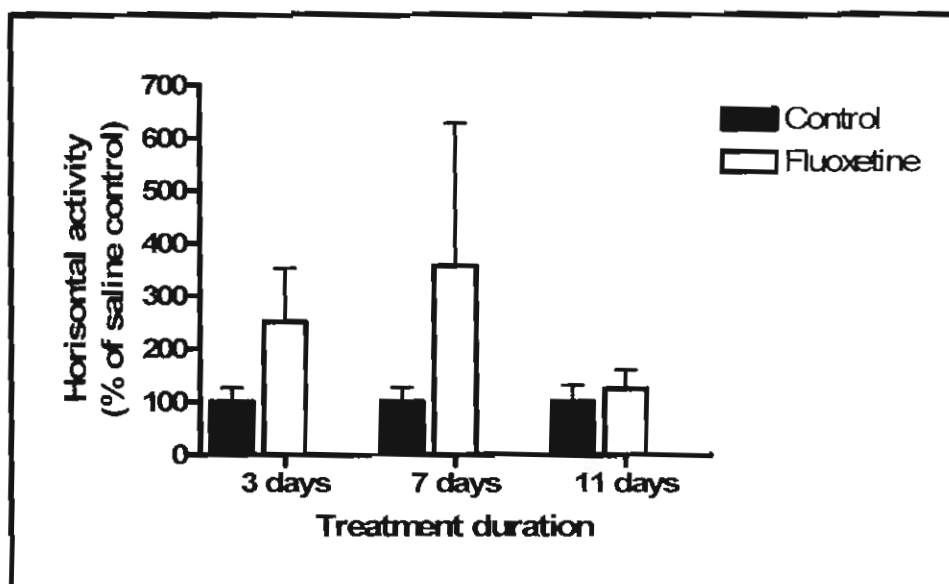


Figure 4-4: The effect of fluoxetine treatment on the locomotor activity of Sprague Dawley rats. Locomotor activity (horizontal activity) is expressed as a percentage of the control (saline). Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with two rats. Data were analysed statistically by performing a two-tailed Student's t-test. No statistical differences were observed.

It can be seen in Figure 4-4 that locomotor activity was not changed significantly with fluoxetine after any treatment period, including 3 days ($100 \pm 26.2\%$ versus $250.3 \pm 102.3\%$; $n = 6$; $p > 0.05$), 7 days ($100 \pm 31.8\%$ versus $357.4 \pm 270.6\%$; $n = 6$; $p > 0.05$) and 11-days ($100 \pm 26.7\%$ versus $103.6 \pm 35.8\%$; $n = 6$; $p > 0.05$). However, it should be noted that the large standard error of, in particular, the bar representing 7 days treatment with fluoxetine suggests a Type II error, so that the number of animals in the study may have been too small for any conclusive evidence.

It has been reported in literature that SSRIs do not influence locomotor activity of rats under any condition (Brocco *et al.*, 2002), which lays the basis for the rest of the conclusions made throughout this dissertation regarding locomotor activity.

4.3.3 Radioligand Binding Studies

Radioligand binding studies were performed on the frontal cortexes of the rats that took part in the forced swim test, to determine the density of β -adrenoceptors (β -ARs). Figure 4-5 depicts the effect of various chronic treatment durations with fluoxetine on β -AR density.

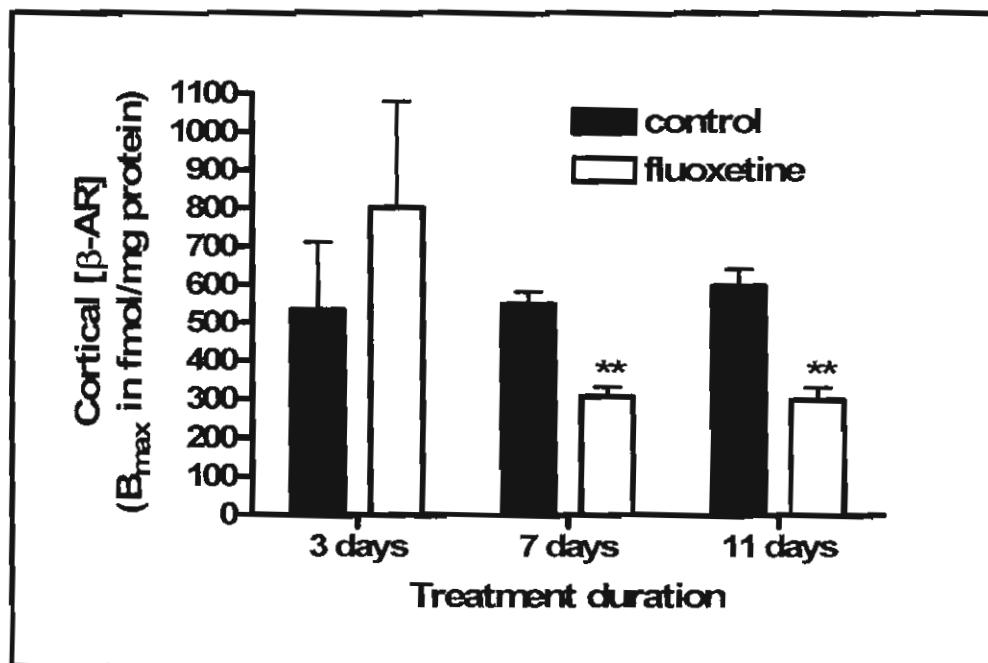


Figure 4-5: The effect of 3, 7 and 11-day treatments with fluoxetine on the β -adrenoceptor density of the frontal cortex of Sprague Dawley rats. β -AR density (B_{max}) is expressed as fmol/mg protein. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments and 5 frontal cortexes from each treatment group were pooled for one experiment. Data were analysed statistically by performing a two tailed Student's t-test with ** indicating $p < 0.01$. [β -AR] = β -adrenoceptor concentration.

According to the results displayed in Figure 4-5 β -AR density was significantly reduced after fluoxetine treatment as compared to the control after 7 days (547 ± 33.2 fmol/mg versus 305 ± 26.2 fmol/mg, $n = 3$; $p < 0.01$) and 11 days (596 ± 44.1 fmol/mg versus 296 ± 33.1 fmol/mg, $n = 3$; $p < 0.01$), but not after 3 days (532 ± 178.7 fmol/mg versus 800 ± 277.9 fmol/mg, $n = 3$; $p > 0.5$). The data correspond with the common observation that antidepressant action is associated with decreased β -AR concentration (see § 2.5). This clearly correlates with the behavioural data in section 4.3.1. The neuroreceptor data therefore support the behavioural data due to the fact that a clear antidepressant-like action is illustrated after 7 and 11 days of treatment during the radioligand binding studies.

