The effect of acute and chronic sildenafil treatment with and without atropine co-administration on anxiety-like behaviour in rats

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Abstract

The neurobiology of anxiety-related disorders is associated with impaired neuroplasticity. The glutamate/NO/cGMP pathway has been proposed to play a key role in neuroplasticity and neurodevelopment. It was demonstrated in recent reports that chronic co-administration of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil and the antimuscarinic agent atropine exerts antidepressive-like activity in rats, and that this effect is related to PDE5 inhibition, with consequent elevation of cGMP levels and enhanced protein kinase G stimulation.

The current study investigated possible anxiolytic effects of the chronic co-administration of sildenafil and atropine in stress-sensitive Flinders Sensitive Line (FSL) rats. FSL rats received vehicle control, fluoxetine (15 mg/kg), atropine (1 mg/kg), sildenafil (10 mg/kg) or sildenafil plus atropine via intraperitoneal administration, either acutely 30 minutes prior to testing (acutely) or daily for 14 days (chronically). FRL control rats received only vehicle. Thereafter anxiety-like behaviour was evaluated in the social interaction test (SIT - acute) and elevated plus maze (EPM - acute and chronic). The current study also compared to different ways to score the EPM, namely the percentage time spend in the open arms of the EPM and both the number of full and half body open arm entries, and also implemented defecation on the EPM as a measure of anxiety.

Vehicle-treated FSL rats exhibited more anxiety-like behaviour than FRL rats in both the SIT and EPM following acute treatment, and in the EPM following chronic treatment. Acute treatment with fluoxetine exerted anxiogenic activity in the SIT and EPM, but anxiolytic activity following chronic administration, as observed in the EPM. In acute treatments neither sildenafil nor sildenafil plus atropine yielded any significant effects on anxiety-like behaviour. However, following chronic treatment, sildenafil exerted anxiolytic activity in the EPM by increasing the time spend in the open arms (45.72% ± 9.94% vs. 20.80% ± 9.94%, $P<0.001$). Atropine exerted a small anxiolytic response (30.71% ± 8.40% vs. 20.80 ±

9.94%), whereas atropine co-administration was additive to sildenafil alone and yielded an enhanced anxiolytic effect in the elevated plus maze (59.56% ± 4.95% vs. 20.80% ± 9.94%, P<0.001), relative to vehicle control. The percentage time spend in the open arms was scored in the EPM, the results suggested that the chronic treatment with sildenafil plus atropine exert an anxiolytic-like effect in FSL rats and the number of fecal droppings did not increase which is also an indication of an anxiolytic-like effects of the treatment.

The current study demonstrated that the chronic treatment with sildenafil, alone or in combination with atropine, exhibit an anxiolytic-like action in stress-sensitive rats. In addition, the data support the clinical potential of using PDE5 inhibitors as antidepressant and anxiolytic strategy and warrant further investigation. Furthermore the study supports the previously proposed key role of the glutamate/NO/cGMP pathway in the neurobiology of anxiety-like disorders, and as an important target for drug development.

Key words: Sildenafil, EPM, FSL rats, anxiolytic and Glutamate/NO/cGMP pathway.

Uittreksel

Die neurobiologie van angsverwante siektetoestande word geassosieer met ingekorte neuroplastisiteit. Daar is voorgestel dat die glutamaat/NO/cGMP-baan 'n sleutelrol speel in neuroplastisiteit en neuro-ontwikkeling. In 'n onlangse studie is daar aangetoon dat die kroniese mede-toediening van die fosfodiësterase-tipe-5- (PDE5-) inhibeerder sildenafil en die antimuskariniese middel atropien antidepressiewe werking in rotte uitoefen, en dat hierdie verwant is aan PDE5-inhibisie, met gevolglike verhoging in cGMP-vlakke en verhoogde proteïenkinase-G-stimulasie.

In die huidige studie word die moontlike angsiolitiese effek van die mede-toediening van sildenafil en atropien in stres-sensitiewe (FSL-) rotte ondersoek. Die FSL-rotte het oplosmiddel-kontrole, fluoksetien (15 mg/kg), atropien (1 mg/kg), sildenafil (10 mg/kg) of sildenafil plus atropien via intraperitoneale toediening ontvang, óf 30 minute voor toetsing (akuut), óf daagliks vir 14 dae (kronies). FRL-kontrolehet slegs oplosmiddel-kontrole ontvang. Hierna is angs-agtige gedrag in die sosiale interaksietoets (SIT- akuut) en verhewe plus-doolhof(*"elevated plus maze"*, EPM - akuut en kronies), waarna die rotte opgeoffer om die hippokampusse te vewyder is. Die relatiewe kwantifisering van PDE5-uitdrukking is bepaal met Western-blotanalises.

Oplosmiddel-kontrolebehandelde FSL-rotte hetmeer angs-agtige gedrag as FRL-rotte in beide die SIT en EPM vertoon na kroniese behandeling. Akute behandeling met fluoksetien het 'n angsiogeniese aktiwiteit uitgelok, maar angsiolitiese aktiwiteit na kronies toediening, soos waargeneem in die EPM. Na kroniese behandeling het sildenafil egter angsiolitiese aktiwiteit in die EPM uitgelok (45.72 ± 9.94 vs. 20.80 ± 9.94, $P<0.001$), terwyl atropien mede-toediening 'n additiewe, verhoogde angsiolitiese effek veroorsaak het (59.56 ± 4.95 vs. 20.80 ± 9.94, $P<0.001$), relatief tot oplosmiddel-kontrole. Western-blotanalises van PDE5-uitdrukking was onsuksesvol, waarskynlik as gevolg van 'n te lae uitdrukkingvlak van

hierdie ensiem in die rotbrein en ’n onvoldoende sensitiwiteit van die tegiek om sulke lae vlakke op te spoor en te kwantifiseer.

Die huidige studie demonstreer dat die kroniese behandeling met sildenafil, alleen of in kombinasie met atropien, angs-agtige werking in stres-sensitiewe rotte vertoon. Ook ondersteun die data die kliniese potensiaal van PDE5-inhibeerders as antidepressiewe en angsiolitiese strategie regverdig verdere ondersoek. Verder ondersteun die resultate van hierdie studie ook die voorheen voorgestelde sleutelrol van die glutamaat/NO/cGMP-weg in die neurobiologie van angsverwante toestande, en as ’n belangrike teiken vir geneesmiddel-ontwikkeling.

Sleutel woorde: Sildenafil, EPM, FSL rotte, anksioliese en Glutamaat/NO/cGMP weg.

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"I do not feel obliged to believe that the same God who has endowed us with sense, reason, and intellect has intended us to forgo their use." - Galileo Galilei (1564-1642)

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Chapter 1 Introduction

This introductory chapter serves as an orientation to the dissertation and study as a whole , describing (1) the article format (i.e. dissertation approach and layout), (2) the problem statement (concise literature overview, which is elaborated on in Chapter 2), (3) study objectives and (4) the study layout (experimental design/approach).

1.1 Dissertation approach and layout

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

- **Problem statement, study objectives and study layout:**

  Chapter 1

- **Literature background**

  Chapter 2 (literature review) and Chapter 3 (article introduction)

- **Materials and methods**

  Chapter 3 (materials and methods for the generation of data presented in the article) and Addendum A (additional materials and methods)

- **Results and discussion**

  Chapter 3 (results and discussion of studies presented in the article) and Addendum B (additional results and discussion)

- **Summary and conclusions**

  Chapter 4 (for the study as a whole, including findings presented in the article and addendum)

1.2 Problem statement

Anxiety is a natural psychophysiological response and a warning adaptation that can be triggered by fearful or stressful situations in humans. However, it becomes a pathological disorder when its manifestation is excessive and uncontrollable, caused by no specific external stimulus and manifesting with a number of physical and affective symptoms, including changes in behaviour and cognition (Rowney et al., 2009). As a cluster, anxiety-related disorders have been estimated to be the most prevalent psychiatric disorders (Dell' Osso et al., 2010), including severely debilitating disease such as major depression, general anxiety disorder, obsessive compulsive disorder, panic disorder, posttraumatic stress disorder and social anxiety disorder (Garner et al., 2009).

The current treatment regimes for the drug treatment of anxiety disorders include different classes of antidepressants, such as the tricyclic antidepressants (TAD), serotonin reuptake inhibitors (SSRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs), as well as benzodiazepines, buspirone, anticonvulsants and antipsychotic drugs. These drugs primarily treat the symptoms, rather than the underlying neuropathology or to reverse compromised neuroplasticity.

The glutamate/NO/cGMP signal-transduction pathway has been demonstrated to play a role in neuroplasticity and the neurobiology of anxiety-related disorders (Puzzo et al., 2008). Inhibitors of the phosphodiesterase type 5 (PDE5) enzyme, such as sildenafil, promote cGMP accumulation in this pathway. These drugs are already in clinical use for the treatment of peripheral disorders, but have also been shown to

exert central effects in rodents and humans (see below) and to intervene with the neuropathology of anxiety-related disorders (Brink et al., 2007, Liebenberg et al., 2010a, and Liebenberg et al 2010b), as explained in more depth below and in par. 2.2.2.

Some of sildenafil's most common adverse effects include headaches, light headedness and dizziness, which are all mediated via action in the CNS. Sildenafil crosses the blood brain barrier, where it causes the accumulation of cGMP (Uthayathas et al., 2007b). A number of post-marketing surveillance reports to the Federal Drug Agency (FDA) of the USA suggest possible neurological, anxiogenic and emotional disturbances associated with the use of sildenafil in men treated for erectile dysfunction. These include reports of enhanced aggressive behaviour such as rape, assault and even murder following the use of sildenafil (Milman et al., 2002). However, while the prevalence of depression, anxiety and psychosocial disturbances are high in men with erectile dysfunction, the administration of sildenafil in these men improved, rather than aggravated, their self-reported mood status (Uthayathas et al., 2007). These contradicting reports therefore seem to prompt further investigation.

Previous studies suggested anxiogenic-like effects of PDE5 inhibitors in rodents, for example acute intraperitoneal administration of 1 mg/kg sildenafil 30 or 35 minutes prior to testing, plus 200 mg/kg *l*-arginine (NO precursor) 25 minutes prior to testing decreased both open arm entries and percentage time spend in the open arms of the elevated plus maze (Volke et al., 2003). From these results it was concluded that the augmentation of the NO-cGMP cascade induces anxiogenic-like effect in male NIH mice (Volke et al., 2003). However, clinical experience suggest that many

psychotropic drugs, such as the antidepressants, achieve onset of therapeutic effect in humans only following 2 to 4 weeks of treatment. Therefore, it may be necessary to evaluate anxiolytic effects of an effective anxiolytic drugs only following chronic administration (Garner et al., 2009).

Our laboratory recently reported that the subchronic (7 days) intraperitoneal co-administration of 10 mg/kg sildenafil plus 1 mg/kg atropine (but neither drug alone) to Sprague Dawley rats exerts an antidepressant-like response comparable to that of fluoxetine in the forced-swim test (Brink et al., 2007). It was concluded that sildenafil possesses an antidepressant-like activity, but which is attenuated due to simultaneous enhancement of muscarinic receptor signalling. Follow-up studies demonstrated that the antidepressant-like activity of sildenafil is related to its inhibition of PDE5 and hence its ability to increase cGMP levels and to activate protein kinase G (Liebenberg et al., 2010b). Furthermore, tadalafil, a structurally unrelated PDE5 inhibitor, yields similar results than those observed with sildenafil, also supporting the involvement of PDE5 inhibition in its antidepressant-like activity (Liebenberg et al 2010a). Finally, we have been able to demonstrate that the pro-cholinergic activity of sildenafil is dose-dependent and that at 3 mg/kg antidepressant-like activity can be observed in the absence of muscarinic inhibition in Flinders Sensitive Line rats (Liebenberg et al., 2010a). Since depression is an anxiety-related disorder, and also in the light of only acute studies with sildenafil in mice reporting on its effect on anxiety-like behaviour, it was now warranted to investigate whether the antidepressant-like activity of chronic sildenafil in rodents is accompanied with any pronounced effect of anxiety-like behaviour.

In the current study we investigated the effect of chronic (14) day administration of sildenafil, atropine or sildenafil + atropine in stress-sensitive rats, as compared to vehicle control and to fluoxetine as positive control, on anxiety-like behaviour in the elevated plus maze (Pellow et al., 1985).

1.3 Study Objectives

The current study aimed to investigate in stress-sensitive rats the effect of:

- the acute administration of sildenafil, with and without cholinergic inhibition, on anxiety-like behaviour;
- the chronic 14 day administration of sildenafil, with and without cholinergic inhibition, on anxiety-like behaviour;

The working hypothesis for this study was that chronic, but not acute treatment with sildenafil, with or without cholinergic inhibition, will exert anxiolytic-like activity in stress-sensitive rats.

1.4 Study Layout

All of the experiments for the current study were performed in the Centre of Laboratory Animals at the Potchefstroom Campus of the North-West University, Potchefstroom, South Africa. For the above mentioned study objectives to be achieved the following study design were followed:

- **Animals:** Male Flinders sensitive line (FSL) rats (a stress-sensitive rat strain), and a corresponding negative control line, the Flinders resistant line (FRL) rats, all weighing 300 ± 10 g on the day of behavioural testing, were used for the study.

- **Drug treatment:** FSL rats received vehicle control, fluoxetine 15 mg/kg (Reneric et al., 2002), atropine 1 mg/kg (Brink et al., 2007), sildenafil 10 mg/kg (Brink et al., 2007) or sildenafil 15 mg/kg plus atropine 1 mg/kg via intraperitoneal administration, either 30 min prior to testing (acutely), or daily for 14 days (chronically).
- **Behavioural testing.** Following the acute or chronic drug administrations, rats were subjected to the behavioural testing initiated an hour after the start of the dark cycle (i.e. 19:00). These included the Elevated Plus Maze (EPM) and Social Interaction test. Defecation was also measured as a measure of anxiety-like behaviour (see Appendix B).

All data were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. Data are expressed as the mean ±S.E.M. and a value of $P<0.05$ was considered to be statistically significant. Chronic treatment studies were performed in triplicate, each with five rats (i.e. 15 rats in total per treatment group), whereas acute studies were performed once with 5 rats per treatment group (i.e. 5 rats per treatment group).

Chapter 2 Literature Review

The current chapter will firstly review scientific literature on the aetiology, classification, manifestation and neurobiology of anxiety disorders, as well as the treatment thereof. Thereafter it will review our current understanding of the role of the glutamate/NO/cGMP pathway (including the role of phosphodiesterase enzymes) in the neurobiology of these disorder, as well as current animal models utilized to investigate anxiety disorders.

2.1 Anxiety disorders

2.1.1 Aetiology of anxiety disorders

Anxiety is a natural reaction that can be triggered by fearful or stressful situations in humans. Anxiety is a pathological disorder when its manifestation becomes excessive and uncontrollable, caused by no specific external stimulus and manifesting with a number of physical and affective symptoms, with also changes in behaviour and cognition (American Psychiatric Association).

In South Africa anxiety disorders have been identified as the most prevalent class of psychiatric disorders, with a prevalence of 15.8% (Stein et al., 2008; Kessler et al., 2005), while in the United States of America (USA) anxiety disorders have an incidence of 18.1% and a lifetime prevalence of 28.8% (Kessler et al., 2005). Furthermore, anxiety disorders account for a $42.3 billion annual cost, with over 50% of the total sum directed towards non-psychiatric medical treatment cost in the USA alone (Garakani et al., 2006). This has an immense impact on global economy and also has a negative impact on the overall wellbeing of affected individuals.

Anxiety disorders are commonly seen by health care professionals in community, primary and secondary health care settings (King et al., 2008; Wittchen et al., 2005). These disorders may (King et al., 2008) persist for many years, and its manifestation is accompanied with a significant amount of personal distress, reduced quality of life, increased morbidity and mortality, and a significant economic burden (Wittchen et al., 2005). Severe anxiety disorders are rigorously debilitating and some clinicians have compared the impairment of quality of live and the decrease in productivity of these patients to those of patients suffering from schizophrenia (Dell'Osso et al., 2010).

Anxiety disorders usually follow a chronic or recurring pattern, in which full symptomatic remission is uncommon. Furthermore, anxiety disorders are associated with the progressive accumulation of comorbid disorders with a significantly increased risk for suicide (Garner et al., 2009).

Currently the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (American Psychiatric Association) distinguishes five main anxiety disorders, including general anxiety disorder, obsessive compulsive disorder, panic disorder, posttraumatic stress disorder and social anxiety disorder. Although simple and specific phobias occur frequently in communities, they are way less devastating and tend to occur less commonly in the clinical setting (Garner et al., 2009).

2.1.2 Classification of Anxiety Disorders

2.1.2.1 General Anxiety Disorder

General anxiety disorder (GAD) occurs when an individual manifests with a prolonged pattern of excessive and chronic worry about a number of actions or events in his/her life, and experiences this worry as difficult to control (Mineka et al., 2008).

GAD is associated with symptoms of restlessness, feeling anxious, being easily fatigued, difficulty with mental concentration, irritability, muscle tension and sleeping disturbances. The anxiety, worry or physical symptoms cause clinically significant distress or impairment of social, occupational and other important areas of functioning (DSM-IV-TR).

2.1.2.2 Obsessive Compulsive Disorder

Obsessive compulsive disorder (OCD) is a debilitating chronic condition, causing severe stress and restricting day to day functioning, and being characterised by obsessions and compulsions (Decloedt and Stein., 2010).

Obsessions can be described as repetitive and persistent thoughts, impulses, or urges that are experienced, at some time during the disturbance, as interfering and inappropriate and that lead to marked anxiety and distress (DSM-IV-TR).

Compulsions are defined as the behaviour or mental acts aimed at preventing or reducing distress, preventing some feared event or situation, and in some instances in response to obsessions (DSM-IV-TR).

OCD is associated with significant suffering that leads to high morbidity. Many aspects of quality of life are negatively affected by OCD, and there is a direct correlation between increased severity of the disease and a decrease in the quality of life (Decloedt and Stein., 2010).

2.1.2.3 Panic Disorder

Panic Disorder is defined by a distinct period of intense fear and discomfort, in which at least four of the following symptoms develop: palpitations, sweating, trembling, sensation of shortness of breath, chest pain, nausea, feeling dizzy, fear of losing control, fear of dying, chills and hot flashes, all which reached a peak within ten minutes from onset. The patients have a constant concern of having additional panic attacks and what the consequences of such an attack would be, leading to considerable changes in behaviour (Mineka et al., 2008).

In some instances Panic Disorder is associated with agoraphobia, which is the fear of being in a place or situation from which escape may be difficult, or help may not be available when having a panic attack (e.g., trains, large crowds). This type of anxiety leads to pervasive avoidance of a variety of situations and may impair an individual's ability to travel to work or to perform day-to-day tasks (DSM-IV-TR).

2.1.2.4 Posttraumatic Stress Disorder

The essential feature of Posttraumatic Stress Disorder (PTSD) is associated with the development of characteristic symptoms following exposure to a traumatic stressor involving direct personal experience of an event associated with actual or threatened death or serious injury of the self or of a loved one. The person's response to the event involves intense fear, helplessness or horror (Mineka et al., 2008). The traumatic event is experienced over and over, intensifying (rather than fading with memory extinction), with recurrent and intrusive recollections of the event, including images, thoughts and perceptions. Intense psychological distress following exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event leads to persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness which was not present before the trauma. The disturbance causes clinically significant distress in social, occupational and other important areas of functioning (DSM-IV-TR).

2.1.2.5 Social Anxiety Disorder

Social Anxiety Disorder can be defined as a marked and persistent fear of social performances or situations in which embarrassment may occur. Exposure to a social performance almost invariably triggers an immediate anxiety response. A patient is diagnosed with Social Anxiety Disorder only if the avoidance, fear, or anxious anticipation of encountering the social situation interferes significantly with the person's daily routine, occupational functioning and/or social life (DSM-IV-TR).

2.1.3 Current Treatment for anxiety

Current psychotropic drug treatment for anxiety includes selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), norepinephrine reuptake inhibitors (NRIs), benzodiazepines, atypical anxiolytic drugs and anticonvulsants (Garner et al., 2009). "The efficacy of these classes of psychotropic drugs has focused attention on the role of enhanced serotonergic and noradrenergic neurotransmission and the altered function of the GABA-benzodiazepine chloride ionophore complex in the neurobiology of anxiety disorders and its pharmacological treatment" (Garner et al., 2009).

2.1.3.1 GAD

A relatively large number of psychotropic drugs from a variety of pharmacological classes are used in the treatment of GAD. The main classes include TAD, SSRIs, and SNRIs, benzodiazepines, buspirone, anticonvulsants and antipsychotic drugs.

Tricyclic antidepressants have been demonstrated to be effective in the treatment of GAD (Zohar et al., 2000), but their clinical use is limited by their overall poor tolerability in comparison with SSRIs and SNRIs. The use of mirtazapine, which is a tetracyclic antidepressant, is supported by a few studies (Gambi et al., 2005) and may also be effective for the treatment of GAD with concomitant major depressive disorder (Feighner., 1999).

The Food and Drug Administration (FDA) in the United States of America has approved the use of the SSRIs paroxetine (Rickels et al., 2003), citalopram (Ball et al., 2005) and escitalopram (Dahl et al., 2001; Davidson et al., 2004) for the treatment of GAD. There are also several clinical trials demonstrating the efficacy and tolerability of sertraline (Dahl et al., 2001) in the treatment of GAD.

Recently the SNRIs have been proposed as first-line treatment option of GAD (Allgulander et al., 2001), following evidence from short- and long-term controlled trials of venlafaxine (Gelender et al., 2000) and duloxetine (Hartford et al., 2007).

Benzodiazepines are generally used during the acute treatment of GAD, particularly in patients affected by somatic symptoms (Rocca et al., 1997). Benzodiazepines in small to moderate doses are more consistently and rapidly effective, although their sustained use is associated with physical and psychological dependence. Due to the delayed onset of action of the antidepressants and to minimize their initial side-effects, the benzodiazepines have been implemented as therapeutic strategy to hasten the onset of therapeutic effect (until the antidepressant becomes effective and initial side-effects subside), whereafter the benzodiazepine is tapered.

Another drug approved by the FDA for treating anxiety disorders is the partial 5-HT_{1a} agonist buspirone, for which efficacy and safety has been demonstrated in the treatment of GAD. Buspirone is effective at a starting dose of 5 mg twice a daily or three times a day.

However, therapeutic response to buspirone may be delayed for at least 2 weeks (Zhan et al., 2004).

The anticonvulsant drugs tiagabine and of pregabalin (Bech et al., 2007), which are structural analogues of GABA, have been demonstrated to be effective in the treatment of GAD

The H_1 receptor-selective antihistamine hydroxyzine has also been shown to be effective in patients with GAD (Llorca et al., 2002).

A few studies have also investigated the use of antipsychotics as mono-therapy in the treatment of GAD. In this regard an open-label trial suggested the benefits of ziprasidone (Snyderman et al., 2005), while another controlled trial has demonstrated the efficacy of flupenthixol in patients with refractory GAD, and a few studies have investigated the tolerability of sulpiride (Chen et al., 1994). Recent controlled studies have demonstrated the efficacy of augmentation therapy with the atypical antipsychotics olanzapine and risperidone in patients with GAD who did not respond to an SSRI, SNRI, BDZ, or another anxiolytic or antidepressant drug (Brawman-Mintzer et al., 2005).

2.1.3.2 OCD

SSRIs and Cognitive Behavioural Therapy (CBT) are considered as first-line treatment of OCD. The efficacy and the tolerability of the SSRIs, fluvoxamine, sertraline, fluoxetine, paroxetine, and citalopram, have been shown in several placebo-controlled studies to be effective (Jenike et al., 1993). However, long-term studies (more than 2-years) of OCD patients treated with SSRIs are rare. Although the SSRI class is better tolerated, still 40% to 60% of patients with OCD do not respond to adequate treatment trials with SSRIs as mono-therapy.

Multiple controlled studies (Leonard et al., 1989) have demonstrated the efficacy of clomipramine in the treatment of OCD. Clomipramine is now recommended as a second-line treatment, and although it shows greater efficacy than SSRIs (Decloedt & Stein,. 2010), it is associated with more unwanted side-effects.

A strategy that has been used to enhance serotoninergic action is the use of alternative routes of administration (Koran et al., 1997), such as intravenous administration (IV). IV treatment with clomipramine or SSRIs has been shown to be effective for OCD patients who do not respond to oral treatment with the same drug (Walsh et al., 2004).

CBT is considered as a first-line therapy in less-severe forms of OCD (Albert et al., 2003), and it should be implemented in addition to a pharmacological treatment in OCD patients with associated personality disorders or dissociative symptoms. For good efficacy in the treatment of OCD, a trial of SSRIs for a long duration (10-12 weeks) and at a high dose (often the maximum recommended dose) is often required. A few studies have demonstrated the efficacy of switching from a SSRI to clomipramine, or to an SNRI such as venlafaxine (Dell'Osso et al., 2010). According to National Institute for Health and Clinical Excellence (NICE) guidelines, the combination of a dopamine antagonist and an SSRI should be effective in treating refractory OCD. Multiple studies have demonstrated the efficacy (McDougle et al., 1990) of SSRI in combination with pimozide (McDougle et al., 1994), haloperidol, risperidone (Saxena et al., 1996), olanzapine (Koran et al., 2000), and quetiapine (Mohr et al., 2002). A few studies have also evaluated the safety and efficacy of valproate, gabapentin, and lamotrigine (Kumar et al., 2000) in combination with an SSRI or a dopamine antagonist.

2.1.3.3 Panic Disorder

In the past three decades, a wide range of pharmacological regimes have been developed for the treatment of Panic Disorder (PD). Imipramine was the first drug (Garakani et al., 2006) used in the treatment of PD and along with clomipramine has been the most studied of the tricyclic antidepressants (TCAs) in the pharmacotherapy of PD (Allgulander et al., 2003).

Most drugs can prevent or greatly reduce anticipatory anxiety, phobic avoidance, and the frequency and intensity of panic attacks. Antidepressants are the drugs of choice in the treatment of PD. The different classes SSRIs, SNRIs, TCAs, and monoamine oxidase inhibitors (MAOIs) are similarly effective in the treatment of this disorder. However, SSRIs and SNRIs

offer the potential advantage of fewer adverse effects when compared to the other classes of antidepressants (Dell'Osso et al., 2010).

At this moment, SSRIs are considered the drug of choice for the treatment of PD, and many studies have demonstrated the efficacy of citalopram, escitalopram (Pelissolo et al., 2008), fluoxetine (Michelson et al., 2001), fluvoxamine (Ansis et al., 2001), paroxetine (Sheehan et al., 2005), and sertraline (Rapaport et al., 2001). There is no data to suggest that there is a difference in efficacy within the SSRI class (Perna et al., 2001), but they are known to present with a difference in side-effect profiles, drug interactions, and half-life (Dannon et al., 2007). In clinical trials escitalopram have been shown to be effective in the treatment of anxiety symptoms associated with depression, PD, and social anxiety disorder (Davidson et al., 2004).

Several studies (Beauclair et al., 1994; Jonas and Cohon., 1993; Susman and Klee ., 2005) have demonstrated the efficacy of high-potency BDZs such as alprazolam and clonazepam in the short-term treatment of this disorder while low potency BDZ for example diazepam may have an anti-panic effect at higher doses than normally prescribed for other anxiety disorders (Menezes et al., 2007). Benzodiazepines have the advantage of a more rapid anxiolytic effect compared to antidepressants but are more likely to cause physical dependence as well as somnolence, ataxia, and decrease in cognitive functioning.

Due to serious side-effects of the irreversible monoamine oxidase inhibitors (MAOIs), such as phenelzine or tranylcypromine, they are generally reserved for patients that are resistant to other treatments (Baumann et al., 2004) and are considered second-line choices (American Psychiatric Association). Data regarding the efficacy of the reversible MAOI moclobemide are inconsistent, and it should be used as a third-line drug treatment.

If a patient with PD does not respond to treatment with an SSRI, the use of another SSRI should be attempted; if that fails, switching to venlafaxine, a TCA, or a benzodiazepine (BDZ) is recommended (Hoffman et al., 2008).

2.1.3.4 PTSD Acute Anxiety

Given the high degree of comorbidity between PTSD and depression, and the common clinical features of PTSD and other anxiety disorders such as anxiety, agoraphobia and panic attacks, it is not surprising that most of the early studies have focused on the efficacy of antidepressants for PTSD (Ronald et al., 2002).

Three controlled trials and several uncontrolled studies examined the efficacy of the TCAs for PTSD symptoms, including studies of imipramine, desipramine, and amitriptyline. Both controlled trials and uncontrolled reports demonstrate the efficacy of MAOIs for the treatment of PTSD, including trials with phenelzine, brofaromine, and moclobemide (Pasquini et al., 2009).

Eight completed, controlled SSRI trials have been reported, but only paroxetine and sertraline have received FDA approval for use in PTSD (Pasquini et al., 2009).

A few controlled studies have examined the efficacy of anticonvulsant (Berlin., 2007) and antipsychotic monotherapy in the treatment of PTSD (Pasquini et al., 2009), and some authors (Kinrys et al., 2003) have suggested the potential efficacy of lamotrigine in PTSD. While two controlled trials have identified the efficacy of adding risperidone and olanzapine with SSRIs, it has been proposed that a combination strategy with an antipsychotic should be recommended if a patient does not respond to treatment with an SSRI or another antidepressant (Pae et al., 2008).

In a placebo-controlled trial conducted by Braun and co-worker (1990), using alprazolam, they reported a positive effect on the well being of the patients taking alprazolam and a marked decrease in anxiety, insomnia, and irritability. However, the treatment with alprazolam did not improve the core symptoms of the syndrome significantly. In another open trail study by Friedman in 1998 using both alprazolam and clonazpam, they arrived at the same conclusions and with a marked increase in withdrawal symptoms. While bolstering GABA mechanisms with acute benzodiazepine treatment may be effective in treating some anxiety disorders, these drugs may actually exacerbate PTSD symptoms

(Gelpin et al, 1996). Therefore, benzodiazepines have little to offer in the effective treatment of PTSD.

2.1.3.5 Social Anxiety Disorder

Very short-term therapy with a benzodiazepine, such as lorazepam (0.5 mg to 1.0 mg), or the β- blocker propranolol (10 mg to 40 mg), are common treatments of choice for SAD, ideally administered about 1 to 2 h before exposure or performance (Menezes et al., 2007).

In many people agoraphobia is also associated with panic disorder, and many of them benefit from drug therapy with an SSRI. SSRIs and benzodiazepines are effective for social phobia, but SSRIs are probably preferable in most cases because, unlike benzodiazepines, they are unlikely to interfere with cognitive-behavioural therapy. β-Blockers are useful for social-related anxiety and phobias related to public performance, because they do not affect cognitive performance (Boadie et al., 2008).

In conclusion, there are currently a great number of psychotropic drugs and a variety of psychotherapy treatments for patients suffering from anxiety disorders, yet the clinical outcome and tolerability is far from satisfactory. In clinical trials, response rates of 40 to 70% and remission rates of 20 to 47% are described (Menezes et al., 2007). Resistance, which include no response or insufficient response, affects approximately one third of patients with anxiety disorders (Menezes et al., 2007). Due to the current treatment regimens being far from optimal, there is a continued need to seek novel drug targets and treatments for these disorders, so that a better understanding of both the underlying neurobiology and neurochemistry of anxiety disorders remain essential objectives.

2.1.4 Neurobiology of anxiety disorders

In recent years great advances have been made in understanding the neurobiological basis of anxiety disorders. In particular, examination and comparisons of stress/fear-related behavioural responses and observations of changes in neurobiological markers have provided key leads for further studies. In addition, significant advances in the spatial and

temporal resolution of brain imaging techniques have helped to clarify the neuronanatomical pathways responsible for processes relevant to fear in humans (Garakani et al., 2006). Animal studies, mostly in rodents, have shown that the amygdala is part of a complex neuronal network (involving the prefrontal cortex, thalamus and hippocampus) that plays an integral part in the multiple aspects of emotional processing, including mediating adaptive and pathological fear responses. Pharmacological studies and brain imaging techniques have defined neural circuits and yielded clues about receptor and gene expression that may elucidate the potential causes and vulnerability to developing an anxiety disorder (Garakani et al., 2006).

2.1.5 Neurochemical basis of anxiety

Most studies have evaluated the role of disturbances of different neurotransmitter systems in the pathogenesis of anxiety disorders, particularly in the limbic system with the amygdala of particular importance (Davis et al., 1997; Garakani et al., 2006; LeDoux J., 1998). Most of the research up to now has focused primarily on the role of the $GABA_A$/BZD complex and the adrenergic and serotonergic systems in anxiety and fear responding (Gorman et al., 2002), but given the shortfalls in clinical efficacy of these agents in the treatment of various anxiety disorders, new targets for drug action are being actively investigated, especially the glutamatergic system and molecular targets within the hypothalamic-pituitary-adrenal axis.

$GABA_A$ receptors modulate anxiety response through projections to limbic areas with a resultant decrease in turnover of monoamines, and to the locus coeruleus and raphe nuclei with suppression of neuronal firing (Kardos et al., 1999). In particular, it has been postulated that down-regulation of the $GABA_A$ benzodiazepine receptor in anxiety disorders would result in a decreased function of endogenous neurotransmitters and the expression of the characteristic symptoms (Garner et al., 2009).

The noradrenergic neurons of the locus coeruleus give rise to diffuse projections in the forebrain, and may play a critical role in mediating fear, stress and arousal responses (Bremner et al., 1996). The central effects of norepinephrine are mediated through pre-

synaptic α_2 receptors on the terminals of the non-noradrenergic neurons, and as such play an important role in mediating the presynaptic inhibition of norepinephrine release (at α_2 autoreceptors) and the release of other neurotransmitters (at α_2 heteroreceptors). These conclusions stem from several lines of evidence. The α_2-adrenergic receptor antagonist, yohimbine, increases the firing of noradrenergic cell bodies in the locus coeruleus and induces anxiety, whereas anxiolytic agents that reduces the firing of these neurons, for example α_2 receptor agonist, clonidine, reduce symptoms of anxiety (Grimsley et al., 1995).

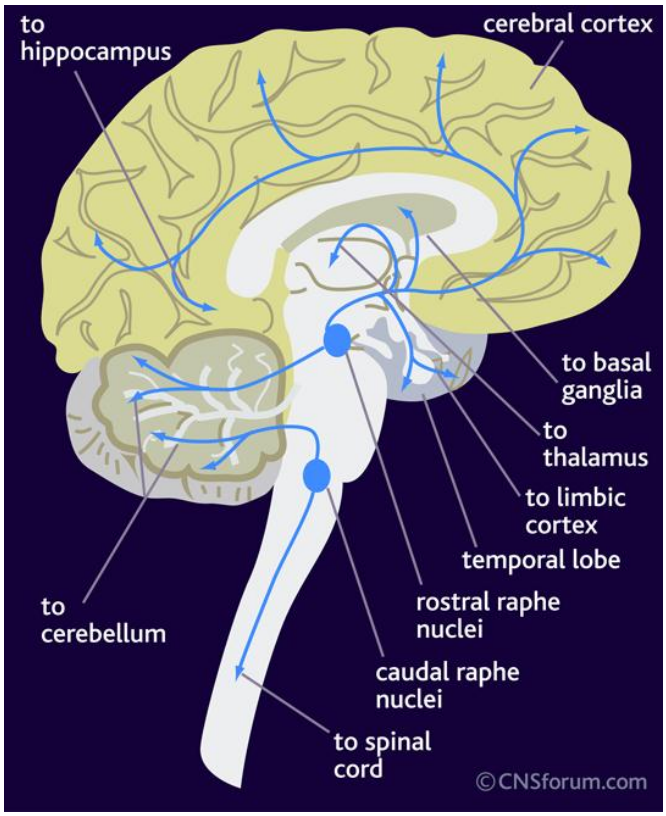

Figure 1. The serotonin pathways in the brain.

Serotonergic neurons located in the raphe nuclei project to large areas in the brain, including the limbic system and hypothalamus, and are integrally involved in the mediation of anxiety responses (Ressler et al., 2000). Presynaptic 5-HT_1 receptors and postsynaptic 5-HT_2 receptors are principally involved in the modulation of anxiety (Salzman et al., 1993). Stimulation of the terminal 5-HT_1 autoreceptors attenuates the release of serotonin at the nerve ending (Stahl et al., 1998). Results from studies of the plasma concentrations of serotonin and its metabolites have suggested a dysfunction of the serotonergic system in anxiety disorders, although these data are highly contradictory; for example, the levels of 5-

HT in the cerebrospinal fluid have been reported to be low in patients with anxiety disorders (Johnson et al., 1995), whereas administration of the non-selective 5-HT_1 and 5-HT_2 agonist, m-chlorophenylpiperazine, leads to an increase in hostility and anxiety in patients with GAD (Germine et al., 1992). It has been proposed that SSRIs act by reducing central serotonergic neurotransmission (following postsynaptic serotonergic receptor down-regulation), implying a state of increased serotonergic neurotransmission in anxiety disorders. In fact, it is believed that two major serotonergic systems, namely one originating from the medial raphe nuclei and the other from the dorsal raphe nuclei, are involved in the neurobiology of anxiety disorders (Deakin et al., 1991). It has been postulated that each system mediates a different aspect of anxiety, and that dysfunction of one or both of these systems would result in different forms of anxiety disorders (Deakin et al., 1991). This could potentially form a neurobiological basis for the sub-classification of anxiety disorders.

2.1.6 Brain structures involved in anxiety disorders

Functional imaging techniques have been used extensively to identify potential neurobiological correlates between core anxiety symptoms and anatomical and neurophysiological alteration in the central nervous system (Kilts et al., 2003). Robust evidence indicates that the amygdala mediates states of increased arousal and fear responses (Ohman et al., 2005). The central nucleus of the amygdala receives information from the visual, auditory, olfactory, nocioceptive and visceral pathways, and mediates the integration of the information and execution of autonomic and behavioural fear responses (Kalin et al., 2004). Two types of fear responses have been described, namely a swift, less finely tuned mode, in response to immediate threats and activated by a direct pathway from the sensory thalamus to the amygdala, and, secondly, a slower response activated by a thalamo-cortico-amygdalo circuit, which allows valuable cortical assessment of threat-related information (Kalin et al., 2004).