The FST will be used during the study, as the test is an appropriate screening test for indicating antidepressant-like action in the current rat model.

4.4 Pilot study 3

Pilot study 3 investigated the antidepressant-like properties of sildenafil in a rat model of depression, and the role of muscarinic acetylcholine receptor sensitisation in this regard.

4.4.1 Forced Swim Test

Figure 4-6 depicts the influence of the various drug treatment regimes (see § Table 3-2) on the immobility of Sprague Dawley rats in the FST, after a 7-day treatment period.

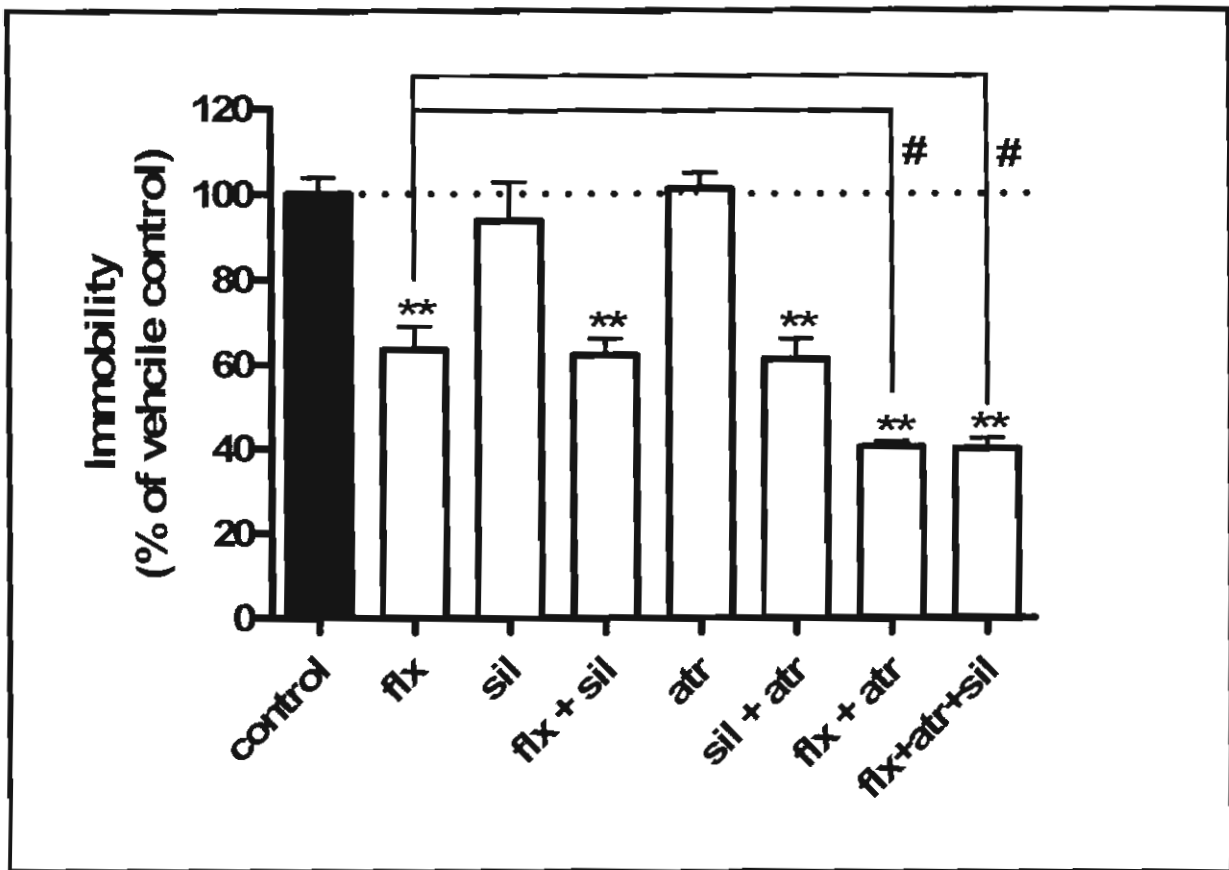


Figure 4-6: The effect of the different treatment regimes on the immobility of Sprague Dawley rats during the forced swim test. Immobility is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. For comparison of data with the control, statistically significant differences were analysed by means of a one-way ANOVA and the Dunnett post-test, with $**p < 0.01$. For comparison of data to each other, statistically significant differences were analysed by means of a one-way ANOVA and the Tukey-Kramer post-test, with $*p < 0.05$. In the graph flx = fluoxetine, sil = sildenafil and atr = atropine.

While Figure 4-6 summarises all the data obtained from the FST for the different drug treatment regimes, the different comparisons will be discussed in separate graphs below (refer to § 4.4.3-4.4.7).

4.4.2 Locomotor Activity

Figure 4-7 illustrates the effect of the various drug treatment regimes on locomotor activity after a 7-day treatment period, during pilot study 3.