The prefrontal cortex and the hippocampus are two other key brain structures known to play an important role in the pathophysiology of anxiety and anxiety-related disorders (Shin et al., 2006). The hippocampus is considered to play a role in the processing of contextual information, differentiating between safe and potentially dangerous situations. During

dysfunction, this may consequently produce an anxiety response to innocuous stimuli with an overestimation of potentially threatening contexts (Shin et al., 2006). The medial prefrontal cortex may play a critical role in the process of fear extinction which, when defined within the context of animal models, is the reduction of conditioned fear responses when a cue is repeatedly presented without the adverse stimulus previously associated with this cue (Milad et al., 2002). Animals with dysfunctions in the medial prefrontal cortex seem to have difficulties in memorizing previous associations between a cue (e.g. a tone signalling a potentially threatening stimulus to follow) and a lack of the adverse stimulus (e.g. electrical shock) (Tamminga et al., 2006).

Structural and functional imaging studies have highlighted the role of different brain areas, such as the temporal lobe, prefrontal cortex, insula and motor striatal regions, in the neural circuitry of panic disorder (Graeff et al., 2008). The most consistent findings suggest:

1. the presence of a left-to-right asymmetry in hippocampal metabolism;
2. hypometabolism in the parieto-temporal areas which may rectify upon treatment, and
3. metabolic changes in anterior cingulate or obito-frontal regions (Dell'Osso et al., 2010).

Studies in patients with PTSD have suggested a hyper-responsivity of the amygdala and deficient activation of the ventral/medial prefrontal cortex and hippocampus (Liberzon et al., 2008). Structural imaging studies have reported smaller volumes of the ventral/medial prefrontal cortex and the hippocampus in patients with PTSD, when compared to healthy controls (Karl et al., 2006).

In SAD, functional imaging studies have shown that patients differ from normal controls in the processing of social threat-related stimuli, and conditioned aversive stimuli (Dell'Osso et al., 2010); in particular, functional magnetic resonance imaging (fMRI) studies have shown the involvement of both the amygdala and the hippocampus (Dell'Osso et al., 2010).

In OCD, obsessions have been associated with over-activity of the frontal cortex, possibly as a consequence of impaired thalamic gating, attributable in turn to deficient striatal function; by contrast, compulsions may be the result of aberrant striatal activity (Saxena et al., 2000). Resting state positron emission tomography (PET) and single photon emission computed tomography (SPECT) studies in patients with OCD showed increased activity in the orbifrontal cortex and striatum, when compared to healthy controls (Whiteside et al., 2004). An fMRI study has found a significant correlation between anxiety and degree of activation of the amygdala (Mataix-Cols et al., 2003).

A PET study found metabolic differences in occipital lobe, limbic regions and basal ganglia in patients with GAD, when compared to healthy controls, after benzodiazepine treatment (Wu et al., 1991). More recently, an fMRI study found that individual differences in the degree of rostral anterior cingulate cortex and amygdala activation predicted better treatment outcomes to venlafaxine (Whalen et al., 2008).

These neuro – imaging findings have led to the hypothesis that anxiety disorders may be classified on neurobiological basis into different subtypes, based on predominant involvement of the amygdala in SAD, the combination of amygdala and cortical involvement in both PTSD and PD, or on predominant involvement of cortico-striatal systems; however, there is at present insufficient consistent evidence to categorize GAD and OCD according to this scheme (Cannistraro et al., 2003). Nevertheless, scientific advances in neurobiology are progressively clarifying fundamental brain mechanisms and the underlying structural causes of anxiety, and this should eventually provide a logical basis for the pharmacological treatment of anxiety disorders.

A robust body of evidence from family, twin and adoptee studies have suggested that a complex genetic component may be involved in the development of anxiety-related traits (Hettema et al., 2001). For example, allelic variation of 5-HT transporter expression and function seems to play a particularly crucial role in the vulnerability to anxiety disorders. In addition, a specific association of the 5-HT transporter polymorphism and amygdala activation is supported by the findings of a recent meta-analysis (Munafo et al., 2008).

The current treatments and research focus on treating the symptoms of psychiatric disorders rather than reversing the underlying abnormalities in neuroplasticity or neurodevelopment that might contribute to psychiatric disorders (Krystal et al., 2009). Thus, novel hypotheses and associated treatment regimens are needed, that will successfully reverse the underlying neuropathology of anxiety disorders.

2.2 Phosphodiesterase

The cyclic nucleotide phosphodiesterases (PDEs), which are widely distributed in mammalian tissue, play a major role in cell signalling by hydrolysing cAMP and cGMP. Due to their diversity and distribution at both cellular and subcellular levels, PDEs can selectively regulate various cellular functions. The PDE superfamily represents 11 gene families (PDE1 to PDE11). Each family include 1 to 4 distinct genes, to give more than 20 genes in mammals encoding for more than 50 different PDE proteins, and of which most are probably synthesised in mammalian cells. Although PDE1 to PDE6 were the first isoforms to be well characterised (due to their wide expression in various tissues and cells), their specific physiological roles and dysregulation in pathophysiology remain unclear. Further research is needed to clarify the roles of many of the PDEs, in particularly the newly discovered PDE7 to PDE11. In many pathologies, such as inflammation, neurodegeneration, and cancer, variation in intracellular signalling related to PDE deregulation may explain the difficulties observed in the prevention and treatment of these pathologies. By selectively inhibiting specific PDEs (when up regulated) with novel inhibitors, it may be possible to restore normal intracellular signalling, thereby providing targeted therapy with reduced adverse effects (Lugnier, 2006). Phosphodiesterases have been identified as important drug targets (Uthayathas et al., 2007), and a number of important drugs used in medicine today act on this group of enzymes, including for example amrinone (for heart failure), theophylline (for asthma), caffeine (as a CNS stimulant), sildenafil (for erectile dysfunction) etc.

2.2.1 The role of the glutamate/NO/cGMP pathway in the CNS

The glutamate/NO/cGMP pathway has been demonstrated to play a role in neuroplasticity and the neurobiology of CNS-related disorders (Puzzo et al., 2008). Intimately associated with this system are the PDE enzymes, particularly type 5 (PDE5), which is responsible for the breakdown of cGMP. Inhibitors of this enzyme are available for clinical use and may also provide a novel way to intervene with the neuropathology of anxiety-related disorders. Recent studies conducted in our laboratory have reported that the PDE5 inhibitors exhibit anti-depressant-like effects in rodents (Brink et al., 2008) (Liebenberg et al, 2010a; 2010b), as will be alluded to below.

Nitric oxide (NO) is an important bio-regulatory molecule in the nervous, immune and cardiovascular systems. NO is synthesized from *l*-arginine, which is converted to *l*-citrulline in the presence of $O_{2,}$ NADPH and tetrahydrobiopterin by nitric oxide synthase (NOS) (Bruckdorfer et al., 2005; Dawson et al., 1994). There are four members of the NOS family:

1. neuronal NOS (nNOS)
2. endothelial NOS (eNOS)
3. inducible NOS (iNOS)
4. mitochondrial NOS (mNOS)

The last of these is an isoform of nNOS present in the inner mitochondrial membrane. nNOS and eNOS are Ca^{+} calmodulin-dependent, constitutively expressed in mammalian cells to generate increments of NO, lasting a few minutes. In contrast, iNOS is a Ca^{+} calmodulin-independent enzyme and its regulation depends on the induction of the enzyme by immune activated cytokines (Alderton et al., 2001).

NO binds and interacts allosterically to the haem-containing soluble guanylyl cyclase (sGC) to increase the synthesis of cyclic guanosine-3', 5-monophosphate (cGMP) from guanosine triphosphate (GTP). This, in turn, promotes cGMP-dependent responses (Bruckdorfer et al., 2005), including the activation of cGMP dependent kinases (PKG), cGMP gated ion channels and cGMP-regulated PDEs (Friebe et al., 2003).

Initial research has focussed on the function of NO in the endothelium, where the eNOS complex is expressed as a constitutively active enzyme (Palmer et al., 1987). However, it soon become apparent that NO had functions outside the vasculature and that it also functioned as a neurotransmitter in both central and peripheral nervous systems. In spite of the abundance of evidence for the latter, NO remains an unusual neurotransmitter and because of its short half-life it is difficult to measure quantitatively. This is mainly due to the fact that NO is a labile free gas that is not stored in synaptic vesicles, does not undergo reversible interaction with receptors and its activation is not terminated by presynaptic re-uptake or enzymatic degradation. Moreover, NO simply diffuses from nerve terminals, as opposed to the exocytosis by which conventional neurotransmitters are released (Dawson and Snyder., 1994). The range of NO diffusion implies that structures in the vicinity of the NO producing cell, both neuronal and non-neuronal, are acting as an effector of the neurotransmitter (Esplugues, 2002). In the CNS, NO is formed following the activation of glutamate receptors, mainly of the N-methyl-D-aspartate (NMDA) subtype. After this activation, Ca^{2+} is transiently increased in the cytosol and forms a complex with calmodulin that binds to and activates constitutively active nNOS (Moncada et al., 2006) (see figure 2 for detailed description).

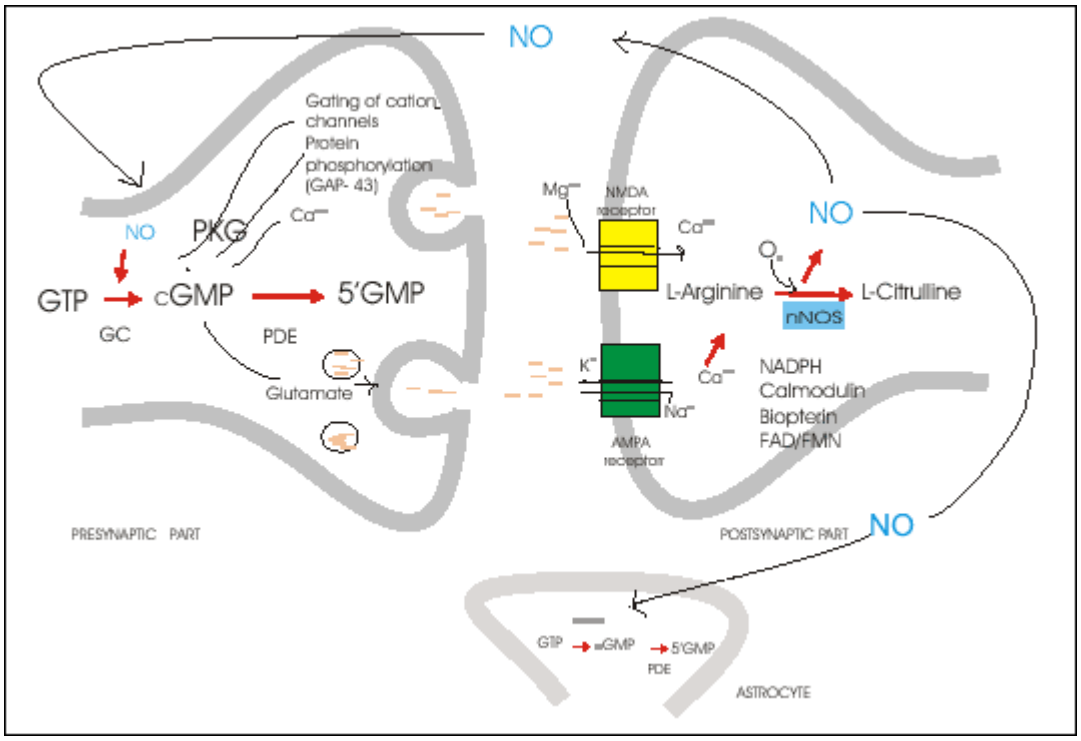

Figure 2 Cyclic nucleotide signalling. Extracellular signals (neurotransmitters, hormones, olfactive and luminous signals) are transferred via membrane-bound transducer molecules, such as G protein-couples receptors, to stimulate the formation of intracellular second messengers, such as the cyclic nucleotides cAMP and cGMP. The formation of cAMP from ATP is catalysed by adenyl cyclase (AC) and of cGMP from GTP by guanylyl cyclase (GC), whereas their inactivation to 5'AMP and 5'GMP, respectively, is mediated by phosphodiesterases (PDEs). Second messengers activate effector proteins such as ion channels or kinases (e.g. protein kinase A (PKA) and protein kinase G (PKG)). The kinases, in turn, phosphorylate other enzymes or transcription factors such as CREB in the nucleus (Puzzo et al., 2008).

NO has been demonstrated to play an important role in several brain functions and or dysfunction, including the regulation of neural excitability, synaptic plasticity, long term potentiation and long term depression (Guimarães et al., 2005). Furthermore, NO has been implicated in other brain functions, such as nociception, learning and memory, anxiety, seizure, feeding, drinking (Uzbay et al., 2001), and regulation of the release and uptake of neurotransmitters such as dopamine, GABA, serotonin and glutamate (Esplugues, 2002). As a result recent studies have found NO to be involved in a number of psychiatric and neurological disorders, including, major depression (Harvey, 1996; Dhir & Kulkarni., 2011), schizophrenia (Harvey., 1996), anxiety disorders (Harvey., 1996), Alzheimers disease and Parkinsons disease (Duncan & Heales., 2005).

2.2.2 Effects of NO on neuronal excitability and firing

NO initiates changes in neuronal function via several routes. NO-mediated activation of soluble guanylyl cyclase, followed by increased levels of cGMP, and the consequent activation of cGMP-dependent protein kinases have been suggested to constitute the main signal transduction pathway of NO (Smolenski et al., 1998). In fact, neuronal cGMP synthesis modulates the function of various cellular functions in the central nervous system, depending on the location and types of neurons involved, including the following:

- Voltage dependent $I_{K\,(Ca)}$ outward current represents the main NO regulated function in hippocampal neurons (Erdemli and Krnjevic, 1995), whereas the inward conductance of a non-selective cation channel is the main target in the locus coeruleus (Pineda et al., 1996).
- NO, through increased cGMP synthesis, reduces the function of $GABA_A$ receptors in the cerebellum (Robello et al., 1996) and that of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors in forebrain, cerebellum and in the horizontal cells of the retina (McMahon and Ponomareva, 1996).

2.2.2.1 Role of nitric oxide in long-term potentiation (LTP) and long-term depression (LTD)

Long-term depression (**LTD**) is an activity-dependent reduction in the efficacy of neuronal synapses lasting hours or longer. LTD has been best characterised in the hippocampus and cerebellum. LTD is thought to result mainly from a decrease in postsynaptic receptor density, although a decrease in presynaptic neurotransmitter release may also play a role. However, it is likely that other plasticity mechanisms play a role as well. Hippocampal LTD may be important for the clearing of old memory traces (Nicholls et al., 2008; Malleret et al 2010). Hippocampal/cortical LTD can be dependent on ionotropic and metabotropic glutamate receptors (Paradiso et al., 2007).

LTD is one of several processes that serve to selectively weaken specific synapses in order to make constructive use of synaptic strengthening caused by LTP. This is necessary because, if allowed to continue increasing in strength, synapses would ultimately reach a ceiling level of efficiency, which would inhibit the encoding of new information (Purves., 2008).

Long-term potentiation (**LTP**) is a long-lasting enhancement in signal transmission between two neurons that results from stimulating them at the same time (Paradiso., 2007). It is one of several phenomena underlying synaptic plasticity. As memories are thought to be encoded by modification of synaptic strength (Bliss & Collingridge., 1993), LTP is widely considered one of the major cellular mechanisms that underlies learning and memory (Paradiso et al., 2007; Bliss & Collingridge., 1993).

At a cellular level, LTP enhances synaptic transmission. It improves the ability of two neurons, one presynaptic and the other postsynaptic, to communicate with one another across a synapse. The precise molecular mechanisms for this enhancement of transmission have not been fully established, in part because LTP is governed by multiple mechanisms that vary by species and brain region. In the most well understood form of LTP, enhanced communication is predominantly carried out by improving the postsynaptic cell's sensitivity to signals received from the presynaptic cell (Malenka & Bear 2004). These signals, in the form of neurotransmitter molecules, are received by neurotransmitter receptors present on the surface of the postsynaptic cell. LTP improves the postsynaptic cell's sensitivity to neurotransmitter in large part by increasing the activity of existing receptors and by increasing the number of receptors on the postsynaptic cell surface (Malenka & Bear 2004).

NO originating from the postsynaptic cells is believed to diffuse through the extracellular space and to induce cGMP formation in the presynaptic nerve ending, thus modulating cellular function leading to LTP. NO is involved in activity-dependent synaptic plasticity in several other brain regions which also possess key roles in cognitive, emotional and behavioural functions. In the cerebellum, NO also seems to be involved in LTD via cGMP synthesis (Paradiso et al., 2007).

Transmission efficacy within neuronal synapses can be modulated in an activity-dependent fashion, such as seen with LTP and LTD. These types of neuronal plasticity were first demonstrated in the hippocampus (Lomo, 1966), where NO acts as a retrograde messenger to influence synaptic transmission in the presynaptic cell and to promote synaptic plasticity.

Consistent with this hypothesis, it has been observed that hippocampal LTP is eliminated (Doyle et al., 1996) or partially blocked (Iga et al., 1993) by NOS inhibitors. The blocking of LTP by NOS inhibitors and LTP facilitation by NO donors have also been observed in the layer V of the auditory neocortex (Wakatsuki et al., 1998) and the medial amygdaloid nucleus (Abe et al., 1996). LTP inhibition is also achieved by injecting NOS inhibitors into the postsynaptic, but not the presynaptic cell (Schuman and Madison, 1991). NO donors, however, potentiate field excitatory postsynaptic potential (fEPSPs) (Bon et al., 1992), increase cGMP mainly in the presynaptic neuronal elements (Boulton et al., 1994) and facilitate LTP when injected into the presynaptic cell (Arancio et al., 1995).

2.2.2.2 The effect of nitric oxide on learning and memory

The effectiveness of the NOS inhibitors in the above-mentioned tasks suggests that NO is involved in the formation of several types of long-term memory. In these tasks, NO preferentially affects memory acquisition, a process which is thought to be related to the induction phase of LTP. In addition, it has been found that, during avoidance learning or exposure to spatial novelty, NOS immuno-reactivity greatly increases in hippocampus, caudate putamen and somatosensory cortex (Bernabeu et al., 1995). In a water-rewarded spatial alternation task, expression of NOS increases in dentate gyrus and frontal cortex (Zhang et al., 1998). These findings indicate that memory acquisition may require up-regulation of NOS activity.

N-methyl-D-aspartate (NMDA) glutamate receptors have long been known to play a major role in learning and memory, also involved in the acquisition (Kim et al., 1991), consolidation (Roesler et al., 1998), reconsolidation (Lee et al., 2006) and extinction (Szapiro et al., 2003) of fear memory. Because of this, approaches targeting the NMDA receptor have been among the first to be used in combination protocols seeking to modulate the effects of psychotherapy. Particularly, D-cycloserine, a partial agonist of the receptor's co-activatory glycine-binding site, has been reported to improve the effects of psychotherapy for various anxiety disorders, including phobias (Ressler et al., 2004), social anxiety (Hoffmann et al., 2006), OCD and panic disorder (Kushner et al., 2007).

2.2.2.3 The effect of nitric oxide on anxiety-related behaviour

In the literature there are a number of reports implicating the NO/cGMP pathway in the modulation of anxiety related behaviours and that this particular pathway is widely spread in the limbic system, supporting the involvement of NO as a modulator of anxiety (Calixto et al., 2009).

Beijamini and co-workers 2006 reported that NO synthase containing neurons in brain areas related to fear and anxiety are activated when rodents is exposed to the elevated plus maze. Depending on the drug treatment, as well as the animal and methodology used to investigate anxiety related behaviour, different results have been obtained with the acute inhibition of NOS using a variety of antagonists showing either anxiolytic- or anxiogenic-like behaviour (Czech et al., 2003; Faria et al., 1997; Vale et al, 1998; Volke et al., 1995). In addition, sildenafil, a selective PDE5 inhibitor which prevent cGMP degradation (thus increasing cGMP levels) exerts an anxiogenic-like effect in the elevated plus maze following acute administration in mice while methylene blue (MB), which inhibits cGMP production via inhibition of guanylyl cyclase, is anxiolytic (Kurt et al., 2004). This emphasizes the central role for this signalling pathway in anxiety.

Several studies exploring the involvement of hippocampal NO in experimental anxiety have reported conflicting results, especially when using the elevated plus maze (Calixto et al 2009). The non-selective NOS inhibitors N-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine-methyl-ester (L-NAME) produced an anxiogenic-like effect in the elevated plus after being injected into the CA1 region of the hippocampus (Monzon et al., 2001 & Roohbakhsh et al., 2007), while other studies using this compound have found it to induce an anxiolytic-like effect when injected into the dentate gyrus of the dorsal hippocampus (Echeverry et al., 2004). However, the administration of 7-nitroindazole (7-NI) a selective nNOS inhibitor, as well as NO donors S-nitro-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP), directly into the ventral hippocampus did not have any effect on anxiety-like behaviour (Ferreira et al., 1999). These data emphasize the variation in findings that may be expected when considering the effects of NO modulation on anxiety levels, and that further study is needed to tease out exactly how, and in which brain region, NO modulation may culminate in a reproducible anxiolytic or anxiogenic response.

2.2.3 Phosphodiesterase 5 inhibitors

Sildenafil was the first oral drug to be approved by the FDA for the treatment of male erectile dysfunction (Nehra et al., 2001). Initial research with this compound was aimed to develop novel drug strategies for the treatment of hypertension and angina pectoris. However, some patients reported an unexpected pharmacological effect in phase I clinical trials, namely restoration of sexual function, in particular the enhancement of physiological penile erection (Jackson et al., 2005).

Sildenafil citrate is a water soluble aromatic compound and its pharmacological effect in the corpus cavernosum is mediated via the selective inhibition of PDE5, thereby bolstering NO-cGMP signalling and resulting in smooth muscle relaxation (vasodilatation) and hence enhanced erectile function (Uthayathas et al., 2007).

Sildenafil is a potent, selective and reversible inhibitor of PDE5 (Uthayathas et al., 2007), thereby effectively blocking the hydrolysis of cGMP and resulting in the accumulation of cGMP, with a consequent enhancement of the biological activity of NO (Goldenberg, 1998).

Since PDE5 is also expressed in the lung, heart and brain (Giordano et al., 2001), it also exerts pharmacological effects via NO-cGMP signalling in these target organs. As such, sildenafil has also been registered for the treatment of pulmonary hypertension. Although various CNS-related effects have been reported in humans and rodents (see below), the drug is not currently indicated or registered for neurological and/or neuro-psychiatric illnesses.

2.2.3.1 The effect of sildenafil on anxiety

The presence of NOS in brain regions associated with anxiety, especially the hypothalamus, amygdala and hippocampus, has provided supportive evidence that NO is implicated in the control of anxiety (Vincent, 1994). Earlier in this dissertation it was described how manipulation of the NO-cGMP system may have important anxiolytic effects.

Clinical studies have reported that some of sildenafil's most common adverse effects include headaches, light headedness and dizziness, all an indication of CNS effects.

Sildenafil crosses the blood brain barrier were it causes the accumulation of cGMP (Uthayathas et al., 2007a). The NO hypothesis (Harvey 1996), and subsequent animal studies (Harkin et al., 1999; Heiberg et al 2002; Harvey et al 2006) that have found antidepressant effects following NOS-cGMP inhibition, as well as evidence for elevated levels of NO metabolites in depressed patients (Suzuki et al, 2001), others would indicate that elevating NO and or cGMP may be depressogenic. Thus, sildenafil may well have the potential to be depressogenic, in agreement with some of these earlier observations. However, it is important to note that depression has also been found to be associated with reduced NO levels, while a similar paradox has been described in animal studies (Harkin et al., 1999), highlighting that NO may have a duel role in regulating mood, and that further work in this regard is needed.

The prevalence of depression, anxiety and psychosocial disturbances are high in men with erectile dysfunction. Despite the above allegations, depressed men treated for erectile dysfunction with sildenafil, in general have experienced alleviation of their depressed mood (Uthayathas et al., 2007b). Although this had initially not been attributed to any central activity of sildenafil, but rather to a secondary response to improved sexual function, it is noteworthy that sildenafil did not aggravate their depressed mood, or in some cases precipitate depressive symptoms.

Initial acute studies in mice suggested that sildenafil may exert anxiogenic-like effects. In one study conducted by Volke et al, (2003), it was demonstrated that an acute intraperitoneal administration of sildenafil (1 mg/kg) 30 to 35 min prior to behavioural testing, in combination with the NO precursor molecule *l*-arginine (200 mg/kg) 25 minutes prior to testing, decrease both open arm entries and percentage time spend in the open arms of the elevated plus maze (EPM). From these results it was concluded that the augmentation of the NO-cGMP cascade induces anxiogenic-like effects in male NIH mice (Volke et al., 2003).

Clinical experience indicates that the onset of therapeutic effect of psychotropic drugs is achieved only after 2 to 4 weeks. Therefore, to observe and evaluate anxiolytic or

anxiogenic properties of a drug, even in animal models, the studies need to consider chronic drug administration.

Therefore, Brink et al, (2008) conducted a sub-chronic drug treatment study in rodents to investigate a postulated antidepressant-like activity for the compound. Based on earlier *in vitro* studies where these authors found that sildenafil bolsters muscarinic receptor signalling (Brink et al., 2008), and that increased muscarinic activity is depressogenic (Janowsky et al., 1972) Brink and colleagues (2008) tested sildenafil in the presence and absence of a central active antimuscarinic drug. They therefore treated male Sprague-Dawley rats for 7 days via intraperitoneal administration with 10 mg/kg sildenafil, 1 mg/kg of the centrally active, antimuscarinic drug atropine, or a combination of the two. In this study fluoxetine was used as a positive control. Although sildenafil was without effect on its own, the drug combination demonstrated a significant antidepressant-like effect in the rat forced swim test (FST), comparable to that of fluoxetine. This study suggested that sildenafil may possess antidepressant-like activity, but which is attenuated or lost due to its ability to increase muscarinic receptor signalling capacity (Brink et al., 2008). In follow-up studies, it was demonstrated in Flinders Sensitive Line (FSL) rats, a genetic rat model of depression, that a lower sildenafil concentration of 3 mg/kg for 14 days also yields an antidepressant-like day but without the need to inhibit cholinergic activity, whereas cholinergic inhibition is needed at doses of 10 and 20 mg/kg (Liebenberg et al., 2010a). Furthermore, the structurally unrelated PDE5 inhibitor tadalafil also yielded antidepressant-like activity at a dose of 10 mg/kg after co-administration with atropine, suggesting that its antidepressant-like activity is indeed related to inhibition of PDE5, and not to a unique pharmacological property of sildenafil (Liebenberg et al., 2010b). From the data in the forced-swim test it was also found that at lower doses the antidepressant-like activity of sildenafil resembles that of drugs that enhance adrenergic neurotransmission (enhanced climbing behaviour), whereas higher doses resembles that of drugs that enhance both adrenergic and serotonergic mechanisms (enhanced climbing and swimming behaviour) (Liebenberg et al., 2010a). In yet another follow-up study, it was demonstrated that the acute intracorticoventricular administration of an cGMP analogue in Sprague Dawley rats yield a similar antidepressant-like response to that observed with sildenafil plus atropine, while the

response to both the cGMP analogue and to sildenafil plus atropine can be inhibited by a protein kinase G (PKG) antagonist. These data provide strong evidence for the involvement of cGMP and its stimulation of PKG in the mechanism of action of sildenafil to yield an antidepressant-like response in rodents (Liebenberg et al., 2010 a).

Although these data provide convincing evidence for the antidepressant activity of PDE5 inhibitors, there are no data of the effect of the chronic administration of sildenafil on anxiety-like behaviour in rodents, while results from acute studies seem to suggest possible anxiogenic activity. This question certainly warrants further research.

2.2.3.2 The effect of sildenafil on neurogenesis

Recent studies have demonstrated that neurogenesis occurs not only in developing organisms, but also occurs continuously into adulthood and throughout life (Taupin, 2006). The ongoing process of neurogenesis is thought to be an important mechanism underlying neuronal plasticity, which influences learning and memory, but which generally declines with age and with neurodegenerative disorders (Uthayathas et al., 2007b).

There is evidence suggesting that nNOS may play a key role in neurogenesis, as well as during the differentiation of neuronal cells (Puzzo et al., 2008). It is known that nNOS is transiently expressed in the cerebral cortical plate and in the hippocampus of the embryonic rat brain during the period of peak cortical neurogenesis (Bredt and Snyder, 1994). In addition, NO production is regulated in an activity-dependent manner in the brain during the period of developmental synaptogenesis (Contestabile, 2000). NO may also promote neurogenesis after brain injury, because administration of a NO donor improves neurological functional recovery in young rats after stroke (Zhang et al., 2002). It is known that cGMP concentration reduces with age (Tadei et al., 2001).

It has been suggested that sildenafil may improve the neurological outcome following stroke. Recent studies have demonstrated that the treatment of local cerebral ischemia in rats with sildenafil promotes cell proliferation, increases angiogenesis and synaptogenesis, and enhances brain plasticity in both young and aged rats (Zhang et al., 2002). Therefore,

the use of PDE5 inhibitors may offer a novel approach to the improvement of brain function in the aged population (Uthayathas et al., 2007b).

2.2.3.3 The memory enhancement effects of sildenafil

In recent findings it was suggested that the systemic administration of sildenafil enhances cognition, as illustrated by the attenuation of learning impairment in rodents induced by blocking of muscarinic acetylcholine receptors, in the complex maze (Devan et al., 2006). As a result, sildenafil may offer a new strategy for memory improvement and a novel therapy for the treatment of Alzheimer's disease (Uthayathas et al., 2007b).

2.2.3.4 The pain relieving effects of sildenafil

Pain is a complex process, which involves both the peripheral and central nervous systems. Pain is a self-protecting mechanism which forces the body to move away from danger and, afterwards, to rest the injured part, giving the body a chance to heal itself (Uthayathas et al., 2007b).

A number of researchers have demonstrated the role of the NO/cGMP signalling pathway in the processing of nociception. It has been shown that the local administration of *l*-arginine, a precursor of NO, produces an antinociception in rats with carregeenin -induced hyperalgesia, and that this effect is blocked by NO inhibitors and methylene blue, an inhibitor of sGC (Duarte et al., 1990). Furthermore, it has been demonstrated that a NO donor and NO synthase inhibitors can reduce pain through a spinal mechanism that involves activation of sGC (Sousa et al., 2001). In addition, there are data to suggest that NO is associated, at least in part, with the antinociception activity of benzodiazepines (Talarek, 2002). Therefore, it has been proposed that sildenafil may also modulate nociceptive processes. It is suggested that the antinociceptive effect of sildenafil is due to a local action, because the contralateral administration of the drug is ineffective in reducing flinching. Data also suggest that the inhibition of PDE5 (and therefore the accumulation of cGMP) is in itself sufficient to produce antinociception (Moreland et al., 1999). In addition, recent studies have provided evidence for an interaction between the PDE5 inhibitor sildenafil and morphine (Jain et al., 2003) in the peripheral nervous system. One study demonstrated that

the local administration of either NO synthase inhibitors or sGC inhibitors block the antinociception produced by morphine (Lorenzetti et al., 1996), while a second study indicated that the NOS inhibitor L-NAME blocks the antinociception produced by a combination of morphine and sildenafil. Importantly, the effect of sildenafil is not modulated by the opioid receptor antagonist naloxone, indicating that sildenafil does not act on the opioid receptor. Jain (2003) suggested that the activation of opioid receptors by morphine produces an increase in NO, which in turn activates cGMP to produce antinociception.

In conclusion, from these studies conducted on both humans and rodents it is clear that sildenafil crosses the blood brain barrier to inhibit intracellular PDE5 and thus to increase cGMP, leading to enhanced biological effects in the brain such neuroplasticity, synaptic plasticity, neurogenesis, antinociception and effects on anxiety and mood. Further research is needed to clarify the exact mechanism(s) of action of sildenafil that are responsible for its central effects.

2.3 Animal models of anxiety

The development of animal models has made it possible to investigate brain-behaviour relationships in order to gain a better understanding of normal and abnormal behaviour and its underlying neurobiological processes. As the predictive (the model should be responsive to the same treatments that are effective antidepressants in humans), face (the model should be reasonably similar to the human condition with respect to symptomatology) and construct (the degree to which the animal model mimics the underlying neurobiology of the disease) validity of these models have improved, it has become possible to extrapolate from these animal models reliable correlates of corresponding human behaviour, psychiatric conditions and their underlying neurobiology (Van der Staay, 2006). Particularly, the availability of suitable stress, fear and genetically modified rodent models have assisted significantly to explain the role of a wide range of pharmacological molecules in the CNS relevant to anxiety. In fact, many promising neurobiological targets were derived from pre-clinical animal models that were later eventually successfully confirmed in clinical trials (Garner et al., 2009). "The translation of anxiety phenotypes into testable measures and

models in animal experiments has also allowed the investigation of interactions between genetic and environmental risk factors, and the resultant changes in brain neurobiology that underlie and confer risk for anxious behaviour" (Garner et al., 2009).

2.3.1 Types of animal models

Most animal models of anxiety study the natural behavioural patterns of rodents to develop aetiologically based behavioural tasks (Rodgers, 1997). These animal models include approach-avoidance tasks (Cryan et al., 2005), in which animals are subjected to an aversive environment such as the open-, elevated arms of the elevated plus maze, light arena and open field tests, with anxiety-like behaviour in each case concluded from increased avoidance. The following are also examples of animal models of anxiety: the social interaction tests (File et al., 2003), punished-based conflict procedures (Vogel et al., 1971), defensive burying test (Jacobson, 2007), predator stress (Blanchard et al., 1971) and the examination of ultrasonic vocalisation induced by maternal separation (Sanchez, 2003).

Novel techniques include the use of radio-telementry to asses physiological parameters in real time, such as core body temperature (Adriaan et al., 2007). These models examine behaviour that is practically relevant and can be directly related to human anxiety, thus showing good face validity.

2.3.1.1 Clinical application of animal models

Pre-clinical models have been used to reveal the anxiolytic properties of a range of neurotransmitters and neuropeptide receptor agonists and receptor antagonists and in many cases have predicted efficacy in human clinical samples. Such an ability to predict human response from an animal model, is referred to as good predictive validity (Garner et al., 2009).

Studies in genetically modified mice have investigated the outcomes of manipulating specific genes, and a few mouse species in which mutations in specific genes encoding for the expression of proteins involved in neurotransmission, including knock-out, knock-in and transgenic mice, have all shown transformed anxiety-related behaviour (Holmes., 2001;

Gross & Hen., 2004; Finn., 2003). "Genetic factors can exert their influence during brain development or in adulthood by modulating neurotransmission. Investigations of the genes involved in the anxiety phenotype have predominantly focused on animal models that target receptor genes, receptor subunits of specific neurotransmitters, with fewer studies examining transporters, neuropeptides and binding proteins, while there have also been very few studies of genes involved in the synthesis of specific neurotransmitters" (Garner et al., 2009). Even though research on targeted genes has yet to contribute directly and significantly to improve the pharmacological treatment of anxiety disorders, it has already allowed researchers to explain epigenetic factors that can modify gene expression through varied mechanisms and confer an increased risk for the development of an anxiety disorder (Garner et al., 2009).

2.3.1.2 Flinders sensitive line (FSL) and Flinders resistant line rat

The Flinders Line rat strains were established by selectively breeding for differential responses, such the hypothermic effects following treatment with the anticholinesterase agent diisopropyl fluorophosphate (DFP). Two lines were bred, namely the Flinders Sensitive Line (FSL) rats that were more sensitive to the hypothermic effects of DFP, and the Flinders Resistant Line (FRL) rats that acted as dominant negative controls and were not more resistant than an outbred control (Overstreet et al., 1979). Early studies ruled out pharmacokinetic differences in the catabolism of acetylcholine to underlie the cholinergic supersensitivity of FSL rats, by showing similar degrees of inhibition of brain acetylcholinesterase activity (Overstreet et al., 1979). The FSL rats were also more sensitive to muscarinic acetylcholine receptor agonists (Overstreet, et al., 1982; Schiller et al., 1992) and exhibited increased muscarinic receptor binding sites in the striatum and hippocampus, but not in the cerebral cortex (Overstreet et al., 1984; Pepe et al., 1988). Other work suggested a dissociation between the sensitivity to cholinergic agonists and the muscarinic acetylcholine receptor elevations, because the increased hypothermic responses to oxotremorine were observed very early in development (10 days of age), but differences in muscarinic binding in the hypothalamus were not observed until the rats reached adulthood (60 days of age) (Daws and Overstreet, 1999). Increased effort was then put in place in order to explore the neurobiological differences between FSL and FRL rats.

The FSL rats were also shown to exhibit other behavioural characteristics which were not necessarily related to altered cholinergic neurotransmission. FSL rats showed a robustly enhanced immobility relative to FRL rats in the forced swim test, a screening test for antidepressant action), which correlated with their enhanced hypothermic response to 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT), but not to oxotremorine (Overstreet et al., 1994). Although the FSL rat line exhibit increases in muscarinic binding in the striatum hippocampus and hypothalamus (Daws and Overstreet., 1999), there seems to be dissociation between the receptor differences and the hypothermic response to cholinergic agents. There are studies showing that severely depressed patients are also more sensitive to cholinergic agents (Janowsky et al., 1994), but binding studies in post-mortem tissues have failed to detect any differences in muscarinic receptors (Overstreet, 2002). In this

regard, following chronic treatment with a dose of DFP that is known to down-regulate muscarinic receptor number, there was also no change in the immobility time in FSL rats (Schiller et al., 1992). This suggested that some of the key behavioural features of FSL rats cannot be explained by differences in cholinergic function.

Differences in dopaminergic function are also unlikely to provide a complete explanation of the behavioural differences between the FSL and FRL rats. When they were treated with the dopamine agonist apomorphine, the FSL and FRL rats exhibited differential responses that varied with the parameter tested: FSL rats showed an increased hypothermic response but a reduced stereotypy response (Crocker and Overstreet., 1991), and there were no differences in dopamine receptor binding in these animals (Crocker and Overstreet., 1991). In another study it was reported that FSL rats have greater levels of dopamine and norepinephrine in several brain areas and that these levels were normalized following chronic treatment with the tricyclic antidepressant, desipramine (Zangen et al., 1998). In addition, FSL rats exhibit a reduced release of dopamine in the prefrontal cortex after exposure to stress, as compared to FRL control rats (Yadid et al., 2001). While these findings suggest that there may be a malfunction of the dopaminergic system in FSL rats, the underlying neurobiological basis of this malfunction is still not clear.