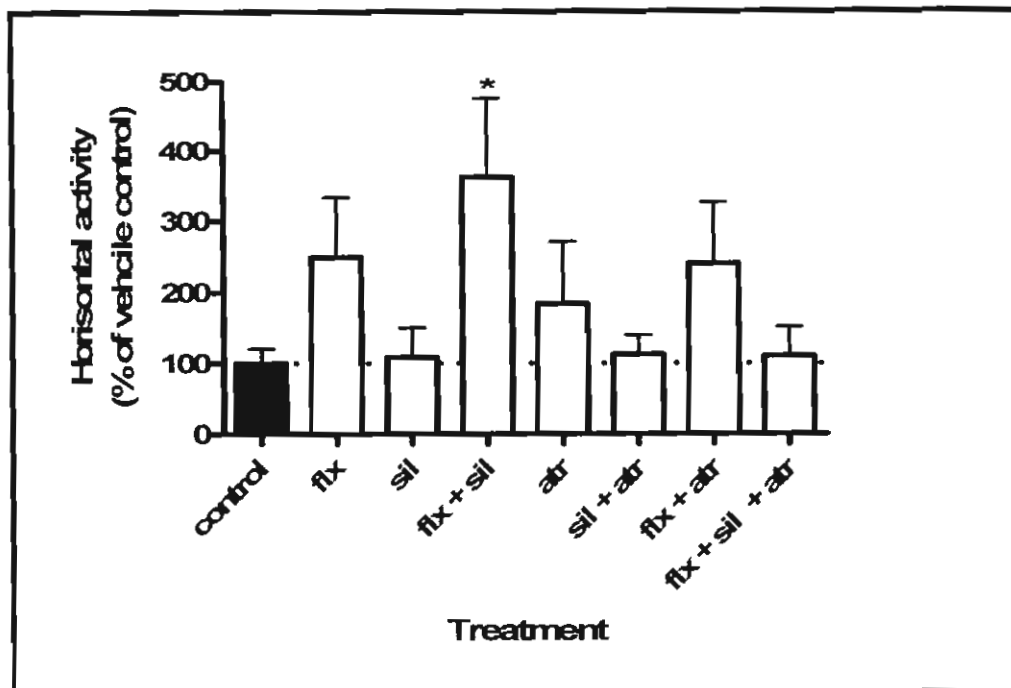


Figure 4-7: The effect of the different drug treatment regimes on the locomotor activity of Sprague Dawley rats. Horizontal activity is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with two rats. . For comparison of data with the control, statistically significant differences were analysed by means of a one-way ANOVA, performing Dunnett post-test, with * indicating $p < 0.05$. In the graph flx = fluoxetine, sil = sildenafil and atr = atropine.

It can be seen in Figure 4-7 that no statistical differences were observed between the various treatment groups. This apart from the combination of Fluoxetine and Sildenafil versus the control ($100.0 \pm 20.89\%$ versus $362.7 \pm 112.5\%$; $p < 0.05$) which clearly depict a significant increase in locomotor activity.

The data from Figure 4-7 therefore suggest that anti-immobility effects induced in the FST by antidepressant treatment, with the exception of the fluoxetine-sildenafil combination, are not secondary to a primary increase in locomotor activity.

4.4.3 Fluoxetine versus Sildenafil

Figure 4-8 illustrates the effect of sildenafil and a combination of fluoxetine and sildenafil on the immobility during the FST as well as the effect of the above-mentioned drugs on β -AR density.

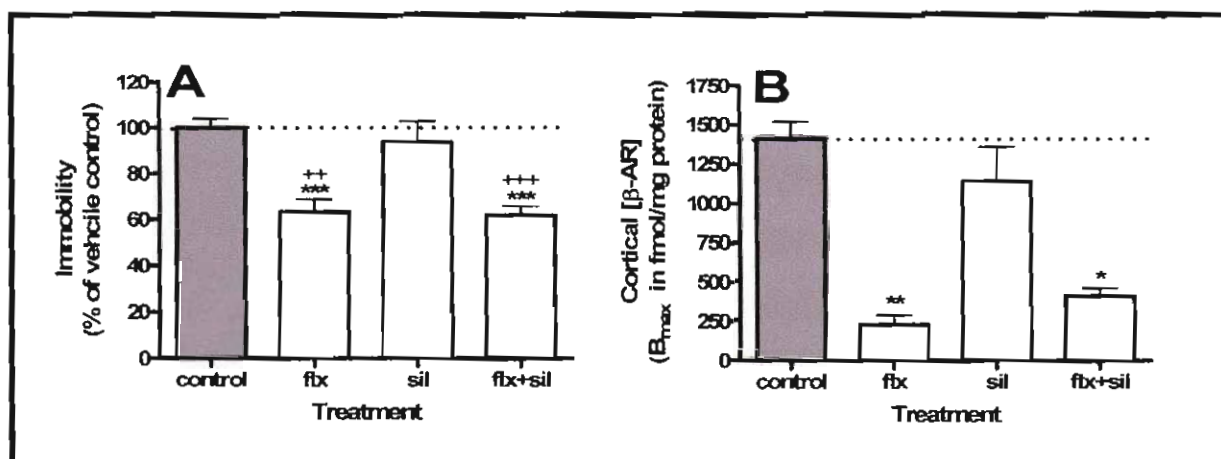


Figure 4-8(A): The effect of fluoxetine, sildenafil and a combination of fluoxetine and sildenafil on the immobility of Sprague Dawley rats in the forced swim test. Immobility is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. Statistically significant differences between data were analysed by means of a one-way ANOVA, performing Tukey-Kramer post-test, with ** indicating $p < 0.01$, *** $p < 0.001$ in comparison to control, and ++ indicating $p < 0.01$, +++ $p < 0.001$ in comparison to sildenafil alone. Figure 4-8(B): The effect of the above-mentioned drugs on the β -AR density. β -AR density (B_{max}) is expressed as fmol/mg protein. Statistically significant differences between data and control were analysed by means of a one-way ANOVA, performing Dunnett post-test, with ** indicating $p < 0.01$ and * $p < 0.05$. In the graph flx = fluoxetine and sil = sildenafil.

It can be seen in Figure 4-8A that sildenafil did not cause a significant reduction in immobility time when administered alone, when compared to the control ($100.0 \pm 3.9\%$ versus $93.8 \pm 9.1\%$; $n = 15$; $p > 0.05$). Neither did it induce a reduction in β -AR density (1409 ± 109.9 fmol/mg versus 1140 ± 218.2 fmol/mg; $n = 3$; $p > 0.05$).

However, the combination of fluoxetine and sildenafil reduced immobility significantly when compared to the control ($100 \pm 21.5\%$ versus $61.9 \pm 5.4\%$; $n = 15$; $p < 0.001$). The radioligand binding studies similarly indicated a statistically significant reduction in β -AR density after treatment with the fluoxetine, sildenafil combination versus the control (1409 ± 109.9 fmol/mg versus 411.1 ± 49.31 fmol/mg; $n = 3$; $p < 0.05$).

The figure also displays a statistically significant reduction in immobility time in the FST when fluoxetine was compared to sildenafil ($93.8 \pm 9.1\%$ versus $61.9 \pm 5.4\%$; $n = 15$; $p < 0.01$). Furthermore, Figure 4-8B illustrates a markable difference in β -AR density between sildenafil and fluoxetine, however, the difference was not of statistical significance.

The combination of fluoxetine and sildenafil reduced immobility significantly when compared to sildenafil ($93.8 \pm 9.1\%$ versus $61.9 \pm 5.4\%$; $n = 15$; $p < 0.001$). This was supported by a significant down regulation in β -AR (1140 ± 218.2 fmol/mg versus 411.1 ± 49.3 fmol/mg; $n = 3$; $p < 0.05$).