Initial data suggested that serotonergic neurotransmission may also be dysfunctional in the FSL rats (Wallis et al 1988), with reports finding an increased responses to 8-OH-DPAT (Overstreet, 2002), a serotonergic 5HT1A receptor agonist. Moreover, cortical binding of ^{3}H-8-OH-DPAT is higher in FSL than in FRL control rats (Schiller et al., 1992), suggesting higher 5-HT_{1A} receptor expression in FSL rats. Also, the levels of 5-HT and 5-HIAA in limbic regions are higher in FSL rats than in the FRL control rats (Zangen et al., 1997). The ability of 5-HT to induce dopamine release was restored in FSL rats treated chronically with desipramine or paroxetine. Thus, both the exaggerated immobility time and the elevated tissue levels of catecholamines and indoleamines are normalized by chronic antidepressant treatment. It should be stressed that the biochemical measures in the outbred rats (FRL) did not change in any brain region after chronic antidepressant treatment (Overstreet, 2002).

However, there are important differences in the observed changes in serotonergic function in the FSL rat as opposed to that found in depressed humans. In particular, FSL rats present with a profound (two times greater) hypothermic response to 8-OH-DPAT than control FRL rats (Overstreet et al., 1994), whereas depressed patients exhibit a blunted hypothermic and hormonal responses to 5-HT_{1A} receptor agonists (Lesch, 1991). However, the increased cortical 5-HT_{1A} receptor binding in FSL rats correlate with a human study showing increased 5-HT_{1A} receptor binding in the frontal cortex after suicide (Arrango et al., 1995).

Very important for the current study, a recent study has demonstrated that chronic mild stress induces enhanced expression of key molecules involved in glutamate-NMDA-NO signalling in FSL, but not FRL rats (Wegener et al., 2010). This study suggests that the glutamate/NO/cGMP pathway may also be dysfunctional in FSL rats, possibly confirming a role for this pathway in increased susceptibility to developing depressive-like behaviours. This finding furthermore supports a role for this pathway in anxiety-related disorders.

2.4 Synopsis

The current treatments of anxiety disorders focus on the role of enhanced serotonergic and noradrenergic neurotransmission and the altered function of the GABA-benzodiazepine chloride ionophore complex. However, response rates of 40 - 70% and remission rates of 20 - 47% are clear indications that current treatment regimes are far from optimal. Current treatments focus on relieving the symptoms of anxiety disorders, rather than reversing the underlying abnormalities in neuroplasticity and neurodevelopment that may be driving the progression to developing an anxiety disorder.

The glutamate/NO/cGMP pathway has been demonstrated to play a role in neuroplasticity and the neurobiology of CNS-related disorders, especially anxiety related disorders. Sildenafil, a selective PDE5 inhibitor crosses the blood brain barrier, thus increasing cGMP levels in the brain leading to enhanced biological effects such as neuroplasticity, synaptic plasticity and mood and anxiety regulation.

Recent studies in rodents have demonstrated antidepressant-like activity following chronic co-administration of PDE5 inhibitors, including sildenafil, with atropine. Acute potentiation of the NO-cGMP pathway, however, was reported to exert anxiogenic activity in mice. It is therefore unclear what the effect of chronic monotherapy and co-administration of sildenafil and atropine would be on anxiety-like behaviour in rodents.

Manuscript Chapter 3

In this chapter, a manuscript titled

"The effects of acute versus chronic treatment with sildenafil on anxiety-like behaviour in rats"

is presented. The paper will be submitted to **Behavioural Pharmacology** as a full-length research report, and prepared according to the specific *Instructions to the Author* for this journal (provided in Addendum D). The references for this manuscript are provided at the end of this chapter.

I, Francois Naudé Slabbert hereby declare that all experimental work, planning, literature research, data capturing and writing of this manuscript was conducted by myself.

As co-authors, Prof CB Brink and Prof BH Harvey we confirm that the above statement by Mr FN Slabbert is true and correct.

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Prof CB Brink Prof BH Harvey

Title Page

Title of article

The effects of acute versus chronic treatment with sildenafil with and without antimuscarinic receptor blockade, on anxiety-like behaviour in rats.

Short title: Anxiolytic activity of sildenafil.

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Abstract

The neurobiology of anxiety-related disorders has been associated with impaired neuroplasticity, in which the glutamate/nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway plays a key role. Previous studies have demonstrated that chronic co-administration of a phosphodiesterase type 5 (PDE5) inhibitor (sildenafil or tadalafil) and the anticholinergic drug atropine exerts anti-depressant-like effects in rodents. However, acute treatment with sildenafil is anxiogenic. We therefore evaluated the anxiolytic-like effects of acute or chronic sildenafil treatment in two rodent anxiety tests, with and without atropine, compared to fluoxetine in male Flinders Sensitive Line (FSL) rats known to present with a heightened stress-response. FSL rats received vehicle control, fluoxetine (15 mg/kg), atropine (1 mg/kg), sildenafil (10 mg/kg) or sildenafil plus atropine via intra-peritoneal administration, either acutely 30 min prior to testing, or daily for 14 days. Flinders Resistant Line (FRL) rats (negative control line) received only vehicle. Thereafter anxiety-like behaviour was evaluated in the elevated plus maze (EPM) (both chronic and acute) and social interaction test (only acute). Drug-naïve FSL rats were clearly anxious compared to their FRL control. Acute administration of fluoxetine or atropine separately were anxiogenic while sildenafil, with or without atropine, did not significantly alter anxiety-like behaviour. Chronic administration of sildenafil alone

(45.7 ± 1.6% vs. 20.8% ± 2.6%, p<0.001) or with atropine (59.6% ± 1.4% vs. 20.8% ± 2.6%, p<0.001) increased the time spent in the open arms of the EPM relative to control, and was comparable to that of fluoxetine. In the social interaction test the acute administration of 15 mg/kg fluoxetine to FSL rats caused a small, yet statistically significant reduction in the time spent in social interaction, relative to vehicle control (49.80 ± 5.89 vs. 69.40 ± 5.32, P<0.01). Sildenafil, alone or in combination with atropine, therefore exerts anxiolytic-like activity in a chronic but not an acute treatment regime, thus congruent with the clinical response to antidepressants in treating anxiety.

Keywords

Sildenafil, elevated plus maze, social interaction, Flinders sensitive line rat, anxiolytic, nitric oxide, cGMP, phosphodiesterase type 5

Introduction

Anxiety is a natural response triggered by fearful or stressful situations in humans. It becomes a pathological disorder when its manifestation is excessive and uncontrollable, caused by no specific external stimulus and manifesting as a number of physical and affective symptoms, including changes in behaviour and cognition (American Psychiatric Association). As a group, anxiety-related disorders have been estimated to be the most common of psychiatric disorders (Dell' Osso et al., 2010), including severely debilitating diseases such as major depression, general anxiety disorder, obsessive compulsive disorder, panic disorder, posttraumatic stress disorder and social anxiety disorder (Garner et al., 2009).

The current options for the drug treatment of anxiety disorders include different classes of antidepressants, such as the tricyclic antidepressants (TAD), serotonin reuptake inhibitors (SSRIs), and serotonin and norepinephrin reuptake inhibitors (SNRIs), as well as benzodiazepines, buspirone, anticonvulsants and antipsychotic drugs. These drug treatments primarily treat the symptoms, rather than treating the underlying neuropathology or reversing compromised neuroplasticity.

The glutamate/NO/cGMP pathway has been demonstrated to play a role in neuroplasticity and the neurobiology of CNS-related disorders (Puzzo et al., 2008). Inhibitors of the phosphodiesterase type 5 (PDE5) enzyme, such as sildenafil, promote cGMP accumulation via this pathway, and are already in clinical use for the treatment of peripheral disorders such as erectile dysfunction and pulmonary hypertension (Uthayathas et al., 2007b). Sildenafil as well

tadalafil have recently been shown to exert central psychotropic effects in rodents (Brink et al., 2008, Liebenberg et al., 2010a, and Liebenberg et al 2010b) and humans (Kennedy et al., 2011).

Some of sildenafil's most common adverse effects include headaches, light headedness and dizziness, which are all an indication of CNS activity. Sildenafil crosses the blood brain barrier where it causes the accumulation of cGMP (Uthayathas et al., 2007a). In addition, sildenafil has been shown to exert pro-cholinergic effects (Devan et al., 2004; Patil et al., 2004; Brink et al., 2008). Although not demonstrated as a group effect with PDE5 inhibitors, pro-cholinergic activity has been associated with the amplification of depressive symptoms (Janowsky et al., 1972; Janowsky et al., 1994), while centrally active muscarinic agonists have found value in treating resistant depression (Veena et al., 2010).

In a few cases men were reported to have developed anxiety following the administration of sildenafil, and a number of case reports have found evidence suggesting neurological and emotional disturbances after taking sildenafil, including aggressive behaviour such as rape and assault (Milman & Arnold., 2002). The prevalence of depression, anxiety and psychosocial disturbances are high in men with erectile dysfunction. Interestingly, the administration of sildenafil in these men improved, rather than aggravated, their self-reported mood status (Uthayathas et al., 2007). The latter data suggests that sildenafil may possess inherent antidepressant activity, which putatively counteracts its pro-cholinergic (depressiogenic) activity.

A recent study demonstrated that sub-chronic administration of sildenafil induces an antidepressant-like response in the rat forced swim test (FST), but only when co-administered with a centrally acting muscarinic acetylcholine receptor antagonist (Brink et al., 2008; 2010a and b). These results suggest that sildenafil possesses antidepressant activity, but which is attenuated by a simultaneous increase in cholinergic neurotransmission. Therefore, the antidepressant activity of sildenafil may only become apparent following the uncoupling of this counteracting depressogenic mechanism.

A number of studies investigating the acute effects of PDE5 inhibitors on anxiety have shown that sildenafil increases anxiety-like behaviour in mice in the elevated plus maze (Volke et al., 2003b; Kurt et al., 2004). In more recent studies the chronic treatment with sildenafil reduced anxiety-like behaviour of rats in the open field test (Solis et al., 2008) as well as in the social interaction test (Liebenberg 2009). Of note, other classes of antidepressants used for the treatment of anxiety disorders, for example the SSRIs, also enhance anxiety following acute administration, whereas chronic treatment ultimately results in an anxiolytic response (Harvey, 1997). Therefore, it appears that the anxiolytic activity of these treatments may be dependent on inducing long-term adaptive changes. In the case of SSRIs, such long-term neuroplastic changes are thought to involve the adaptive down-regulation of serotonergic receptors that predict subsequent anxiolytic and antidepressant activity (Harvey, 1997).

Of special note is that the pro-cholinergic activity of sildenafil is dose-dependent since a 3 mg/kg dose displays antidepressant-like activity in the absence of muscarinic inhibition in depressiogenic Flinders Sensitive Line rats (Liebenberg et al., 2010a). Since depression and

anxiety-related disorders are highly co-morbid, and that both disorders respond to chronic but not acute SSRI treatment, this argues strongly that given the antidepressant-related effects of sildenafil, as well as its cholinergic dependency (Liebenberg et al 2010a), these same properties may be extended to its pharmacological effects on anxiety. Since there is now controversy surrounding the anxiogenic versus anxiolytic actions of these drugs, and whether these have anything to do with inherent cholinergic status as well as duration of treatment, it was incumbent on us to further investigate how acute or chronic sildenafil treatment modifies anxiety-like behaviour.

The FSL rats are a well defined and validated genetic animal model of depression (Overstreet et al. 2005). This animal model of depression has several characteristics that correlate directly with clinical depression in humans. FSL rats are highly stress responsive, with increased levels of anxiety, while they only yield an antidepressive-like response in the force swim test (FST) following chronic treatment with an antidepressant but not following acute treatment (Overstreet et al., 2005).

The underlying neurobiology of the FSL rats is consistent with the current hypothesis of depression, and in particular involves disturbed glutamatergic, serotonergic and cholinergic functioning (Yadid et al., 2001; Wegener et al., 2010), all of which are implicated in the neurobiology of depression (Harvey, 1997; Harvey et al, 2006). The involvement of nitric oxide in mood disorders is supported by evidence that both the serotonergic (Harvey et al., 2006) and cholinergic systems (Brink et al., 2008) interact with the NO cascade. Thus, 5-HT active drugs

(i.e. SSRI's) and NO-cGMP modulators (i.e. sildenafil) may similarly have anxiolytic effects in animals after chronic but not acute treatment.

In the current study we investigated the effect of acute (once) versus chronic (14 days) administration of sildenafil, atropine or sildenafil + atropine in stress-sensitive rats, as compared to vehicle control and to fluoxetine (positive control) treated animals, on anxiety-like behaviour as determined in the social interaction test and elevated plus maze (Pellow et al., 1985).

Methods

Animals: Male Flinders sensitive line (FSL), and a corresponding negative control line, the Flinders resistant line (FRL) rats, all weighing 300 ± 10 g on the day of behavioural testing, were housed under conditions of constant temperature (22 °C) and humidity (50%) with a 12:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum. All animal procedures were approved by the Ethics Committee of the North-West University, ethics approval no. NWU – 00039 – 09 – S5, comply with national legislation and were in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals.

Drug treatment. Drugs were obtained from reputable sources, including fluoxetine (Aspen, Port Elizabeth, South Africa), atropine (Merck, Darmstadt, Germany) and sildenafil (10 mg/kg, a kind gift from Pfizer Global Research and Development, Kent, United Kingdom). All drugs were dissolved in 0.5 ml/injection vehicle containing 5% dimethylsulfoxide (DMSO) in saline. Both FSL and FRL rats received drugs via intraperitoneal administration at doses previously reported as follows: vehicle control, fluoxetine 15 mg/kg (Reneric et al., 2002), atropine 1 mg/kg (Brink et al., 2007), sildenafil 10 mg/kg (Brink et al., 2007) or sildenafil plus atropine, either acutely 30 min prior to testing (acutely), or daily for 14 days (chronically), with at least 15 rats per treatment group. Infection following chronic intraperitoneal administrations was limited by following sound protocol with sterile needles and by altering the injection site daily. For the chronic drug administration, the last dose was given 12 hours before the behavioural experiments.

Behavioural testing. Behavioural testing was performed one to five hours after the start of the dark cycle. Prior to testing all the animals were moved in their home cages to the experimental room and allowed to acclimatise for 1 hour before the start of the experiments. All the acutely treated animals were subjected to the social interaction tests and the elevated plus maze, whereas chronically treated animals were subjected only to the elevated plus maze.

The social interaction test was conducted after an acclimatization period of an hour. Two rats of the same treatment group and similar body weight was placed in the centre square of the square test arena (1 m × 1 m, marked with lines to render sixteen 25 cm × 25 cm blocks). The following actions were recorded as social interaction: grooming, licking, sniffing and crawling over or under one another. Social interaction was recorded with a digital video camera over a period of 5 minutes, individually scored for each rat and expressed as the percentage time spent in social interaction.

After the social interaction test the animal was left to acclimatise for one hour before the start of the elevated plus maze.

The EPM apparatus consists of a plus-shaped platform of 1 m x 1 m, elevated 50 cm above the floor level, with two opposing 10 cm wide open arms (with a 5 mm ledge to prevent slipping and falling) and two opposing closed arms (30 cm high opaque side-walls) placed at right angles to one another. The maze was placed in an isolated room. A 40 W blue lamp was positioned 1 m above the maze and a 60 W desk lamp pointed away from the maze toward one wall. A digital video camera was positioned directly above the maze. After acclimatising to the room

for 1 hr, the rats were placed individually in the centre square of the EPM facing the open arm and allowed to explore the maze for 5 min under continuous video surveillance. After the test, each rat was immediately returned to its home cage and the maze wiped clean with methanol to eliminate any odour. During scoring of the test, an arm entry was defined as the entry of all four paws into the arm.

Statistical analysis. GraphPad Prism® version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used to process the experimental data. All data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test. Data are expressed as the mean ± S.E.M. and a value of $P<0.05$ was considered to be statistically significant. The Student's t-test was used to compare two groups. Behavioural experiments from chronic studies were performed in triplicate, each with five rats/group (i.e. 15 rats in total per treatment group), whereas acute studies were performed using five rats per treatment group.

Results

Acute study: Social interaction:

Figure 3-1A demonstrates a significant decrease in the amount of time spent in social interaction in acute vehicle-treated FSL rats, compared to correspondingly treated FRL rats (69.40 ± 5.32 vs. 121.00 ± 9.70, $P<0.001$). The acute administration of 15 mg/kg fluoxetine to FSL rats 35 minutes prior to testing caused a small, yet statistically significant reduction in the time spent in social interaction, relative to vehicle control (49.80 ± 5.89 vs. 69.40 ± 5.32, $P<0.01$).

In figure 3-1B it can be seen that the acute administration of 1 mg/kg atropine to FSL rats significantly increased the time spent in social interaction, relative to vehicle control (174.40 ± 11.08 vs. 69.40 ± 5.32, $P<0.001$), whereas neither 10 mg/kg sildenafil alone nor sildenafil plus atropine induced any change in the time spent in social interaction. No significant difference in time spent in social interaction was observed with acute sildenafil- and sildenafil plus atropine-treated FSL rats, as compared to the corresponding vehicle-treated FSL rats.

From figure 3-1C it can be seen that the number of line crossings of vehicle-treated FSL rats are significantly lower than that of FRL rats (23.20 ± 3.70 vs. 72.60 ± 12.99, $P<0.001$). No significant difference in the line crossings were observed following the acute administration of fluoxetine in FSL rats, as compared to vehicle-treated FSL rats.

From the data presented in figure 3-1D it can be seen that neither the acute administration of 1 mg/kg atropine, nor of 10 mg/kg sildenafil alone in FSL rats altered the number of line crossings

relative to vehicle control, whereas a small, yet statistically significant increase in line crossings is observed following the acute co-administration of sildenafil plus atropine, relative to vehicle control (30.8 ± 5.07 vs. 23.20 ± 3.70, $P<0.05$). This increase was also statistically significant relative to both atropine alone (30.80 ± 5.07 vs. 17.20 ± 2.58, $P<0.001$) or sildenafil alone (30.80 ± 5.07 vs. 18.60 ± 2.96, $P<0.001$).

Acute study: EPM

Figure 3-2A shows a significant decrease in the percentage of time spent in the open arms of the EPM, when acute vehicle-treated FRL rats are compared to corresponding FSL rats (16.76 ±4.20 vs. 27.19 ± 4.52, $P<0.01$). While there was a trend for the acute administration of 15 mg/kg fluoxetine in FSL rats to reduce the percentage of time spent in the open arms, this did not reach statistical significance. Similarly, it can be seen from figure 3-2B that none of the acute treatments with sildenafil, atropine or sildenafil plus atropine induced any significant changes in the percentage of time spent in the open arms of the EPM. A small but significant difference between the sildenafil alone and sildenafil plus atropine co-administration group could be demonstrated (22.12 ± 7.35 vs. 10.20 ± 8.00, $P< 0.05$).

Chronic study: EPM

Figure 3-3 depicts the percentage time in the open arms of the EPM following chronic drug administration. It can be seen in figure 3-3A that chronic vehicle-treated FSL rats spent a significantly lower percentage of time in the open arms of the EPM compared to FRL rats (20.80 ± 9.94 vs. 60.22 ± 7.78, $P<0.001$). The chronic administration of 15 mg/kg fluoxetine to FSL rats

exerted a significant increase in the percentage time spend in the open arms compared to vehicle-treated FSL rats (65.02 ± 6.61, vs. 20.80 ± 9.94, P<0.001), and comparable to the behaviour observed with vehicle-treated FRL rats. Of note is that FRL, but not FSL rats, tend to spend more time in the open arms following chronic treatment than following acute (single injection) treatment with vehicle control (compare figures 2A & 3A: FRL 27.19 ± 2.02 vs. 60.22 ± 2.08, P<0.001; FSL 16.76 ±1.88 vs. 20.80 ± 2.57, vs.; Student's t-test).