Figure 4-8A and B displays no statistically significant variation between the fluoxetine test group and the fluoxetine and sildenafil combination test group.

Sildenafil does not possess any antidepressant-like properties in a rat model of depression. The fact that there was no statistical difference between the fluoxetine, sildenafil combination and fluoxetine indicated that sildenafil did not enhance the antidepressant properties of fluoxetine during our trial period. The data also suggest that sildenafil is not able to reverse the antidepressant effects of fluoxetine on the FST and β -AR.

4.4.4 Fluoxetine versus Atropine

Figure 4-9 illustrates the effect of atropine and a combination of atropine and fluoxetine on immobility in the FST and β -AR density.

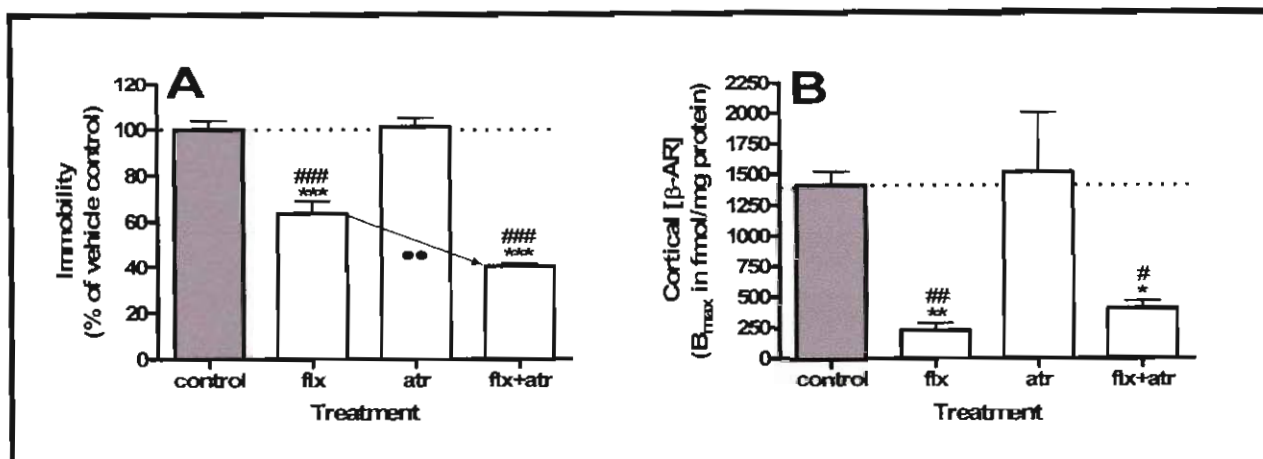


Figure 4-9(A): The effect of fluoxetine, atropine and a combination of fluoxetine and atropine on the immobility of Sprague Dawley rats in the forced swim test. Immobility is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. **Figure 4-9(B):** the effect of the above-mentioned drugs on the β -AR density. β -AR density (B_{max}) is expressed as fmol/mg protein. Statistically significant differences between data were analysed by means of a one-way ANOVA, performing Tukey-Kramer post-test, with * indicating $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ in comparison to the control. # indicates $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ in comparison to atropine and •• indicates the statistical difference ($p < 0.01$) between fluoxetine and the combination of fluoxetine and atropine. In the graph fix = fluoxetine and atr = atropine.

It can be seen in Figure 4-9A that atropine did not reduce immobility in a statistically significant manner when compared to the control ($100.0 \pm 3.9\%$ versus $101.3 \pm 3.8\%$; $n = 15$; $p > 0.05$). These findings were supported by the radioligand binding studies with no statistically significant reduction in β -AR density (1409 ± 109.9 fmol/mg versus 1516 ± 404.3 fmol/mg; $n = 3$; $p > 0.05$) after atropine treatment.

Figure 4-9A also demonstrates a statistically significant reduction in immobility time when the combination of fluoxetine and atropine was compared to the control ($100.0 \pm 3.9\%$ versus $40.5 \pm 1.3\%$; $n = 15$; $p < 0.001$). A statistically significant reduction in β -AR was also observed when the combination of fluoxetine and atropine was compared to control (1409 ± 109.9 fmol/mg versus 404.3 ± 59.5 fmol/mg; $n = 3$; $p < 0.05$).

A statistically significant reduction in immobility time was observed when atropine was compared to fluoxetine ($101.3 \pm 3.8\%$ versus $61.9 \pm 5.4\%$; $n = 15$; $p < 0.001$) and the combination of fluoxetine and atropine ($101.3 \pm 3.8\%$ versus $40.5 \pm 4.9\%$; $n = 15$; $p < 0.001$).

This was supported by data retrieved from the radioligand binding studies with statistically significant reductions in β -AR density between: atropine versus fluoxetine (1516 ± 486.1 fmol/mg versus 227.6 ± 59.9 fmol/mg; $n = 3$; $p < 0.01$) and atropine versus fluoxetine and atropine (1516 ± 486.1 fmol/mg versus 404.3 ± 59.5 fmol/mg; $n = 3$; $p < 0.05$).

The combination of fluoxetine and atropine reduced the immobility time in a statistically significant manner when compared to fluoxetine ($61.9 \pm 21.1\%$ versus $40.5 \pm 4.9\%$; $n = 15$; $p < 0.01$); however, this was not supported by a significant reduction in β -AR density as illustrated in Figure 4-9B.

The results indicate that atropine does not possess antidepressant-like characteristics. This correlates with findings of Borsini and co-workers (1988) that anticholinergic mechanisms alone are not sufficient to influence immobility time during the FST. Atropine enhances the antidepressant-like properties of fluoxetine in the FST, however, it was not supported by the radioligand binding studies.

4.4.5 Fluoxetine versus the combination of fluoxetine, atropine and sildenafil

Figure 4-10 illustrates the effect of the fluoxetine, atropine and sildenafil combination on immobility in the FST as well as β -AR concentration.

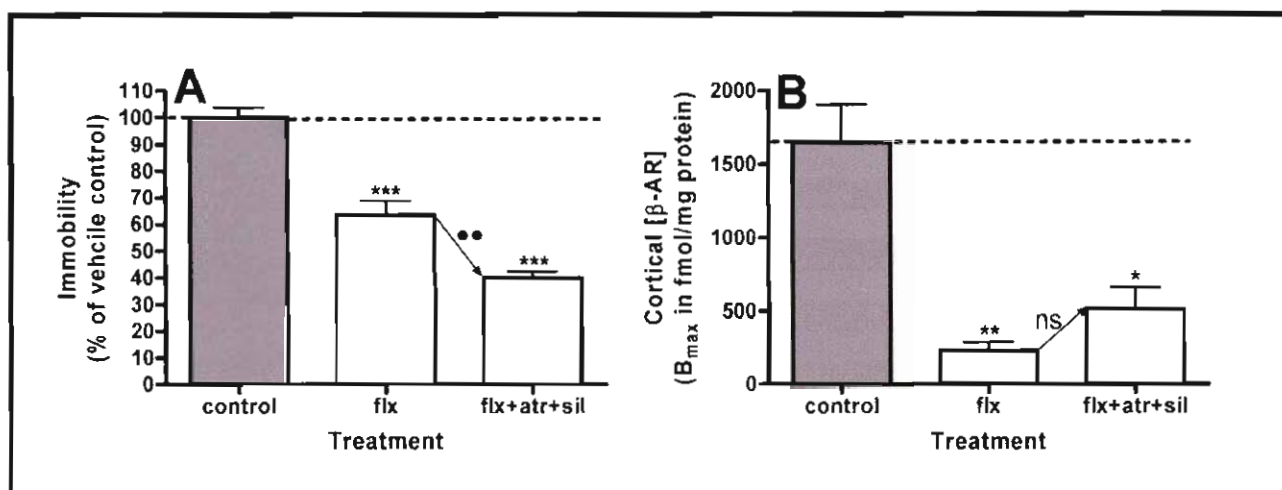


Figure 4-10(A): The effect of fluoxetine and a combination of fluoxetine, sildenafil and atropine on immobility in the forced swim test. Immobility is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. **Figure 4-10(B):** The effect of the above-mentioned drugs on the β -AR density. β -AR density (B_{max}) is expressed as fmol/mg protein. Statistically significant differences between data were analysed by means of a one-way ANOVA, performing Tukey-Kramer post-test, with * indicating $p < 0.05$, ** $p < 0.01$, *** 0.001 in comparison with the control and ** indicates the statistical difference ($p < 0.01$) between fluoxetine and the combination of fluoxetine, atropine and sildenafil. In the graph flx = fluoxetine, sil = sildenafil and atr = atropine.

In Figure 4-10 it can be seen that a statistically significant reduction in immobility was observed when the combination of fluoxetine, atropine and sildenafil was compared to the control ($100 \pm 3.9\%$ versus $39.9 \pm 2.5\%$; $n = 15$; $p < 0.001$). This correlates with data retrieved from the radioligand binding studies which led to a significant reduction in β -AR density (1409 ± 109.9 fmol/mg versus 514.1 ± 144.4 fmol/mg; $n = 3$; $p < 0.05$).

Figure 4-10A also illustrates a significant reduction in immobility time when fluoxetine was compared to the combination of fluoxetine, atropine and sildenafil ($61.9 \pm 5.4\%$ versus $39.9 \pm 2.5\%$; $n = 15$; $p < 0.01$). However, as seen in Figure 4-10B this was not supported by the radioligand binding studies, as no statistically significant difference was observed in β -AR concentration between fluoxetine and the combination of fluoxetine, atropine and sildenafil (227.6 ± 59.9 fmol/mg versus 511.1 ± 144.4 fmol/mg; $n = 3$; $p > 0.05$).

The combination of fluoxetine, atropine and sildenafil does possess antidepressant-like properties in a rat model of depression. The data also indicate that atropine and sildenafil have

the ability to enhance the antidepressant-like properties of fluoxetine in the FST, however, this was not supported in the radioligand binding studies.

4.4.6 Sildenafil and atropine versus the combination of sildenafil and atropine

Figure 4-11 illustrates the effect of the combination of atropine and sildenafil on immobility time in the FST, as well as the effect of the above-mentioned drugs on β -AR density.

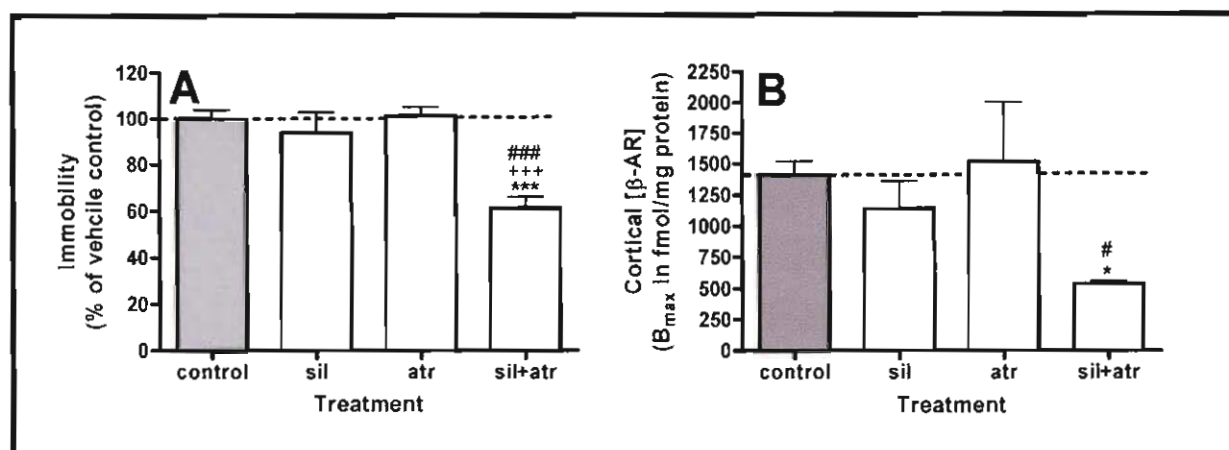


Figure 4-11(A): The effect of sildenafil, atropine and a combination of sildenafil and atropine on immobility in the forced swim test. Immobility is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. **Figure 4-11(B):** The effect of the above-mentioned drugs on the β -AR density. β -AR density (B_{max}) is expressed as fmol/mg protein. Statistically significant differences between data were analysed by means of a one-way ANOVA, performing Tukey-Kramer post-test, with * $p < 0.05$ and *** indicating $p < 0.001$ in comparison with the control. # indicates $p < 0.05$ and ## $p < 0.01$ in comparison with atropine and +++ indicates a significant difference of $p < 0.001$ in comparison with sildenafil.