From figure 3-3B it can be seen that the chronic administration of 1 mg/kg atropine alone induced a small but statistically significant increase in the percentage of time spent in the open arms of the EPM, relative to vehicle control (30.71 ± 8.40 vs. 20.80 ± 9.94, P<0.01). Likewise, the chronic administration of 10 mg/kg sildenafil alone induced a significant increase in the percentage of time spent in the open arms of the EPM, relative to vehicle control (45.72 ± 5.92 vs. 20.80 ± 9.94, P<0.001), and also significantly more than that achieved with atropine alone (45.72 ± 5.92 vs. 30.70 ± 8.40, P<0.001). The co-administration of sildenafil plus atropine in FSL rats yielded an additive response, significantly different from vehicle-treated control (59.56 ± 4.95 vs. 20.80 ± 9.94, P<0.001), as well as from atropine alone (59.56 ± 4.95 vs. 30.70 ± 8.40, P<0.001) and from sildenafil alone (59.56 ± 4.95 vs. 45.72 ± 5.92, P<0.001). In fact, the behavioural response following the co-administration of sildenafil plus atropine (Figure 3-3B) was comparable to the behaviour observed in vehicle-treated FRL rats (figure 3-3A), and with fluoxetine-treated FSL rats (figure 3-3A) of the chronic treatment groups.

Discussion

In keeping with the stress-sensitive phenotype of these animals, the current study clearly demonstrates that Flinders Sensitive Line (FSL) rats display enhanced anxiety-like behaviour compared to their FRL controls in both the social interaction test (figure 3-1A) and the elevated plus maze (figures 3-2A and 3-3A). This difference is not affected by whether the rats were acutely (once) or chronically (14 days) exposed to intraperitoneal injections with vehicle control, although there is an indication that chronically treated FRL rats, but not FSL rats, spend more time exploring the open arms of the elevated plus maze.

Secondly, acute administration of fluoxetine produced an anxiogenic-like response in the social interaction test (figure 3-1A) and a trend toward such an effect in the elevated plus maze (figure 3-2A). These observations are in line with prevailing opinion that acute SSRI treatment is not anxiolytic, being more likely to increase anxiety at the start of treatment (Harvey, 1997). Indeed, acute administration or an overdose of fluoxetine is known to cause side effects such as anxiety, nervousness and agitation (Den Boer and Westenberg, 1988). Moreover, an early therapeutic response in both depression and anxiety disorders is highly unlikely since a major limitation of the SSRIs is their delayed onset of action (Susman and Klee 2005).

However, chronic administration of fluoxetine displayed a pronounced anxiolytic-like effect in FSL rats (figure 3-3A). Such a result was expected since antidepressants, and including the SSRIs, take between 2 and 4 weeks to achieve therapeutic effects in the treatment of anxiety.

The data from this study further demonstrated that the acute administration of sildenafil, with or without atropine, does not significantly alter anxiety-like behaviour in either the social interaction test (figure 3-1B) or the elevated plus maze (figure 3-2B), while acute atropine treatment alone increased anxiety-like behaviour (figure 3-1B). This latter effect was not accompanied by a corresponding change in line crossings (figure 3-1D), so that locomotor activity cannot explain the observed response. Atropine is a competitive antagonist of acethycholine (ACh) at muscarinic receptors. In a high dosage atropine causes cortical stimulation in the CNS which leads to restlessness, agitation and hallucination (Rang et al., 2007). Despite earlier studies demonstrating an anxiogenic response following acute treatment with sildenafil (Kurt et al., 2004 and Volke et al., 2003), our data indicate that unlike acute fluoxetine, acute sildenafil is not anxiogenic. Fluoxetine, and in general SSRIs, are believed initially to inhibit 5-HT re-uptake, but which over time leads to a change in 5-HT function due to 5-HT receptor desensitization (Wegener et al., 2003), and thus plays a more direct role in generating anxiety at the start of treatment that is dependent on receptor state. This is especially the case in depression which is associated with upregulated 5-HT receptors (Van de Kar 1989). However, since cGMP is a down-stream subcellular target, it is devoid of the problem of non-physiological flooding of post-synaptic 5HT1a and 2a/c receptors, and thus directly activating guanylyl cyclase-cGMP with sildenafil would not be liable to inducing agitation or anxiety.

Figure 3-3 A and B show an almost equivalent anxiolytic-like effect between the chronic fluoxetine and sildenafil plus atropine treated FSL rats. Chronic treatment with sildenafil alone

decreased anxiety significantly but not as effectively as fluoxetine. Human and animal data indicate that SSRIs may affect anxiety related behaviours, which is in keeping with the role of central 5-HT in the modulation of these behaviours (Griebel, 1995; Handley, 1992). In humans, SSRIs are prescribed for panic disorders (Westenberg and den Boer, 1988), obsessive–compulsive disorders (Insel et al., 1985), social phobia, and anxious depression (Sheehan et al., 2005), suggesting that despite their tendency to increase anxiety at the start of treatment, SSRI's are effective in a number of anxiety disorders after chronic treatment (Harvey, 1997). Furthermore, previous reports have suggested that positive modulators of the glutamate/NO/cGMP pathway may be anxiogenic (Volke et al., 2003 and Kurt et al., 2004), although these findings are from acute studies in rodents. The current study demonstrates that the effect of chronic administration with sildenafil on anxiety-like behaviour is markedly different from that observed following acute administration. Indeed, in agreement with data described recently by Solis et al (2008), sildenafil engendered a significant anxiolytic response in FSL rats after chronic administration. These actions may involve the down-regulation of supersensitive 5HT receptors after chronic use, thus acting to increase serotonergic signalling in a similar way to SSRI's albeit via a different mechanism. Indeed, recent work has confirmed that chronic sildenafil treatment is associated with an increase in serotonergic-mediated behaviours, as determined using a behavioural sampling technique in the rat FST (Liebenberg et al, 2010b).

Both NO and 5-HT play a role in anxiety and affective disorders (Harvey 1996), while they also may modulate each others effects (eg. Harvey et al., 2006). NO and cGMP can modify neuronal

release of 5-HT (Wegener et al., 2000), while glutamate and NO exert important actions on cellular and neuronal plasticity (Puzzo et al., 2008). Thus cGMP-directed effects on 5HT release and neuroplasticity may be driving the antidepressant- and anxiolyic like effects of sildenafil, a mechanism that is probably also responsible in part for the pharmacological effects of the SSRI's in depression and anxiety.

In conclusion, the glutamate/NO/cGMP pathway has been postulated to play a key role in the neurobiology of anxiety-related disorders, which is further supported by these data. Most antidepressants currently used display clinically effective anxiolytic activity, but only following chronic treatment. Our data concur that the anxiolytic effects of sildenafil are also only evident following chronic treatment, thus providing some degree of predictive validity for these observations. Unlike its previously reported antidepressant effects where it requires concomitant suppression of central muscarinic receptors for efficacy, the anxiolytic effects of sildenafil occur without the co- administration of atropine and thus appears to occur independent of the cholinergic system. Thus although the inhibition of PDE5 evokes both antidepressant-like and anxiolytic-like effects in rodent models, these actions may ensue via different mechanism(s), especially with regard to the cholinergic system (Liebenberg 2009). Finally, unlike acute SSRI treatment, acute sildenafil treatment is not anxiogenic as has been noted earlier by other investigators. This discrepancy may be due to the differences in anxiety tests used, and further study is therefore warranted.

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Legends for figures

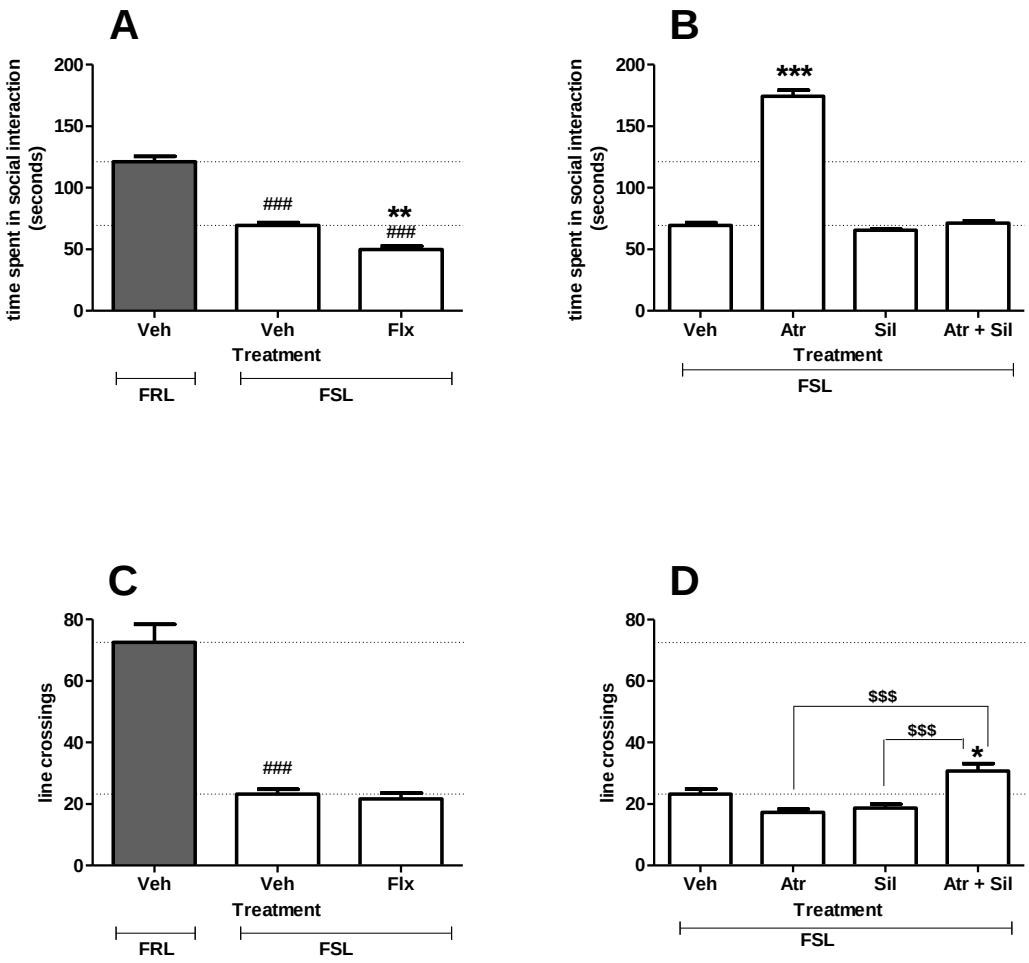
Figure 3-1. (A) The time spent in social interaction following the acute administration of vehicle (Veh) in Flinders Sensitive Line (FRL) and Flinders Sensitive Line (FSL) rats, as well as following the acute administration of 15 mg/kg fluoxetine (Flx) in FSL rats. (B) The time spent in social interaction following the acute administration of Veh, 1 mg/kg atropine (Atr), 10 mg/kg sildenafil (Sil) or sildenafil plus atropine (Sil+Atr) in FSL rats. (C) The number of line crossings following the acute administration Veh in FRL and FSL rats, as well as following the acute administration of Flx in FSL rats. (D) The number of line crossings following the acute administration of Veh, Atr, Sil or Sil+Atr. The data represent the mean ± S.E.M., with 15 rats per treatment group. Data was analyzed with a two-way ANOVA, and when the absence of an interaction between the treatments was demonstrated, the data was further analyzed with a one-way ANOVA followed by a Tukey-Kramer multi comparison post-test. Statistical significance was accepted when the error was less than 5% ($^{###}$P<0.001 relative to FRL control; *P<0.05, **P<0.01, ***P<0.001 relative to the FSL control; $^{\$\$\$}$P<0.001 between indicated treatment groups).

Figure 3-2. (A) The percentage time spent in the open arms in the elevated plus maze following the acute administration of vehicle (Veh) in Flinders Sensitive Line (FRL) and Flinders Sensitive Line (FSL) rats, as well as following the acute administration of 15 mg/kg fluoxetine (Flx) in FSL rats. (B) The percentage time spent in the open arms in the elevated plus maze following the acute administration of Veh, 1 mg/kg atropine (Atr), 10 mg/kg sildenafil (Sil) or sildenafil plus atropine (Sil+Atr) in FSL rats. The data represent the mean ± S.E.M., with 15 rats per treatment

group. Data was analyzed with a two-way ANOVA, and when the absence of an interaction between the treatments was demonstrated, the data was further analyzed with a one-way ANOVA followed by a Tukey-Kramer multi comparison post-test. Statistical significance was accepted when the error was less than 5% ($^{\#\#}P<0.01$ relative to FRL control; $^{\$}P<0.05$ between indicated treatment groups; ns = not significant).

Figure 3-3. (A) The percentage time spent in the open arms in the elevated plus maze following the chronic (14 days) administration of vehicle (Veh) in Flinders Sensitive Line (FRL) and Flinders Sensitive Line (FSL) rats, as well as following the acute administration of 15 mg/kg fluoxetine (Flx) in FSL rats. (B) The percentage time spent in the open arms in the elevated plus maze following the chronic administration of Veh, 1 mg/kg atropine (Atr), 10 mg/kg sildenafil (Sil) or sildenafil plus atropine (Sil+Atr) in FSL rats. The data represent the mean ±S.E.M, with 15 rats per treatment group. Data was analyzed with a two-way ANOVA, and when the absence of an interaction between the treatments was demonstrated, the data was further analyzed with a one-way ANOVA followed by a Tukey-Kramer multi comparison post-test. Statistical significance was accepted when the error was less than 5% ($^{\#\#\#}P<0.001$ relative to FRL control; $^{**}P<0.01$, $^{***}P<0.001$; $^{\$\$\$}P<0.001$ between indicated treatment groups).

Figure 3-1


A
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100
50
0
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Veh
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Treatment
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time spent in social interaction (seconds)
200
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50
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Veh
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Sil
Atr + Sil
Treatment
FSL
C
line crossings
80
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40
20
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Veh
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Treatment
FRL
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line crossings
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Atr + Sil
Treatment
FSL

Figure 3-2

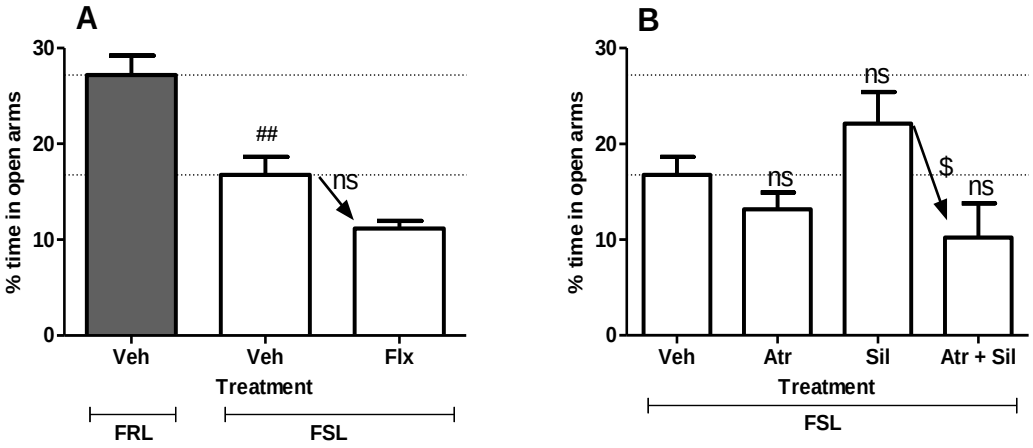
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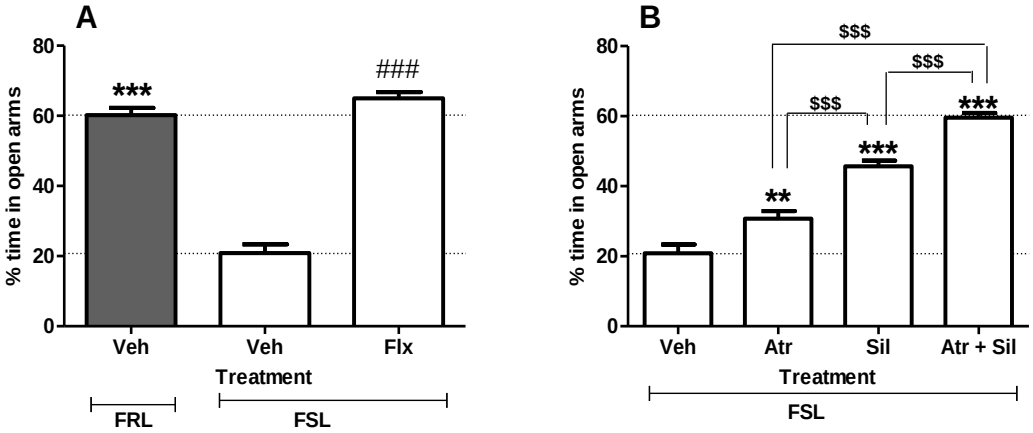
Figure 3-3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Summary, Discussion, Conclusions and Recommendations

Chapter 4

The results of this study are presented in chapter 3 and addendum B of this dissertation. This chapter will summarize the results from this study in order to formulate a comprehensive conclusion to the study, together with recommendations for prospective studies.

4.1 Abridged Summary

Table 4-1 displays the comparison between FSL and FRL rats in the elevated plus maze and the social interaction tests for both the acute and chronic studies.

Table 4-1. Summary of the anxiety-like behaviour of FSL vs. FRL rats following acute and chronic vehicle and drug treatment groups (↔) = no effect, (↑) = increased anxiety-like behaviour and (↓) = decreased anxiety-like behaviour. Social interaction test (SIT), Elevated plus maze (EPM) and the drug treatment include fluoxetine (Flx), atropine (Atr) and sildenafil (Sil).

Treatment Groups		**Anxiety-like behaviour**						
		Acute Study				**Chronic Study**		
Rat	**Drug**	**SIT**	**EPM**			**EPM**		
			% time spent in open arms	**Open arm entries**	**Defecation**	**% time spent in open arms**	**Open arm entries**	**Defecation**
FRL	**Veh**	↓↓↓	↓↓↓	-	-	↓↓↓	-	-
FSL	**Veh**	reference group						
	Flx	↑	↑	↔	↔	↓↓↓	↔	↔
	Atr	↑↑	↔	↔	↔	↓	↔	↔
	Sil	↔	↔	↔	↔	↓↓	↔	↔
	Sil + Atr	↔	↔	↔	↔	↓↓↓	↔	↔

Key findings described in Table 4-1:

1. The acute fluoxetine treated FSL rats displayed a small but significant anxiogenic-like response in the EPM, but with no significant effect on total arm entries. However, the chronic fluoxetine treated FSL rats displayed a significant decrease in anxiety-like behaviour.

2. Acute atropine treatment produced no significant effect on the behaviour of the animals, however the chronic treatment produced a slight but significant anxiolytic-like effect.

3. In the SIT the acute FLX treated FSL rats displayed a significant increase in anxiety-like behaviour.

4. Acute sildenafil treatment did not significantly alter anxiety-like behaviour in either the EPM or the SIT. Chronic sildenafil treatment alone decreased anxiety-like behaviour significantly.

5. As with the acute sildenafil treatment alone the combination of acute sildenafil plus atropine did not affect anxiety-like behaviour significantly in the EPM or the SIT. However in the chronic study sildenafil plus atropine yielded a significant anxiolytic-like response equivalent to that of fluoxetine and sildenafil alone also produced a significant anxiolytic-like effect.