Figure 4-11A illustrates a statistically significant reduction in immobility between the atropine and sildenafil combination versus the control ($100.0 \pm 3.9\%$ versus $61.2 \pm 4.8\%$; $n = 15$; $p < 0.001$). A statistically significant reduction in β -AR density was also observed after the treatment with the atropine and sildenafil combination versus the control (1409 ± 109.9 fmol/mg versus 524.8 ± 22.2 fmol/mg; $n = 3$; $p < 0.05$).

Figure 4-11A also depicts a statistically significant difference in immobility time between sildenafil and the combination of atropine and sildenafil ($93.8 \pm 9.1\%$ versus $61.2 \pm 4.8\%$; $n =$

15; $p < 0.001$), however, this was not supported by a statistically significant reduction in β -AR density during the radioligand binding studies.

In Figure 4-11A a statistically significant reduction in immobility was witnessed between the combination of sildenafil and atropine versus atropine ($100.0 \pm 3.9\%$ versus $61.2 \pm 4.8\%$; $n = 15$; $p < 0.001$). These findings were supported by the significant reduction in β -AR density after treatment with sildenafil and atropine when compared to atropine (1516 ± 486.1 fmol/mg versus 534.8 ± 22.2 fmol/mg; $n = 3$; $p < 0.05$).

The combination of atropine and sildenafil does possess antidepressant-like properties in a rat model of depression. The results from this experiment also indicate that the ability of sildenafil to potentiate mAChR as reported by Eager (2004), is unmasked with the use of an anticholinergic drug (atropine – which did not demonstrate any antidepressant-like properties when administered on its own). This indicates that the ability to potentiate the mAChR function is responsible for masking an antidepressant-like property of sildenafil, and that the sildenafil and atropine combination may be effective as an antidepressant in clinical trials, which may result in a new treatment regime for depression with improved efficacy, reduced side effects and faster onset of action.

4.4.7 Fluoxetine vs. sildenafil and atropine combination

Figure 4-12 illustrates the effect of fluoxetine and the combination of sildenafil and atropine on immobility in the FST, as well as on cortical β -AR concentration.

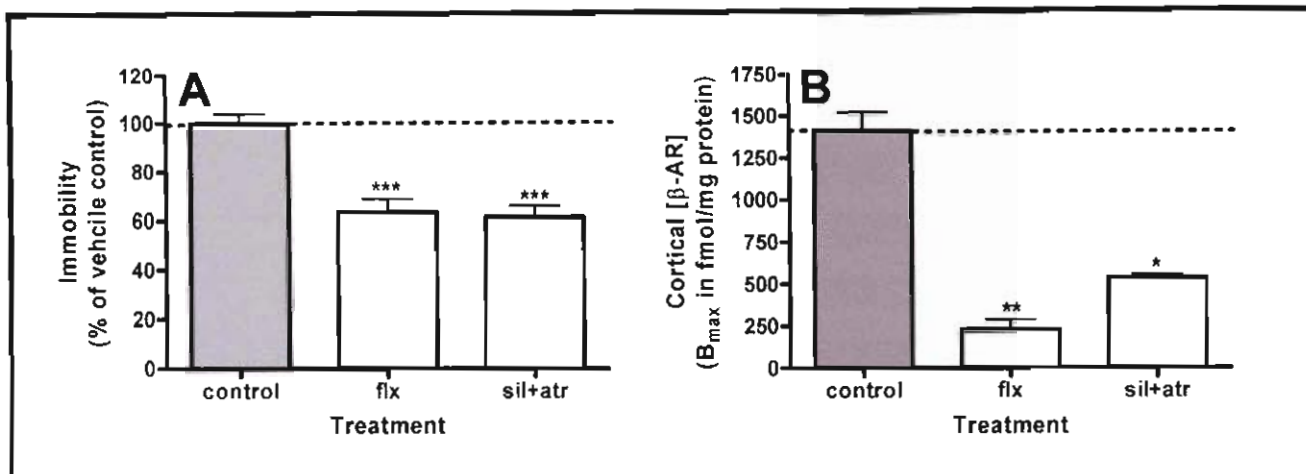


Figure 4-12(A): The effect of fluoxetine and combination of sildenafil and atropine on immobility during the FST. Immobility is expressed as a percentage of the vehicle control. Data points represent averages \pm standard error of the mean (S.E.M) from three independent experiments, each with five rats. **Figure 4-12(B):** The effect of the above-mentioned drugs on the β -AR density. β -AR density (B_{max}) is expressed as fmol/mg protein. Statistically significant differences between data and the control were analysed by means of a one-way ANOVA, performing Dunnett post-test, with * $p < 0.05$, ** $p < 0.01$ and *** indicating $p < 0.001$.

No statistically significant differences in immobility time ($61.9 \pm 5.4\%$ versus 61.2 ± 4.85 ; $n = 15$; $p > 0.05$) and β -AR concentration (227.6 ± 59.9 fmol/mg versus 534.8 ± 22.2 fmol/mg; $n = 3$; $p > 0.05$) were observed between fluoxetine and the combination of sildenafil and atropine.

This indicates that the antidepressant-like properties of the combination of sildenafil and atropine are comparable to that of fluoxetine in a rat model of depression.

5.1 Summary of results

The main objective of the current study was to determine the effects of sildenafil pre-treatment on a rat model of depression. The aim was to establish whether sildenafil possesses any antidepressant-like properties that may be masked by muscarinic cholinergic sensitisation. Sprague-Dawley rats were pre-treated with fluoxetine (20 mg/kg), atropine (1 mg/kg), sildenafil (10 mg/kg) and various combinations of these drugs (see § Table 3-2), where after rat behaviour (immobility) was measured, employing the rat forced swim test (FST), and cortical β -adrenoreceptor (β -AR) density was measured, employing standard radioligand binding studies.

The effects of the drugs used during pilot study 3 on immobility and β -AR density are summarised in Table 5-1

Table 5-1 A summary of the behavioural and neuroreceptor changes observed during pilot study 3. (↓ = decrease; ↔ = no change)

Treatment	Results	
	Effects on immobility in FST	Effects on β -AR density
fluoxetine	↓↓	↓↓
sildenafil	↔	↔
atropine	↔	↔
fluoxetine + sildenafil	↓↓	↓
fluoxetine + atropine	↓↓↓	↓
sildenafil + atropine	↓↓	↓
fluoxetine + atropine + sildenafil	↓↓↓	↓

Note: Three arrows indicate a more significant effect than one arrow. Results illustrated were obtained by means of a one-way ANOVA, performing a Tukey-Kramer post-test.

The results ultimately suggest that atropine and sildenafil do not possess any antidepressant-like properties in a rat model of depression when administered alone. However, when combined the combination illustrates an antidepressant-like effect comparable to that of fluoxetine. The combination of fluoxetine and sildenafil also produces antidepressant-like behavioural changes, but the effect achieved with this combination was not greater than the effect achieved with fluoxetine alone, which indicated that sildenafil did not potentiate the antidepressant-like ability of fluoxetine. The combination of fluoxetine and atropine, however, did illustrate a statistically significant difference in immobility time during the FST when compared to fluoxetine, which may be the result of a potentiation of the antidepressant-like properties of fluoxetine by atropine. However, this was not supported by radioligand binding studies. The possible potentiation of the antidepressant-like properties of fluoxetine in the FST was also witnessed with the combination of fluoxetine, atropine and sildenafil. This was, however, not supported by radioligand binding studies, measuring β -AR concentration.

5.2 Conclusion

In this study an antidepressant-like effect by sildenafil is unmasked by an antimuscarinic drug. However, many questions remain unanswered and some data need to be confirmed with more specific techniques.

Sildenafil alone did not elicit any antidepressant-like response in both the FST and radioligand binding studies. This might be explained by findings of Eager (2004), who reported that sildenafil displayed the ability to potentiate the signalling mechanism of mAChR, which may result in a depressogenic action. This is also supported by findings of Prast and co-workers, who investigated the role of cGMP in the process of enhancing the releasing effect of acetylcholine. The results indicated that the enhanced release of acetylcholine by NO donors DEA/NO and linsidomine was abolished by LY-83,583 (Prast *et al.*, 1995), an inhibitor of guanylyl cyclase in the basal forebrain (Schmidt *et al.*, 1985; Malta *et al.*, 1988). Since the inhibition of GC may decrease cGMP concentration, these data suggest that cGMP may be involved in the NO-mediated release of acetylcholine. In the nucleus accumbens, the release of acetylcholine elicited by DEA/NO and linsidomine was also abolished by the guanylyl cyclase inhibitors ODQ and LY-83,583 (Prast *et al.*, 1994). Due to the fact that the increase in acetylcholine release is activated through a cGMP dependent mechanism, sildenafil might increase the release of acetylcholine with an increase of cGMP through the inhibition of PDE5.

Central cholinomimetics display antimanic properties as opposed to antidepressant effects of anticholinergics (Fritze & Beckmann, 1998) and it 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 patients is super-sensitive compared to those without depression (Daws & Overstreet, 1999).

These data taken together show the great importance of the cholinergic system and mAChR function in mood disorders like depression. In this regard, the potentiation of the central cholinergic system by sildenafil may potentially exacerbate depression. The FDA also reported various neurological, emotional and psychological disorders associated with sildenafil in 2001, which included depression and anxiety. This could possibly be linked to the above-mentioned ability of sildenafil to sensitise the cholinergic muscarinic signalling system.

However, several studies have reported a secondary alleviation of depression, in patients treated for erectile dysfunction caused by SSRIs (Stuart *et al.*, 2001) as well as in patients with Parkinson's disease (Raffaele *et al.*, 2002). The oral administration of sildenafil to rats has also been shown to increase brain levels of cGMP and evoke neurogenesis (Zhang *et al.*, 2002). This indicates that sildenafil is not a depressogenic substance and has the ability to alleviate depression, but these properties are masked by its ability to potentiate the muscarinic cholinergic pathway.

The main objective of this study was to investigate the possibility that an antidepressant property of sildenafil may be unmasked by an antimuscarinic drug (e.g. atropine) that displays no effect on immobility when administered alone (Borsini *et al.*, 1988). This led to a positive antidepressant-like result in the animal model of depression used in this study. Various mechanisms could explain the antidepressant-like action that was witnessed during pilot study 3 with the use of sildenafil. The following are some of the more important mechanisms.

Firstly, through stimulation of serotonin transport in the synapse. Serotonin signalling has been implicated in a wide range of disorders including anxiety, obsessive-compulsive disorder and depression (Lucki, 1998). The inactivation of serotonin following the release at synapses is mediated by antidepressant-sensitive serotonin transporter (SERT). Sildenafil has the ability to increase surface SERT binding and as a result increases serotonin transport, lowering synaptic serotonin (Zhu *et al.*, 2004). This ability of sildenafil correlates with the mechanism of action of tianeptine (an antidepressant with proven clinical efficacy) which also stimulates the uptake of serotonin in the synapse (Fattacini *et al.*, 1990).

In contrast to the above-mentioned, sildenafil may increase serotonin release. Reports have suggested that serotonin release is enhanced by NO donors in a biphasic way (Lorrain & Hull, 1993; Guevara-Guzman *et al.*, 1994). Hypothalamic superfusion with low concentrations of linsidomine, DEA/NO, SNAP, SNOG or SNP decreases serotonin outflow, whereas higher concentrations of these NO donors enhance the release of the monoamine. Moreover, both the inhibitory and the enhancing effects elicited by low and high linsidomine concentration respectively are abolished, when the cGMP synthesis is inhibited by 1H-(1, 2, 4) oxadiazolo (4,3- α) guinoxalin-1-one (ODQ), a sGC inhibitor. Thus, in the hypothalamus, NO-induced modulation of serotonin release is mediated by cGMP (Kaehler *et al.*, 1999). Thus, the increase in cGMP concentration induced by sildenafil may increase the release of serotonin, leading to the alleviation of depression-like symptoms.

Another approach is neurogenesis, which entails the formation of new neurons. The inhibition of neurogenesis, or neurodegeneration has been recently suggested to be involved in the pathogenesis of depression and Alzheimer disease (Wen *et al.*, 2004; Reif *et al.*, 2004). Therefore, drugs that stimulate neurogenesis in the brain may improve the above-mentioned illnesses and may provide new ways of treating these diseases. Hindley and co-workers (1997) reported that NO stimulates neurite growth through a cGMP dependent mechanism and Zhang *et al.* (2003) reported that sildenafil, which causes intracellular accumulation cGMP, increased neurogenesis and promoted functional recovery after stroke in rats.