Acute fluoxetine-treated FSL rats displayed an increase in anxiety-like behaviour in both the EPM and SIT, but demonstrated anxiolytic-like behaviour in the chronic study. The atropine caused a medium increase in anxiety-like behaviour in FSL rats in the SIT but not in the EPM, while in the chronic study it induced a small decrease in anxiety-like behaviour. Sildenafil alone had no significant effect on the anxiety-like behaviour in the acute study, but caused a medium decrease in anxiety-like behaviour following chronic treatment. The combination of sildenafil

with atropine did not affect anxious behaviour in the rats following acute treatment, but produced a statistically significant decrease in the anxiety-like behaviour following chronic treatment.

None of the treatment regimes used in this study had a significant effect on the locomotion of the animals, as suggested by a lack of effect on total arm entries in the EPM. These data confirm that the current treatments do not affect the natural activity of the animals.

Lastly, faecal defecation was also used as an indirect parameter of anxiety in the current study. There was no significant differences observed between the different treatment groups, suggesting that this parameter may not be sensitive enough to detect differences in anxiety as observed in the EPM (see Table 4-1).

4.2 Discussion and Conclusion

In the current study the acute administration of fluoxetine, sildenafil or sildenafil plus atropine did not yield any anxiolytic-like response in either the Social Interaction test or the Elevated Plus Maze. In fact, fluoxetine even exerted an anxiogenic-like response in this scenario. These results were expected for fluoxetine. Firstly since the SSRIs (the drug class of choice in the treatment of anxiety disorders (Durand et al., 1999) are known to have a delayed onset of action in the treatment of anxiety disorders, taking up to 2 to 4 weeks for initial and up to 6 weeks for full response in humans (Susman and Klee, 2005), and secondly, the initial response to SSRIs may include agitation and enhanced anxiety (Boadie et al., 2008). Previous animal studies revealed that the acute administration of fluoxetine does indeed cause anxiogenic-like behaviour in various animal models (Petkov et al., 1990; Handley & McBlane, 1992 & Kshama et al,. 1995). In another animal study, Silva and co-workers (1999) showed that the acute administration of fluoxetine in Wistar rats causes anxiogenic-like effects in the elevated plus maze (Silva et al., 1999). Thus, the findings of the current acute study are in line with data from earlier studies and observations reported in literature.

A delayed onset of action may also explain the lack of anxiolytic-like response to sildenafil or to sildenafil plus atropine following acute treatment. However, opposite to that described for fluoxetine, an anxiogenic-like response was not observed following the acute administration of sildenafil or sildenafil plus atropine in FSL rats. These results are opposite to that reported in mice (Volke et al., 2003 and Kurt et al., 2004), where the acute administration of sildenafil at the same dose one hour before testing was found to exert anxiogenic-like effects. These data therefore suggest that the effects of sildenafil on anxiety-like responses following acute administration may be species-dependent.

In the EPM the total number open arm entries can be taken as a secondary indicator of locomotor activity (Dawson & Tricklebank., 1995), which indicate the effect of a drug on the natural activity of an animal (Lister, 1990). The data in figure B-1 (addendum B) suggest that none of the current treatment regimes affected locomotor activity of the animals, but can also be interpreted as anxiolytic-like behaviour, when the number of open arm entries remain constant while the percentage time spend in the open arms increases (Lister, 1990). Furthermore fecal defecation is also a secondary indicator of anxiety-like behaviour in the EPM (Daniels et al., 2004). The exposure to a fearful situation simulates the autonomic nervous system resulting in increased defecation (Hall, 1934; Lister, 1990). The fecal dropping data in figure B-2 (addendum B) display no significant increase between all of the treatment groups which can be taken to suggest a lack of anxious behaviour.

Importantly, chronic (14 days) administration of sildenafil alone, as seen also with the positive control fluoxetine, exerted a significant anxiolytic-like response relative to vehicle control in FSL rats. These results are markedly different from that observed following acute administration. Chronic administration of the antimuscarinic agent atropine alone also exerted a small, yet statistically significant anxiolytic response, but smaller than that of sildenafil alone. This anxiolytic-like response of sildenafil and atropine co-administration was additive and comparable to that of fluoxetine. Taken together, these data suggest that chronic administration of sildenafil yields an anxiolytic-like response in stress-sensitive rats and that this

response will be augmented by simultaneous anticholinergic treatment. These results support previous findings by Liebenberg (2009) and Solis et al. (2008) that chronic treatment with sildenafil reduces anxiety-like behaviour in the social interaction and open field tests, respectively. Furthermore, these results provides context to seemingly conflicting findings regarding the putative anxiogenic effects of sildenafil in rodents (Devan et al., 2006; Kurt et al., 2004; Prickaerts et al., 2002). That the anxiolytic activity of chronic co-administration of sildenafil plus atropine is as effective as fluoxetine in FSL rats should be interpreted together with previous findings from our laboratory that the antidepressant-like activity of chronic sildenafil plus atropine co-administration is comparable to that of fluoxetine in the forced swim test (Brink et al., 2007). This implies that both sildenafil alone and sildenafil plus atropine display an anxiolytic-like and antidepressant-like effects in FSL rats when administrated chronically.

In conclusion, the following are the primary findings of the current study:

- Acute administration of fluoxetine in FSL rats is anxiogenic in the elevated plus maze.
- Acute administration of both sildenafil and sildenafil plus atropine was not anxiogenic in the social interaction test, although produced an inconclusive result with respect to anxious behaviour in the elevated plus maze.
- Chronic treatment of FSL rats with either sildenafil or atropine produced an anxiolytic response, with sildenafil plus atropine being additive and yielding an anxiolytic-like effect comparable to that of fluoxetine in the EPM, but without affecting the locomotor activity.

4.3 Recommendations

While the current study supports previous findings regarding the anxiolytic activity of the current treatment regime, there are several remaining questions, that should be addressed in prospective studies. These are discussed below:

4.3.1 Recommendations

- Quantitative polymerase chain reaction (qPCR) may be more sensitive to detect changes in the levels of biomarkers of neuroplasticity. Changes in gene expression, as represented by mRNA expression in qPCR, does not necessarily translate into altered protein expression, but is more sensitive and even small changes is easier to detect levels at cellular level.

- While FSL rats represent a genetic model of depression and are known to exhibit enhanced stress sensitivity, previous studies have indicated that stressed and stress-naïve animals do not respond in the same manner, such that only following chronic, mild stress may more meaningful behavioural changes be observed.

- While the current findings report on the antidepressant-like and anxiolytic-like activities of PDE5 inhibitors following chronic administration in rats, since sildenafil is already used clinically for the treatment of erectile dysfunction and pulmonary hypertension, it may be useful to consider testing sildenafil's psychotropic effects in higher order animals, even primates, and eventually to evaluate these effects in clinical trials.

Materials and Methods

Addendum A

This addendum provides a compilation of all the materials, validations and methods used in this study, including for yielding results not used in the manuscript (Chapter 3), but described in Addendum B.

1.1 Animals

The study protocol was approved by both the Research Committee and the NWU Ethics committee (ethics approval number NWU – 00039 – 09 – S5), and performed in accordance with the guidelines stipulated by the ethics committee for the use animals in experimental work at the North West University. All animals were maintained according to a code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

Male FRL and FSL rats were supplied by the Animal Research Centre on the North West University, weighing approximately 300 g ± 10 g at the day of testing. The rats were randomly assigned to groups consisting of 5 rats per group.

Rats were housed at the North West University Animal Research Center, under conditions of constant temperature (22 °C) and humidity (50%) with a 12:12-h light/dark cycle (lights on 06:00 to 18:00). Food and water were provided ad libitum.

1.2 Materials

List of reagents used in the treatment of the rats.

- Fluoxetine (a kind gift from Aspen, Port Elizabeth, South Africa), atropine (Merck, Darmstadt, Germany), sildenafil (a kind gift from Pfizer Global Research and Development, Kent, United Kingdom), methanol (Sigma-Aldrich, Johannesburg, South Africa), dimethyl sulfoxide (Saarchem,

Gauteng, South Africa), sodium chloride (Sigma-Aldrich, Johannesburg, South Africa), double distilled water (LAMB, laboratory, Northwest University).

- 1 ml disposable insulin syringes (Local pharmacy), latex gloves (local pharmacy), Sony HDR-XR150 digital video camera.

2.1 Additional parameters in the elevated plus maze

Figure A-1. The elevated plus maze .

In 1955 Montgemery reported that open elevated alleys evoked greater avoidance than closed alleys. This model was later refined by (Handley & Mithani., 1984), since then the model was extensively validated pharmacologically, physiologically and behaviorally (Pellow et al., 1985).

Either forced or voluntary entry into the open arms is accompanied by elevated corticosterone levels, increased freezing and production of fecal boli, hormonal and

behavioural changes all of which are indications of increased anxiety (Pellow et al., 1985). Normal exploratory behaviour is to stay in the closed arms, and this tendency to stay in the closed arms can be increased by using compounds that increase aversion towards the anxiety provoking open arms. In contrast anxiolytic compounds reduce natural aversion of the open arms and increase the exploration thereof (Pellow et al., 1985)

Before the start of the experiment the animals was left for an hour to acclimatise. The EPM apparatus consists a plus-shaped platform of 1 m x 1 m, and 10 cm arm width, elevated 50 cm above the floor level, with two open arms (with a 5 mm ledge to prevent slipping and falling) and two closed arms (30 cm high opaque side-walls). The maze is placed in an isolated room. A 40 W blue lamp was positioned 1 m above the maze and a 60 W desk lamp pointed away from the maze toward one wall. A digital video camera was positioned directly above the maze. The rat was placed in the centre square of the EPM each time facing the open arm. The animals were allowed to explore the maze for 5 min under continuous surveillance. After the test, the rat was immediately placed back in its home cage and the maze wiped thoroughly with methanol to eliminate any odour. During scoring of the test, an arm entry was defined as the entry of all four paws into the arm (Lister, 1987). This model has previously been validated and used as a measure of anxiety by various authors (Pellow et al., 1985 & Hogg, 1996)

Locomotor activity is seen a secondary indication (Dawson & Tricklebank., 1995) of the natural activity in animals tested on the EPM to determine whether the drug that's being tested have sedating or stimulating effects (Lister, 1990). The total number of both full body and half body open arm entries was used as an index of general activity (Pellow et al., 1985). Open arm entries also serves as an index of anxiety in the EPM, if the number of open arm entries remain consistent but the percentage of time spend in the open arms increase then is suggest anxiolytic-like behaviour (Lister, 1990).

2.1.1 Defecation as measure of anxiety in the elevated plus maze

Defecation was first described in 1934 by Hall, to be an index of anxiety in rodents (Hall, 1934). However, Bindra and Thompson (1953) reported that there is no significant relation between fearfulness and defecation as measured in the open field test though they regarded defecation in a novel environment as signs of nervousness. Defecation is often used as a measure of anxiety, but their validity as measures of anxiety has been questioned (Lister, 1990). In a study conducted by (Daniels et al., 2004), they describe defecation as an additional indicator of anxiety in the elevated plus maze.

After each rat was removed from the EPM after 5 minutes the number of fecal droppings was counted and documented, thereafter the maze was thoroughly wipes with a damp methanol cloth.

3.1 The social interaction test

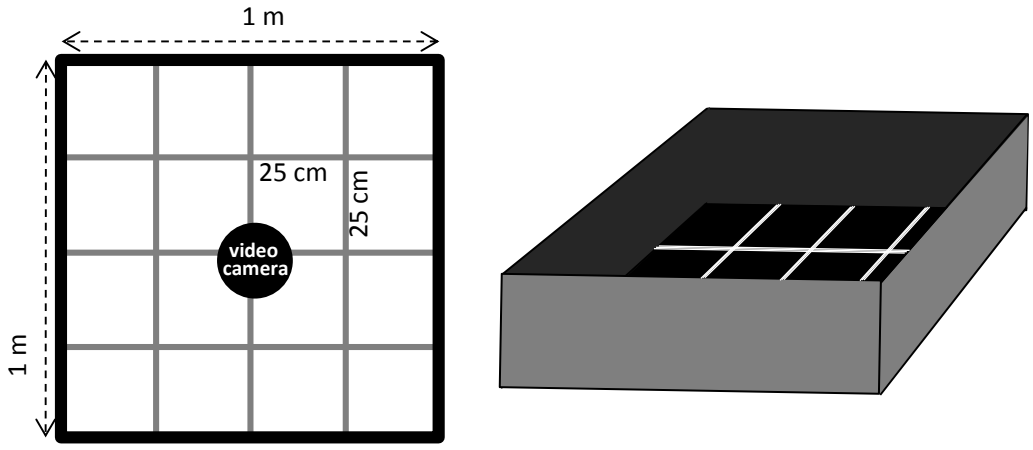

Figure A-2. Social interaction test arena.

The social interaction test (SIT) was first developed by File and Hyde in 1978. The SIT is based upon the time spent by pairs of male rats novel to each other, in active social interaction, measured in a test arena (Lister., 1990). The time spend in social interaction between two rats is seen as an index of anxiety. The increase in social

interaction means a decrease in anxiety-like behaviour. Locomotor activity assists in determining whether changes in social interaction are due to a general stimulant or sedative effect (Lister, 1990). The social interaction test has been validated in our laboratory for the evaluation of anxiety-like behaviour (Liebenberg, 2009).

In the current study, the social interaction test was carried out as described previously for FSL rats (Overstreet and Griebel 2004). Two rats of the same treatment group and similar body weight was placed to the centre square of the square test arena (1 m × 1 m, marked with lines to render sixteen 25 cm × 25 cm blocks). The following actions were recorded as social interaction: grooming, licking, sniffing and crawling over or under, and the total number of line crossings (when the rats crossed a line with all four paws it was seen as a single line crossing) were recorded as an indication of locomotor activity. The social interaction was recorded with digital video camera over a period of 5 minutes, individually scored for each rat and expressed as the percentage time spent in social interaction.

Additional Results

Addendum B

This addendum will present additional data of experimental work performed, that was not included in the manuscript (Chapter 3).

1.1 Additional Elevated plus maze results

This addendum represents results from additional EPM parameter. Firstly, full body and half body open arm entries and the number of fecal defecation on the EPM.

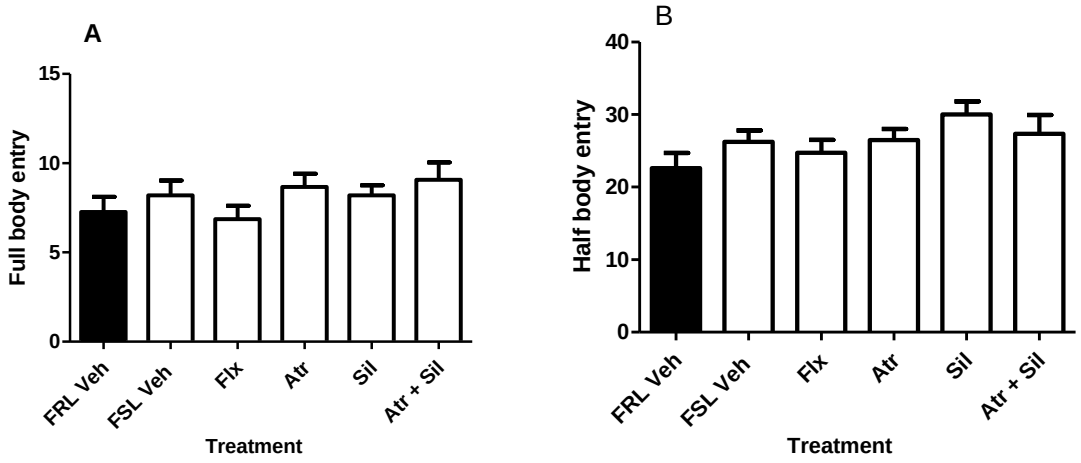

Figure B-1. (A) The effect of a chronic treatment regime on vehicle treated FRL rats and FSL rats with vehicle, fluoxetine 15 mg/kg, atropine 1 mg/kg, sildenafil 10 mg/kg and atropine 1 mg/kg plus sildenafil 10 mg/kg in the elevated plus maze. (B) The effect of a chronic treatment regime on vehicle treated FRL rats and FSL rats with vehicle, atropine 1 mg/kg, sildenafil 10 mg/kg and atropine 1 mg/kg plus sildenafil 10 mg/kg in the elevated plus maze. The data represent the number of half body open arm entries during a 5 minute period the mean ± S.E.M. for 15 rats per group.

Figure B-1 a represent the data obtained from the full body open arm entries in the EPM. In figure B-1 A there is no significant difference in the number of full body arm entries between the vehicle treated FRL and FSL rats. Although the atropine and sildenafil plus atropine treatment groups display an slight increase in full body arm entries when compared to the FSL vehicle treated group the increase remain statistically insignificant. While the fluoxetine and sildenafil alone treatment groups display a decrease in full body arm entries when compared to the FSL control group the decrease is insignificant.

Figure B-1 B represents the data obtained from half body open arm entries in the elevated plus maze. When the FRL and FSL vehicle treated rats are compared the FSL rats display non-significant increase in half body open arm entries. When the FSL vehicle treated group is compared to the other treatment groups the data display an upward trend, but there are no significant difference between any of the treatment groups.

The following figure B-2 represents the defection data as obtained in the EPM

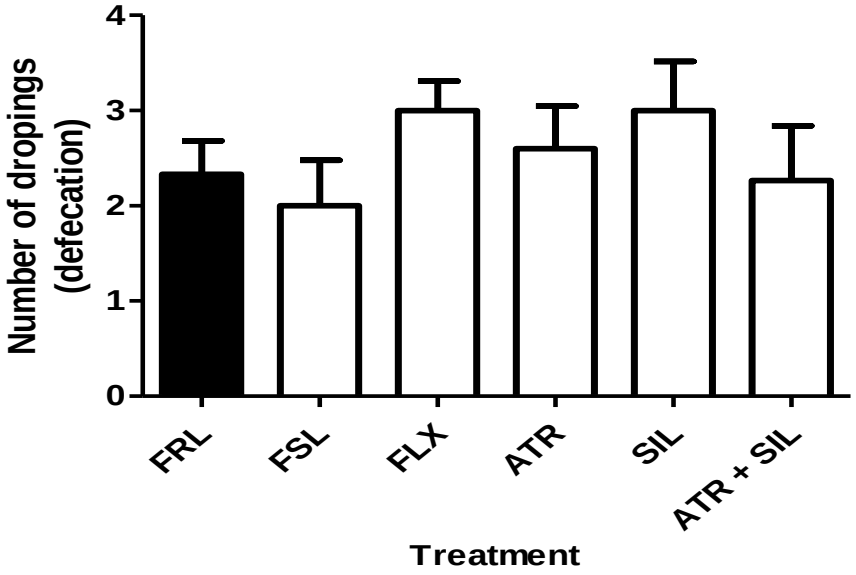

Figure B-2. The effect of a chronic treatment regime on vehicle treated FRL rats and FSL rats with vehicle, fluoxetine 15 mg/kg, atropine 1 mg/kg, sildenafil 10 mg/kg and atropine 1 mg/kg plus sildenafil 10 mg/kg in the elevated plus maze. The data represent the number of droppings (defecation) made during a 5 minute period the mean ±S.E.M. for 15 rats per group.