The sildenafil and atropine combination also possesses the ability to down-regulate β -AR density, which is comparable to that of fluoxetine and concurs with the consensus that these receptors are down-regulated following antidepressant treatment. The above-mentioned interaction between the NO/cGMP pathway and the noradrenergic pathway could possibly be explained by the fact that NO donors increase the release of noradrenalin under *in vitro* and *in vivo* conditions (Lonart et al., 1992; Satoh et al., 1996, 1997) as well as that *in vitro* release of *l*-norepinephrine, stimulated by NMDA or 3, 4-diaminopyridine can be enhanced by NO donors or *l*-arginine (Lauth et al., 1993; Stout and Woodward, 1994; Jones et al., 1995).

The combination of fluoxetine and atropine as well as the combination of fluoxetine, atropine and sildenafil reduced immobility time in a statistically significant manner when compared to fluoxetine. The mechanisms through which the above-mentioned combinations exert a superior effect to fluoxetine have not yet been established and also need further investigation. The fact that sildenafil, which potentiates the cholinergic muscarinic system which leads to depressive-like symptoms, did not reverse the antidepressant effects of fluoxetine on FST and β -AR is another interesting finding, that needs further investigation. This might be an indication of separate mechanisms of action, and the potentiation of the cholinergic muscarinic with sildenafil is reversed with the use of a SSRI's.

In conclusion

The current study suggests that extensive muscarinic cholinergic activation masks an antidepressant-like property of sildenafil in a rat model of depression. The antidepressant properties of sildenafil in combination with atropine are comparable to that of fluoxetine in an animal model of depression. The combination of fluoxetine with atropine, in addition enhances the antidepressant-like properties of fluoxetine, while the triple combination is as effective as the fluoxetine and atropine combination. The above mentioned findings might give rise to new ideas in the prevention of depression.

5.3 Recommendations

The following recommendations are formulated to clarify some of the findings during this study and to provide answers to some of the questions left unanswered.

- Studies should be launched to determine whether the antidepressant properties of sildenafil, illustrated during this study, can be linked to the inhibition of PDE5 or another property of the drug. The link between the antidepressant properties and PDE5 inhibition can be established by utilising other PDE5 inhibitors (e.g. zaprinast, tadalafil and vardenafil).

- The link between sildenafil or the NO/cGMP pathway and the cholinergic system need to be clarified with further research. The influence of the sildenafil on muscarinic cholinergic receptor density in the frontal cortex of an animal model of depression also needs to be determined.
- The mechanism by which sildenafil exerts its antidepressant-like properties as well as the link between the modulating effects of sildenafil on β -adrenoreceptors density, especially as this receptor type is associated with antidepressant properties, would justify further research.
- The further investigation of the superior efficacy of the atropine and fluoxetine combination as well as the combination of atropine, fluoxetine and sildenafil is warranted.
- *In vitro* studies can be conducted to support the findings of this study and to further our understanding of these antidepressant-like properties that were observed in an animal model of depression.
- *In vivo* studies to determine the onset of antidepressant action of the sildenafil and atropine combination as well as the combination of fluoxetine and atropine, and the combination of atropine, sildenafil and fluoxetine, and whether it might have a faster onset of action than other conventional treatments of depression are recommended. This could be examined by using the olfactory bulbectomy (The bilateral removal of the olfactory bulbs of a rat results in a complex constellation of behavioural, neuroendocrine, neurochemical and neuroimmune alterations, many of which are correlated with changes observed in major depression (Kelly *et al.*, 1997)) model of depression.
- The investigations into receptor modulating effects of sildenafil may be extended to other central receptor systems, for example serotonergic, dopaminergic, NMDA and GABAergic receptors.

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

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Abbreviations

5-HT	:	Serotonin or 5-hydroxy-tryptamine
AC	:	Adenylate Cyclase
Ach	:	Acetylcholine
ACPC	:	1-aminocyclopropanecarboxylic acid
AMP	:	Adenosine Monophosphate
AMPA	:	α -amino-3-hydroxy-5-methyl-4-isoxazole
atr	:	Atropine
Ca ²⁺	:	Calcium Ions
cAMP	:	Cyclic Adenosine Monophosphate
cGMP	:	Cyclic Guanosine Monophosphate
CGP-37849	:	DL-(E)-2-amino-4-methyl-5-phosphono-3-pentonic acid
CNS	:	Central Nervous System
CREB	:	cAMP Response Element Binding Protein
CYP3A4	:	Cytochrome P450 3A4
DEA/NO	:	Diethylamine/nitric oxide
DSM-IV	:	See references
eNOS	:	Endothelial NOS
FDA	:	Food and Drug Administration
flx	:	Fluoxetine

FST	:	Forced Swim Test
GABA	:	Aminobutyric Acid
GTP	:	Guanosine Triphosphate
iNOS	:	Inducible, Ca ²⁺ -dependent NOS
IP _x	:	Inisitol Multiphosphates
K ⁺	:	Potassium Ions
KA	:	Kainate or Kainic Acid
L-NAME	:	N ^G -nitro-L-arginine Methyl Ester
mAChR	:	Cholinergic Muscarinic Receptor
MB	:	Methylene Blue
MeCh	:	Metacholien
MED	:	Male Erectile Dysfunction
Memantine	:	1-amino-3,5-dimethyladamantane
Mg ²⁺	:	Magnesium Ions
mV	:	milli Volt
Na ²⁺	:	Sodium Ions
NAc	:	Nucleus Accumbens
NADP	:	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NMDA	:	N-methyl-D-aspartate
nNOS	:	Neuronal Nitric Oxide Synthase
NO	:	Nitric Oxide
NO/cGMP	:	Nitric Oxide / Cyclic Guanosine Monophosphate
NOS	:	Nitric Oxide Synthase

O ₂	:	Oxygen
ODQ	:	1H-(1,2,4)oxadiazolo(4,3- α)guinoxalin-1-one
PDE1	:	Phosphodiesterase Type 1
PDE2	:	Phosphodiesterase Type 2
PDE3	:	Phosphodiesterase Type 3
PDE5	:	Phosphodiesterase Type 5 inhibitor
PDE6	:	Phosphodiesterase Type 6
PKC	:	Protein Kinase C
PKG	:	Protien Kinase G
PTSD	:	Post traumatic Stress Disorder
RAS	:	Reticular Activating System
sGC	:	Soluble Guanylyl Cyclase
sil	:	Sildenafil
SNAP	:	S-nitroso- <i>N</i> -acetylpenicillamine
SSRIs	:	Serotonin Reuptake Inhibitors
TCA	:	Tricyclic Antidepressants
TRIM	:	1-(2-trifluoromethylphenyl)imidazole
TuJ1	:	betalll-tubulin
β -AR	:	Beta Adrenoceptor