In figure B-2 the vehicle treated FSL rats display a non-significant decrease in the number of fecal droppings when compared to the vehicle treated FRL rats. The fluoxetine treated rats display a large but non-significant difference in the number of fecal dropping when compared to the vehicle treated FSL rats. There is no significant difference between the atropine treated group and the FSL control group. Sildenafil had no significant effect on the number of fecal droppings in the EPM when compared to the vehicle treated FSL rats. Lastly, there was also no statistical significance between the sildenafil plus atropine treated group and the FSL vehicle treated group.

1.2 Discussion

The rodent elevated plus maze (EPM) is a well-established and robust behavioural test paradigm with a very reliable predictive validity for anxiolytic drug activity, supported by a host of experimental data from various laboratories, accumulated over several decades (Rodgers, 1997). In the EPM the most commonly used parameters of anxiety-like behaviour scored include the number of open arm entries and the time spend in the open arms as a percentage of the total time (Lister, 1990). Scoring is usually done manually from either by direct observation (real-time scoring) or from digital video recordings from above.

In chapter 3 (manuscript) we have reported the anxiolytic-like effects of sildenafil and sildenafil plus atropine in the elevated plus maze by only measuring the percentage time spend in the open arms. However, for comparison we have also measured both full body and half body open arm entries (Lister, 1990). However, although a direct correlation have been suggested to exist between the percentage time spent in the open arms and the number of open arm entries, this is not always true. For example, “if a treatment regime increases an animal’s preference for the open arms without altering the total number arm entries, this is still taken to reflect an anxiolytic action” (Lister, 1990; Pellow 1985; Dawson & Tricklebank., 1995; Hogg 1996 & Daniels et al., 2004). In the current study both sildenafil and sildenafil plus atropine (fig B-1 A and B) did not alter the locomotor activity and at the same time

increase the time spend in the open arms significantly (fig 3-3 B) which is an indication of anxiolytic-like behaviour.

The number of open arm entries in the EPM can also be utilised as an indirect (secondary) measure of locomotor activity (Pellow, 1985; Dawson & Tricklebank., 1995). Inhibition of locomotor activity would adversely affect the reliability of the test to detect anxiolytic activity. Therefore, this delineation of locomotor activity following the indicated drug treatment regimens was also implemented in the current study to detect whether the current treatment regimens affect the natural activity of the rodents. The data in figure B-2 A and B suggest that the current treatments did not alter the locomotor activity in any of the groups.

Lastly, we also measured defecation (the number of faecal droppings left on the maze during the 5 minute test period) in the EPM, which is regarded by some researchers as an indication of anxiety (Hall, 1934, Lister, 1990 & Daniels et al., 2004). An increase in anxiety activates the autonomic nervous system, consequently leading to enhanced defecation (Lister 1990). Therefore, an increase in the number of faecal droppings suggests an increase in anxiety in the elevated plus maze

Thus, the data in figure B-2 indicate the number faecal dropping counted on both the open and closed arms of the elevated plus maze. Fearful or anxiety causing situations activate the autonomic nervous system, and that will cause an animal to defecate (Lister 1990; Daniels et al., 2004). In other words, in animals with high levels of anxiety the number of fecal dropping will increase significantly. However the results in figure B-2 display the opposite, there is no significant difference between the different treatment groups, which suggest that the current treatment regimes in this study reveal an anxiolytic-like effect and this finding further support the data in chapter 3 (manuscript).

Instructions for Authors

Addendum C

Guidance for Authors on the Preparation and Submission of Manuscripts to Behavioural Pharmacology

Note: These instructions comply with those formulated by the International Committee of Medical Journal Editors. For further details, authors should consult the following article: International Committee of Medical Journal Editors. ''Uniform Requirements for Manuscripts Submitted to Biomedical Journals'' N Engl J Med 1997;336:309-315. The complete document appears at http://www.icmje.org.

Full details concerning many of the points below, including standard forms, are provided in the guidelines on the journal's web site (www.behavioural pharm.com).

Scope

Behavioural Pharmacology accepts original full and short research reports in diverse areas ranging from ethopharmacology to the pharmacology of schedule-controlled operant behaviour, provided that their primary focus is behavioural. Suitable topics include drug, chemical and hormonal effects on behaviour, the neurochemical mechanisms under-lying behaviour, and behavioural methods for the study of drug action. Both animal and human studies are welcome; however, studies reporting neurochemical data should have a predominantly behavioural focus, and human studies should not consist exclusively of clinical trials or case reports. Preference is given to studies that demonstrate and develop the potential of behavioural methods, and to papers reporting findings of direct relevance to clinical problems. Papers making a significant theoretical contribution are particularly welcome and, where possible and merited, space is made available for authors to explore fully the

theoretical implications of their findings. Reviews of an area of the literature or at an appropriate stage in the development of an author's own work are welcome. Commentaries in areas of current interest are also considered for publication, as are Reviews and Commentaries in areas outside behavioural pharmacology, but of importance and interest to behavioural pharmacologists.

Points to consider before submission

We have prepared a standard covering letter (available from the journal website) to accompany your submission. Whether you use this letter or your own wording, please think carefully about the following points and make the appropriate declarations.

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Ethics committee approval

You must state clearly in your submission in the Methods section that you conducted studies on human participants must with the approval of an appropriate named ethics committee. Please also look at the latest version of the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm). Similarly, you must confirm that experiments involving animals adhered to ethical standards and must state the care of animal and licensing guidelines under which the study was performed.

The editors will welcome the submission of work in any area of behavioural pharmacology. However, studies that subject animals to pain or stress require special consideration and three principles will be used to judge the ethics of such work. First, experiments of this kind will be required to reach a higher threshold of scientific quality in order to be publishable. Second, the utility of the study will be addressed, in terms of the balance between the distress inflicted and the likelihood of benefit. Third, it must be demonstrated that the objectives of the experiment could not have been achieved by the use if less stressful procedures; the fact that a particular procedure has been used in previously published work will not in itself suffice. The

editors believe that behavioural pharmacologists apply strict ethical criteria to their work, and will have no difficulty in meeting these requirements.

Authorship

All authors must sign the letter accompanying their submission to confirm that they have read and approved the paper, that they have met the criteria for authorship as established by the International Committee of Medical Journal Editors, that they believe that the paper represents honest work, and that they are able to verify the validity of the results reported. You might also be interested to read the debate on authorship in general in the British Medical Journal's Authorship collection (http://bmj.com/cgi/collection/authorship). Many of the points covered above are discussed in the New England Journal of Medicine's collection of papers entitled 'Editorials on Journal Policy' (http://authors.nejm.org/Misc/Policies.asp).

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A number of research funding agencies now require or request authors to submit the post-print (the article after peer review and acceptance but not the final published article) to a repository that is accessible online by all without charge. As a service to our authors, LWW will identify to the National Library of Medicine (NLM) articles that require deposit and will transmit the post-print of an article based on research funded in whole or in part by the National Institutes of Health, Wellcome Trust, Howard Hughes Medical Institute, or other funding agencies to PubMed Central. The revised Copyright Transfer Agreement provides the mechanism.

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Four types of manuscripts will be considered: Research reports; Short reports; Reviews; and Commentaries. Short Reports should be of no more than 1500 words and two tables or figures. Please contact one of the editors to discuss the suitability of topics for Review --- up to 7500 words, Commentary --- up to 2500 words, or other material falling outside the usual categories.

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following sections, each starting on a separate page: Title Page, abstract and keywords, text, acknowledgements, references, individual tables and captions. Margins should be not less than 3 cm. Pages should be numbered consecutively, beginning with the Title Page, and the page number should be placed in the top right hand corner of each page. Abbreviations should be defined on their first appearance in the text; those not accepted by international bodies should be avoided. Submit the required number of paper copies and keep copies of everything submitted.

Presentation of papers

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The Title Page should carry the full title of the paper and a short title, of no more than 45 characters and spaces, to be used as a 'running head' (and which should be so identified). The first name, middle initial and last name of each author should appear. If the work is to be attributed to a department or institution, its full name should be included. Any disclaimers should appear on the Title Page, as should the name and address of the author responsible for correspondence concerning the manuscript and the name and address of the author to whom requests for reprints should be made. Finally, the Title Page should include the sources of any support for the work in the form of grants, equipment, drugs, or any combination of these. Disclose funding received for this work from any of the following organizations: National Institutes of Health (NIH); Wellcome Trust; Howard Hughes Medical Institute (HHMI); and other(s).

Abstracts

The second page should carry a structured abstract of no more than 200 words. The abstract should state the Objective(s) of the study or investigation, basic Methods (selection of study subjects or laboratory animals; observational and analytical methods), main Results (giving specific data and their statistical significance, if possible), and the principal Conclusions. It should emphasise new and important aspects of the study or observations.

Key Words

The abstract should be followed by a list of 3–10 keywords or short phrases which will assist the cross-indexing of the article and which may be published. When possible, the terms used should be from the Medical Subject Headings list of the Index Medicus (http:// www.nlm.nih.gov/mesh/meshhome.html).

Text

Full papers of an experimental or observational nature may be divided into sections headed Introduction, Methods (including ethical and statistical information), Results and Discussion (including a conclusion), although reviews may require a different format.

Acknowledgements

Acknowledgements should be made only to those who have made a substantial contribution to the study. Authors are responsible for obtaining written permission from people acknowledged by name in case readers infer their endorsement of data and conclusions.

References

References are to be cited in the text by author and year, as Black and White (1991) or (Black and White, 1991). A series of references in the text should appear in chronological order, e.g. White and Black, 1989; Black and White, 1991. References having three or more authors are cited Black et al., (1991). References to papers by the same authors in the same year are distinguished by letters a, b, etc. (e.g. 1988a,b). Publications having no obvious authors are cited as Anon, (1991) in the text and bibliography. References with three or more authors should be placed in chronological order after taking account of the names of the first and second authors. Some sample reference styles follow:

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Standard journal article:

Sanger D, Willner P, Bergman J (2003). Transgenic mouse models of Alzheimer's

disease: phenotype and application. *BehavPharmacol***14**: 363-7.

More than seven authors:

Sahraei H, Poorheidari G, Foadaddini M, Khoshbaten A, Asgari A, Noroozzadeh A, et al. (2004). Effects of nitric oxide on morphine self-administration in rat.*PharmacolBiochemBehav***77**:111-6.

Books

Book:

Besner D, Humphreys GW, editors (1991). *Basic processes in reading: visual word recognition*. Hillsdale, NJ: Erlbaum.

Chapter in a book:

Sanger DJ (1986). Drug taking as adjunctive behaviour. In: *Behavioural analysis of drug dependence*. Goldberg R, Stolerman IP (editors). New York: Academic Press; pp. 123-160.

Personal communications and unpublished work should not feature in the reference list but should appear in parentheses in the text. Unpublished work accepted for publication but not yet released should be included in the reference list with the words 'in press' in parentheses beside the name of the journal concerned. References must be verified by the author(s) against the original documents.

Either British or American spellings are acceptable, but please be consistent.

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Each table should be typed on a separate sheet in double spacing. Tables should not be submitted as photographs. Each table should be assigned an Arabic numeral, e.g. (Table 3) and a brief title. Vertical rules should not be used. Place explanatory matter in footnotes, not in the heading. Explain in footnotes all non-standard abbreviations that are used in each table. Identify statistical measures of variations, such as standard

deviation and standard error of the mean.

Be sure that each table is cited in the text. If you use data from another published or unpublished source, obtain permission and acknowledge the source fully.

Illustrations

References to figures and tables should be made in order of appearance in the text and should be in Arabic numerals in parentheses, e.g. (Fig. 2). TIFF and EPS files, with fonts embedded, are preferred. If scanned, line art should be at a resolution of 800 dpi, and halftones and colour at 300 dpi. All colour values should be CMYK. If hard copies are submitted they should have a label pasted to the back bearing the figure number, the title of the paper, the author's name and a mark indicating the top of the figure. Illustrations should be presented to a width of 82 mm or, when the illustration demands it, to a width of 166 mm. Photomicrographs must have internal scale markers. If photographs of people are used, their identities must be obscured or the picture must be accompanied by written consent to use the photograph. If a figure has been published before, the original source must be acknowledged and written permission from the copyright holder for both print and electronic formats should be submitted with the material. Permission is required regardless of authorship or publisher, except for documents in the public domain. Figures may be reduced, cropped or deleted at the discretion of the editor. Colour illustrations are acceptable but authors will be expected to cover the extra reproduction costs (for current charges, contact the publisher).

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Captions should be typed in double spacing, beginning on a separate sheet of paper. Each one should have an Arabic numeral corresponding to the illustration to which it refers. Internal scales should be explained and staining methods for photomicrographs should be identified.

Units of measurement

Measurements of length, height, weight, and volume should be reported in metric units (metre, kilogram, or litre) or their decimal multiples. Temperatures should be given in degrees Celsius. Blood pressures should be given in millimetres of mercury.

All haematologic and clinical chemistry measurements should be reported in the metric system in terms of the International System of Units (SI). Editors may request that alternative or non-SI units be added by the authors before publication.

Abbreviations and symbols

Use only standard abbreviations. Avoid abbreviations in the title and abstract. The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement. Drug names should be generic, although authors may add brand names in parentheses if they wish.

Offprints

Offprints may be purchased using the appropriate form that will be made available with proofs. Orders should be sent when the proofs are returned; orders received after this time cannot be fulfilled.

Conference Contribution

Addendum D

In this addendum the study abstract that was presented at the congress of the South African Basic and Clinical Pharmacology Society (2010, Cape Town, South Africa) Young Scientist Category, is shown.

The effects of sildenafil, with and without atropine, on anxiety-like behaviour in rats and on the expression of phosphodiesterase type 5 in rat brain

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Purpose:

Antidepressants, amongst other treatments, are currently used to treat anxiety and anxiety-related disorders. The neurobiology of these disorders has been associated with impaired neuroplasticity, in which the glutamate/nitric oxide (NO)/ cyclic guanosine monophosphate (cGMP) pathway plays a key role. Since modulators of this pathway has been demonstrated to alter mood and anxiety, and since the co-administration of a phosphodiesterase type 5 (PDE5) inhibitor (sildenafil or tadalafil) together with the anticholinergic drug atropine, has been demonstrated to exert anti-depressant-like effects in rodents, we evaluated the effects of these drugs in a rodent test of anxiolytic drug action.

Methods:

Flinder's Sensitive Line (FSL – stress sensitive) and Flinder's Resistant Line (FRL) male rats, weighing ± 300 g on the day of behavioural testing, was provided by the Centre for Laboratory Animals of the Potchefstroom Campus of the North-West University. FSL rats received via intraperitoneal administration vehicle control, fluoxetine (15mg/kg), atropine (1mg/kg), sildenafil (10mg/kg) or sildenafil plus atropine, either acutely 30 min prior to testing, or daily for 14 days (n = 15 per treatment group). FRL rats received only vehicle. Thereafter rat behaviour was evaluated in the elevated plus maze (EPM) and the % time spent in open arms within 5 minutes was scored. Rats were then decapitated, the frontal cortex and hippocampi dissected and the tissue stored at -80°C until biochemical analysis. Western blot analyses were used to determine the active (phosphorylated) and total PDE5 protein expression.

Results:

In both the acute and chronic studies the FSL rats spent less time in the open arms of the EPM than FRL rats (acute: 17 ± 2% vs 27 ± 2%, $p<0.01$; chronic: 21 ± 3% vs 60 ± 2%). In the acute study, fluoxetine reduced time spent in the open arms, while neither sildenafil alone or in combination with atropine altered time spent in open arms significantly relative to vehicle control. However, in the chronic study, fluoxetine increased the % time spent in the open arms of the EPM relative to the vehicle-treated group (65 ± 6% vs 21 ± 10%, $p<0.001$). Sildenafil plus atropine co-administration increased the time spent in the open arms of the EPM relative to the vehicle-treated group (59% ± 5% vs 21% ± 10%, $p<0.001$), and comparable to the effect of fluoxetine. Although less pronounced, sildenafil alone also increased the time spent in the open arms (46 ± 6% vs 21 ± 10%, $p<0.001$).

Conclusion

The results suggest that FSL rats display an enhanced anxiety-like behaviour relative to FRL rats, in line with their established stress-sensitive characteristics. Furthermore our data suggest that chronic administration of PDE5 inhibitors exert anxiolytic actions in FSL rats, opposite to the anxiogenic effects following acute administration described by others. In our study, such an anxiolytic-like effect is potentiated by the co-administration of the antimuscarinic drug atropine. These data are supportive of the antidepressive-like effects of these drugs reported earlier by our laboratory.

List of Abbreviations	*Addendum E*

A

AC	adenyl cyclase
Ach	acethylcholine
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ATP	adenyl-tri-phosphate
ANOVA	one-way analysis of variance
Atr	atropine

B

BDNF	Brain-derived neurotrophic factor
BDZ	benzodiazepine
BSA	Bovine serum albumin

C

Ca^{+}	Calcium
cAMP	cyclic adenyl-monophosphate
CBT	Cognitive Behavioural Therapy
cGMP	cyclic guanosine-3', 5-monophosphate
CNS	Central nervous system
CREB	cAMP response element-binding

D

ddH_2O	Double Distilled Water
DFP	diisopropyl fluorophosphate
DMSO	dimethylsulfoxide
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders, 4^{th} Edition, Text Revision

E

eNOS	endothelial NOS
EPM	elevated plus maze

F

fEPSP	potentiatefiled excitatory postsynaptic potential
FDA	Food and Drug Administration
Flx	fluoxetine
fMRI	functional magnetic resonance imaging
FRL	Flinder's Resistant Line
FSL	Flinders Sensitive Line
FST	forced swim test

G

GC	guanylyl cyclase
GABA	γ-amino butyric acid
GAD	General anxiety disorder
GTP	Guanosine triphosphate

H

HHMI	Howard Hughes Medical Institute

I

iNOS	inducible NOS
IV	intravenous administration

L

L-NOARG	N-nitro-L-arginine
L-NAME-	NG-nitro-L-arginine-methyl-ester
LTP	
LTD	
LAMB	Laboratory for Applied Molecular Biology

M

MB	methylene Blue
mAb	mouse antibody
MAOI	Mono amine oxidase inhibitor
mNOS	mitochondrial NOS
mRNA	messenger RNA

N

NOS	nitric oxide synthase
NICE	National Institute for Health and Clinical Excellence
NIH	National Institutes of Health
NLM	National Library of Medicine
NMDA	N-methyl-D-aspartate
nNOS	neuronal NOS
NO	nitric oxide
NRF	National Research Foundation
NRIs	norepinephrine reuptake inhibitors

O

O_2	oxygen
OCD	Obsessive compulsive disorder

P

PKA	protein kinase A
PKG	protein kinase G
PDE	phosphdiesterase

PD	Panic Disorder
PDE5	phosphodiesterase type 5
PET	positron emission tomography
PTSD	Posttraumatic Stress Disorder
PVDF	polyvinylidene difluoride

Q

qPCR	Quantitative polymerase chain reaction

S

SNAP	S-nitro-N-acetylpenicillamine
SNP	sodium nitroprusside
sGC	soluble guanylyl cyclase
SAD	social anxiety disorder
SDS	Sodium dodecyl sulphate
SI	the International System of Units
Sil	sildenafil
SIT	social interaction test
SNRIs	serotonin and norepinephrine reuptake inhibitors
SPECT	single photon emission computed tomography
SSRIs	selective serotonin reuptake inhibitors

T

TAD	tricyclic antidepressants
TCAs	tricyclic antidepressant
TEMED	Tetramethylethylenediamine

V

Veh	vehicle

X

XRS	ChemiDoc

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
7-NI	7-nitroindazole
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)-tetralin

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